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MicroRNA-31 Activates the RAS Pathway and Functions as an Oncogenic MicroRNA in Human Colorectal Cancer by Repressing RAS p21 GTPase Activating Protein 1 (RASA1)*[S]

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Background: MicroRNAs are important for colorectal cancer signal transduction.
Results: miR-31 stimulates colorectal cancer cell proliferation and tumorigenesis by directly targeting RASA1.
Conclusion: miR-31 activates the RAS pathway and functions as an oncogenic microRNA in human colorectal cancer.
Significance: Learning how miRNAs participate in tumor signaling is crucial for understanding tumor signal transduction and cancer therapy.

MicroRNAs (miRNAs) are known to play a vital role in colorectal cancer. We found a widespread disruption in miRNA expression during colorectal tumorigenesis using microarray and quantitative RT-PCR analysis; of the 161 miRNAs altered in colorectal cancer compared with normal adjacent tissue samples, miR-31 was the most significantly dysregulated. We identified candidate targets of miR-31 using bioinformatics approaches and validated RAS p21 GTPase activating protein 1 (RASA1) as a direct target. First, we found an inverse correlation between miR-31 and RASA1 protein levels *in vivo*. Second, *in vitro* evidence demonstrated that RASA1 expression was significantly decreased by treatment with pre-miR-31-LV, whereas anti-miR-31-LV treatment increased RASA1 protein levels. Third, a luciferase reporter assay confirmed that miR-31 directly recognizes a specific location within the 3′-untranslated region of *RASA1* transcripts. Furthermore, the biological consequences of miR-31 targeting RASA1 were examined by the cell proliferation assay *in vitro* and by the immunodeficient mouse xenograft tumor model *in vivo*. Taken together, our results demonstrate for the first time that miR-31 plays a significant role in activating the RAS signaling pathway through the inhibition of RASA1 translation, thereby improving colorectal cancer cell growth and stimulating tumorigenesis.

Colorectal cancer (CRC)[4] is the second most frequent cause of cancer-related death in the United States and Europe (1). The development of CRC involves a multistep process with the accumulation of both genetic and epigenetic changes, including changes in the RAS pathway (2); however, other signaling pathways also appear to show accumulated alterations. This suggests that alternative factors contribute to CRC and that underlying levels of regulation exist to control the complex cross-talk among different signal transduction pathways.

Recently, the classical family of protein-coding genes recognized as tumor suppressors and oncogenes has been expanded to include a type of non-protein-coding RNA molecule known as microRNAs (miRNAs) (3). miRNAs play critical roles in the negative regulation of gene expression by base pairing to complementary sites on target messenger RNAs (mRNAs), thus causing a block in translation or triggering the degradation of the target mRNAs (4). miRNAs modulate various critical biological processes including cell proliferation, differentiation, apoptosis, tumorigenesis, and the immune response (5).

miRNAs have been studied most intensively in the field of oncological research, and emerging evidence suggests that the dysregulation of certain miRNAs that regulate the translation of oncogenes and tumor suppressors is involved in the pathogenesis of cancers (6). For instance, miR-17–92, which targets HIF-1α in lung tumors (7), and miR-21, which targets PTEN, TPM1, and PDCD4 in breast tumors (8), have been shown to be oncogenic, whereas miR-15a and miR-16, which target BCL-2 in chronic lymphocytic leukemia (9), and the let-7 family, which target RAS in lung tumors (10) are tumor suppressive. In CRC, multiple miRNAs have aberrant expression patterns (11, 12), and several have been shown to directly target oncogenes or

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[4] The abbreviations used are: CRC, colorectal cancer; miRNA, microRNA; ncRNA, scrambled negative control RNA; NC-LV, lentivirus carrying noncoding sequence; NAT, normal adjacent tissue; qRT-PCR, quantitative reverse transcription polymerase chain reaction; PCNA, proliferation cell nuclear antigen; pre-miR-31-LV, lentivirus carrying pre-miR-31 precursor; pre-NC-LV, lentivirus carrying pre-noncoding sequence; anti-miR-31-LV, lentivirus carrying anti-miR-31 inhibitor; anti-NC-LV, lentivirus carrying anti-noncoding sequence.

tumor-suppressor genes. These include miR-21, which targets PDCD4 (13), miR-143, which targets KRAS (14), and miR-10b, which targets BCL2L11 (15). Among the aberrantly expressed miRNAs, miR-31 has been reported to be up-regulated in CRC compared with non-tumoral mucosa samples, and stage IV tumors have a significant increase in miR-31 levels compared with stage II tumors (12). These results suggest a possible role for miR-31 in colorectal tumorigenesis.

However, the previously discovered roles of miR-31 in tumors are complicated, as its expression level is increased in head and neck cancer (16), hepatocellular carcinoma and squamous cell carcinoma (17), but significantly decreased in breast cancer (18), gastric cancer (19), prostate carcinoma (20), and urothelial carcinomas (21). Furthermore, target research also reveals that on certain occasions, miR-31 can behave as either a tumor suppressor or an oncogenic miRNA. For example, it targets LATS2 and PPP2R2A in lung cancer (22), thus acting as an oncogene, whereas it exerts a tumor-suppressor function in breast cancer by targeting RHOA (18). Therefore, it is necessary to investigate the roles of miR-31 in an extended range of different tumors, including CRC, for a clearer view of its dynamic behavior.

In this study, we performed miRNA microarray analyses and quantitative reverse transcription-PCR (qRT-PCR) on colorectal tissues and normal adjacent tissues from patients to identify the key miRNAs involved in CRC tumorigenesis. *In silico* analysis revealed a key tumor suppressor gene, RAS p21 GTPase activating protein 1 (RASA1), as the target of miR-31. Direct inhibition of RASA1 translation by miR-31 and a potential role of miR-31 as an oncogene in colorectal tumorigenesis were experimentally validated.

EXPERIMENTAL PROCEDURES

Clinical Samples and Cell Lines—Human CRC tissue and paired normal adjacent tissue (NAT) samples were obtained from patients who underwent radical resection at Tianjin Medical University Cancer Institute and Hospital (Tianjin, P.R. China), Jinling Hospital (Nanjing, P.R. China) and Jiangsu Province Hospital of TCM (Nanjing, P.R. China) from 2008 to 2012. All samples were gathered according to the Institutional Review Board-approved protocol and the written informed consent from each patient. For each case, samples from the primary tumor and the corresponding normal colorectal mucosa were collected for comparison and were moved to liquid nitrogen within 30 min after operation. The patient information (gender, age, tumor site, and TNM stage) was obtained from surgical and pathological records from the hospitals and is shown in supplemental Tables S1 and S2.

Caco-2 and HT-29 human colorectal adenocarcinoma cells were obtained from the Institute of Cell Biology at the Chinese Academy of Sciences (Shanghai, P.R. China). Caco-2 cells were cultured in Dulbecco's modified essential medium with high glucose (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 1% of the MEM non-essential amino acid ×100 solution (Invitrogen), whereas HT-29 cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS. All cells were cultured in a humidified incubator at 37 °C with 5% CO_2.

Total RNA Isolation—Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA quality was determined by formaldehyde-agarose gel electrophoresis, and the concentration of RNA was determined using an Eppendorf BioPhotometer plus (Eppendorf AG, Hamburg, Germany).

Microarray and qRT-PCR Assays—A microarray-based approach was used to identify miRNAs that were differentially expressed between human CRC tissue and NAT (23). The commercially available Mammalian miRNA Array containing 924 non-redundant microRNA probes, Service version 3.0, was purchased from CapitalBio Corporation (Beijing, China). Labeling, hybridization, washing, and scanning were performed according to the standard operating procedure provided by CapitalBio Corporation. Briefly, 25–50 μg of total RNA was purified using a MirVana® miRNA Isolation Kit (Ambion, Austin, TX) to enrich the small RNA fraction. After the quality of small RNA was detected by formaldehyde-agarose gel electrophoresis, the RNAs were fluorescently labeled with CU-Cy3 using T4 RNA ligase. Hybridization and washing were then performed. Finally, array scanning was performed using a confocal LuxScan scanner (CapitalBio Corp). The scanning setting was adjusted to obtain an equal visualized intensity of U6 spots across arrays. Data analysis based on the TIFF images obtained was performed using SpotData Pro software (CapitalBio Corp.).

Quantitative RT-PCR assays to investigate differences in the expression of the miRNAs of interest were performed on a 7300 Sequence Detection System (Applied Biosystems) using EvaGreen Dye (Biotium, Hayward, CA) (23). Briefly, 2 μl of total RNA (1 μg/μl) was reverse transcribed into cDNA using avian myeloblastosis virus reverse transcriptase (Takara Bio, Shiga, Japan) and a stem-loop RT primer (Invitrogen) under the following conditions: 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min. The conditions for the PCR were as follows: 95 °C for 5 min, 95 °C for 15 s, and 60 °C for 1 min, for 40 cycles. All reactions, including the no template controls, were run in triplicate. After the reactions were complete, the C_T values were determined using fixed threshold settings. miRNA expression was normalized to U6 snRNA expression in this study. The amount of miRNA to relative to the internal control U6 was calculated using the equation $2^{-\Delta CT}$, in which $\Delta C_T = C_{T,\mathrm{miRNA}} - C_{T,\mathrm{U6}}$.

Overexpression or Knockdown of miR-31 by Lentivirus Infection—Recombinant lentiviruses carrying pre-miR-31 precursor (pre-miR-31-LV), pre-noncoding sequence (pre-NC-LV), anti-miR-31 inhibitor (anti-miR-31-LV), or anti-noncoding sequence (anti-NC-LV), were obtained from GenePharma (Shanghai, China). Each lentivirus contained a GFP sequence so that the infection efficiency could be monitored by the fluorescence. They were individually added to HT-29 cells of 30% confluence in 6-well plates or 10-cm dishes at a multiplicity of infection of 25 together with Polybrene at a final concentration of 5 μg/ml. Cells were then harvested at 3 days post-infection for Western blotting, qRT-PCR, or animal experiments.

Plasmid Constructs and Luciferase Reporter Assay—The whole human *RASA1* 3′ untranslated region (3′-UTR) sequence (1032 bp), obtained from the GenBank database, was amplified by PCR using a human genomic DNA template made from Caco2

cells. The PCR products were inserted into the SpeI/HindIII sites of the *p*-MIR-reporter plasmid (Promega, Madison, WI) using the following primers: forward, 5′-CTGACTAGTCAGCCTTCGCCCCAGTG-3′, and reverse, 5′-CTGAAGCTTGCAAGATATCCCTTGGTTTTATTTTA-3′. Efficient insertion was confirmed by sequencing.

For the luciferase reporter assays, Caco2 cells were cultured on 6-well plates. For each well, cells at 70–80% confluence were transfected with 1 μg of firefly luciferase reporter plasmid, 0.5 μg of β-galactosidase expression vector (Promega), and 100 pmol of precursor oligonucleotides (pre-miR-31), antisense oligonucleotides (anti-miR-31), or scrambled negative control RNA (ncRNA) (each from Ambion, Grand Island, NY) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

The cells were assayed using luciferase assay kits (Promega) at 24 h post-transfection. The β-galactosidase plasmid was co-transfected and used for normalization. Each transfection experiment was performed in triplicate.

Protein Extraction and Western Blotting—The HT-29 cells on 6-well plates were rinsed twice with cold PBS and lysed in RIPA lysis buffer (Sunshine Technology, Nanjing, China) containing a protease inhibitor mixture (Sigma) at 1:100 dilution on ice for 30 min. Tissue samples were frozen solid using liquid nitrogen in a mortar, ground vigorously and rapidly, and lysed in RIPA lysis buffer containing a protease inhibitor mixture at 1:100 dilution on ice for 30 min. Insoluble components of cell lysates or tissue homogenates were removed by centrifugation (4 °C, 12,000 $\times$ *g*, 10 min), and protein concentrations were measured using a Pierce BCA protein assay kit (Thermo Scientific). The RASA1 protein level was quantified by Western blotting analysis of 100 μg of cell extract or tissue extract using the antibody against RASA1 (B4F8) (1:100 or 1:200 dilution, mouse monoclonal antibody sc-63, Santa Cruz Biotechnology, Santa Cruz, CA). ERK1/2 protein or phospho-ERK1/2 protein levels were quantified by Western blotting analysis of 100 μg of cell extract using antibodies against MAPK (ERK1/2) (p44/p42) (1:1,000 dilution, rabbit polyclonal antibody number 4695, Cell Signaling, Boston, MA) or against phospho-ERK1/2 (Thr^{202}/Tyr^{204}) (1:800 dilution, rabbit polyclonal antibody BS5016, Bioworld Technology, St. Louis, MN). The RGS4 protein was examined by Western blotting analysis of 100 μg of cell extract or 300/450 μg of tissue extract using the anti-RGS4 (C-17) polyclonal antibody (1:100 dilution, Santa Cruz sc-6203). The LATS2 protein was detected by Western blotting analysis of 100/300 μg of cell extract or tissue extract using the anti-LATS2 monoclonal antibody (1:1,000 dilution, Proteintech Group 20276-1-AP; Proteintech, Chicago, IL). Equal loading was confirmed by blotting using the HRP-conjugated anti-glyceraldehyde-3-phosphate dehydrogenase antibody (1:10,000 dilution, KangChen Bio-tech, Shanghai, P.R. China). Immunocomplexes were visualized using an enhanced chemiluminescence kit (Cell Signaling Technology) according to the manufacturer's protocol.

Active RAS Pulldown Assay—An active Ras pulldown assay was performed with an active RAS pulldown and detection kit (Thermo Scientific) following the manufacturer's instructions. Briefly, HT-29 cells were seeded onto 10-cm dishes at 2×10^6 cells each dish. After incubation for 24 h, the cells were infected, respectively, with pre-miR-31-LV, pre-NC-LV, anti-miR-31-LV, or anti-NC-LV, then lysed in 500 μl of lysis buffer containing protease inhibitors at 3 days after infection. Cell lysate was spun at 16,000 $\times$ *g* at 4 °C for 15 min, and the supernatant (total lysate) was used in a protein assay using Pierce BCA Protein Assay. A 1-mg total lysate sample was then mixed with 80 μg of GST-Raf1-RBD bound to glutathione-agarose beads and incubated at 4 °C for 1 h with gentle rocking for affinity precipitation of RAS-GTP. After the beads were washed twice, 50 μl of reducing sample buffer was added to each pulldown reaction. The samples were incubated at room temperature for 2 min before analysis by Western blotting with the anti-RAS antibody (1:200 dilution, Thermo Scientific, included in the RAS pulldown and detection kit). The total RAS protein level in the lysates was quantified by Western blotting analysis using an anti-RAS antibody (1:1000 dilution, BD Biosciences).

Cell Proliferation Assay—HT-29 cells were seeded onto 6-well plates at 2×10^5 cells/well. After incubating for 24 h, the cells were infected, respectively, with pre-miR-31-LV, pre-NC-LV, anti-miR-31-LV, or anti-NC-LV. At 60 h post-infection, the cells were harvested and the viable cells were seeded at a density of 6×10^3 each well onto 96-well plates. The number of cells was determined using a Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) at 12, 24, 36, 48, 60, and 72 h. The absorbance at 450 nm, representing the relative viable cell number, was measured at each time point.

The growth of HT-29 cells with miR-31 overexpression or knockdown was also assessed by proliferation biomarkers KI-67 and proliferation cell nuclear antigen (PCNA). The primers used in qRT-PCR for detection of *KI-67* and *PCNA* were as follows: *KI-67*, forward, 5′-ATGGATAAGCGCACGGATGAAT-3′, and reverse, 5′-TCGGGCTGCCAGATAGAGTC-3′; *PCNA*, forward, 5′-CAGGGCTCCATCCTCAAGAA-3′ and reverse, 5′-TCTTCATTGCCGGCGCATT-3′.

Immunodeficient Mouse Xenograft Tumor Model—All animal experiments were performed according to the regulations of P.R. China and Nanjing University, and approved by the animal care and use committee of Nanjing University. HT-29 cells, which were infected with pre-miR-31-LV, pre-NC-LV, anti-miR-31-LV, or anti-NC-LV, were harvested by a brief trypsinization (0.05% trypsin, 0.02% EDTA in Ca^{2+}/Mg^{2+}-free PBS) then washed twice in PBS and re-suspended in medium without serum to count the number of viable cells by trypan blue staining using a hemocytometer.

Athymic BALB/c male nude (nu/nu) mice, ~4 weeks old on arrival, were obtained from the Model Animal Research Center of Nanjing University (Nanjing, China) and maintained under pathogen-limited conditions. They were equally divided into 4 groups (7 mice/group), and injected subcutaneously with 1×10^7 viable tumor cells infected, respectively, by pre-miR-31-LV, pre-NC-LV, anti-miR-31-LV, or anti-NC-LV 3 days later.

After subcutaneous implantation of cells, animals were observed daily for tumor growth and subcutaneous tumors were measured on 8, 12, 15, 18, and 21 days. Each tumor volume was calculated according to the following equation: V (mm) = D^2 (mm^2) $\times$ L (mm)/2, where D and L are the smallest and the

largest perpendicular tumor diameters, respectively. The mice were sacrificed and photographed at 21 days post-implantation; then xenograft tumors were excised, also photographed, and weighted.

Statistical Analysis—All experiments were performed in triplicate, and all results are presented as the mean ± S.D. Hierarchical Cluster analysis was performed using Gene Cluster software (Stanford University). Differences/correlations between groups were calculated using Student's *t* test. $p < 0.05$ was defined as statistical significance. Significance analysis of microarrays was carried out. To exclude extreme outliers, the miRNAs with expression lower than a threshold ($C_{T,\mathrm{miRNA}} - C_{T,\mathrm{U6}} > 15$, mean fold-change >1.5 or <0.67) were eliminated.

RESULTS

Aberrant miRNA Expression in Colorectal Cancer—To identify aberrantly expressed miRNAs, we screened the miRNA expression levels in 12 CRC samples and 9 non-tumor NAT samples through a microarray-based approach (for patient information, refer to supplemental Table S1). The miRNAs with significantly different expression in the CRC samples compared with the NAT samples were identified by statistical analysis including significance analysis of microarrays and Student's *t* test. The miRNAs with an expression fold-change greater than 1.5 or less than 0.67 were identified. Based on these principles, significance analysis of microarrays analysis generated a list of 87 miRNAs that were differentially expressed in the CRC samples compared with NAT samples (Table 1). Among them, miR-31 was the most significantly up-regulated miRNA (6.13-fold up-regulation) in the CRC samples. miR-31 also had a relatively high score in significance analysis of microarrays analysis (significance analysis of microarrays score = 2.76, *q* value = 0.00, *p* value = 0.00). Notably, the set of differentially expressed miRNAs described here, which includes miR-31, miR-203, miR-142–5p, miR-143, and miR-145, is similar to those reported in previous papers (11, 14, 24, 25). These studies, together with our results, firmly support the notion that miRNA expression profiling could generate a unique molecular signature for CRC. Differences in the miRNA expression pattern between the CRC and NAT samples were further revealed by unsupervised clustering analysis of CRC and NAT samples blinded to their clinical annotations. Hierarchical cluster analysis generated a dendrogram with two major branches in both columns (CRC *versus* NAT) and rows (up-regulated *versus* down-regulated genes) (Fig. 1*a*).

To further validate the microarray data, we performed qRT-PCR to quantify the levels of certain differentially expressed miRNAs in 6 new pairs of CRC and NAT samples. The qRT-PCR analysis revealed the same trends in expression for these miRNAs (Fig. 1*b*) (for patient information, refer to samples 1 to 6 in supplemental Table S2).

Identification of RASA1 as a Target of miR-31—It is well known that a single miRNA can affect multiple targets via distinct mechanisms (26). To study the mechanisms responsible for colorectal cancer tumorigenesis, we performed bioinformatics analyses to search for miR-31 target mRNAs. Targetscan (27), Miranda (28), and Pictar (29) were used independently to predict miR-31 targets. Because individual computer-aided algorithms generate a high number of false positives, we used a combination of these three approaches to provide a more accurate assessment of the miRNA targets.

TABLE 1

Aberrantly expressed miRNAs in colorectal cancer revealed by a microarray scan

SAM and *t*-testanalysis generated a list of miRNAs that were differentially expressed in the CRC samples compared to the NAT samples.

Name	Fold Change	Score	q-value (SAM)	p-value (t-test)	Name	Fold Change	Score	q-value (SAM)	p-value (t-test)
Positive Genes (Fold change≥1.5)					miR-151	1.780	1.658	1.667	0.05
miR-31	6.134	2.762	0.000	0.01	miR-27b	1.675	4.951	0.000	0.00
miR-203	6.001	3.038	0.000	0.00	miR-17-3p	1.632	2.095	0.000	0.00
miR-224	5.401	2.168	0.000	0.01	miR-200a	1.632	4.382	0.000	0.00
miR-429	3.823	4.728	0.000	0.00	miR-186	1.617	1.260	3.226	0.02
miR-20b	3.674	2.970	0.000	0.00	miR-181d	1.595	1.107	4.384	0.04
miR-223	3.606	2.926	0.000	0.00	let-7d	1.594	1.344	2.951	0.16
miR-98	3.428	2.031	0.000	0.01	miR-362	1.536	1.293	2.951	0.03
miR-18a	3.427	3.522	0.000	0.00	miR-193b	1.520	1.178	3.810	0.09
miR-18b	3.266	2.294	0.000	0.00	miR-128a	1.506	1.304	2.951	0.02
miR-142-5p	3.176	3.239	0.000	0.00	miR-30e-5p	1.505	1.383	2.951	0.02
miR-20a	3.053	4.497	0.000	0.00	miR-16	1.503	2.474	0.000	0.03
miR-221	3.035	4.071	0.000	0.00					
miR-19b	2.812	7.800	0.000	0.00	Negative Genes (Fold change≤0.67)				
miR-17-5p	2.784	8.321	0.000	0.00	miR-601	0.178	-2.749	2.308	0.03
miR-182	2.757	2.162	0.000	0.00	miR-486	0.195	-1.874	4.384	0.13
miR-193a	2.738	2.229	0.000	0.01	miR-575	0.209	-2.744	2.308	0.01
miR-335	2.714	2.448	0.000	0.00	miR-610	0.220	-2.216	4.384	0.04
miR-34a	2.695	3.638	0.000	0.00	miR-202	0.232	-1.901	4.384	0.02
miR-106a	2.531	7.451	0.000	0.00	miR-632	0.313	-2.044	4.384	0.00
miR-19a	2.423	2.830	0.000	0.00	miR-627	0.327	-1.534	5.063	0.04
miR-222	2.414	3.839	0.000	0.00	miR-557	0.337	-2.542	2.308	0.04
miR-210	2.375	2.210	0.000	0.02	miR-602	0.375	-2.169	4.384	0.02
miR-423	2.343	2.619	0.000	0.00	miR-665	0.382	-2.174	4.384	0.06
miR-107	2.339	4.421	0.000	0.00	miR-650	0.392	-1.874	4.384	0.01
miR-130b	2.291	2.524	0.000	0.00	miR-720	0.416	-5.520	0.000	0.00
miR-146b	2.226	3.355	0.000	0.00	miR-572	0.439	-1.611	5.063	0.06
miR-27a	2.174	8.055	0.000	0.00	miR-612	0.453	-1.644	5.063	0.07
miR-15b	2.165	1.967	0.000	0.03	miR-663	0.455	-2.294	2.308	0.06
miR-106b	2.149	3.591	0.000	0.00	miR-133a	0.464	-1.708	4.675	0.05
miR-146a	2.145	2.900	0.000	0.00	miR-638	0.481	-3.802	0.000	0.01
miR-331	2.144	3.352	0.000	0.00	miR-491	0.487	-1.249	5.814	0.22
let-7e	2.118	1.500	2.308	0.07	miR-523	0.505	-1.187	5.909	0.04
miR-128b	2.076	2.355	0.000	0.00	miR-574	0.507	-2.140	4.384	0.00
miR-148b	2.069	1.293	2.951	0.01	miR-133b	0.513	-1.329	5.647	0.12
miR-368	2.053	1.679	1.667	0.00	miR-622	0.514	-1.162	5.909	0.11
miR-15a	2.031	3.068	0.000	0.00	miR-422b	0.537	-2.039	4.384	0.08
miR-452	2.010	1.299	2.951	0.02	miR-145	0.549	-5.031	0.000	0.00
miR-338	1.990	1.711	1.667	0.04	miR-432	0.555	-1.532	5.063	0.06
miR-141	1.972	2.323	0.000	0.02	miR-134	0.556	-1.328	5.647	0.11
miR-103	1.893	4.682	0.000	0.00	miR-659	0.557	-1.332	5.647	0.08
miR-130a	1.861	1.578	2.308	0.07	miR-422a	0.560	-1.454	5.122	0.19
miR-101	1.816	2.122	0.000	0.01	miR-594	0.564	-3.625	0.000	0.01
miR-93	1.816	3.079	0.000	0.00	miR-484	0.601	-2.610	2.308	0.01
miR-365	1.809	1.050	4.384	0.15	miR-671	0.634	-0.873	7.416	0.05

Because the expression of miR-31 was dramatically increased in several CRC tissue samples and cell lines, it is considered an important miRNA for colorectal tumor formation (30, 31). Therefore, we focused our efforts on the identification of candidate tumor suppressor genes regulated by miR-31 and selected several candidates, including RASA1, LATS2, and RGS4.

When studying LATS2 as a target of miR-31 in CRC, we found its protein levels in both NAT and CRC too low to be detected by Western blotting (supplemental Fig. S1*a*), even when loading up to 300 μg of tissue lysate (supplemental Fig. S1*b*), which is consistent with previous reports that LATS2 mRNA and protein levels are extremely low in colon (32, 33). Meanwhile, we also found that the RGS4 protein level showed no significant difference between CRC and NAT samples (overall fold-change −7.54%, $p = 0.105$) (supplemental Fig. S1*c*). Most importantly, although miR-31 in HT-29 cells was significantly overexpressed or down-regulated by lentivirus infection (Fig. 3*a*), the RGS4 protein level detected by Western blotting had no significant difference (supplemental Fig. S1*d*).

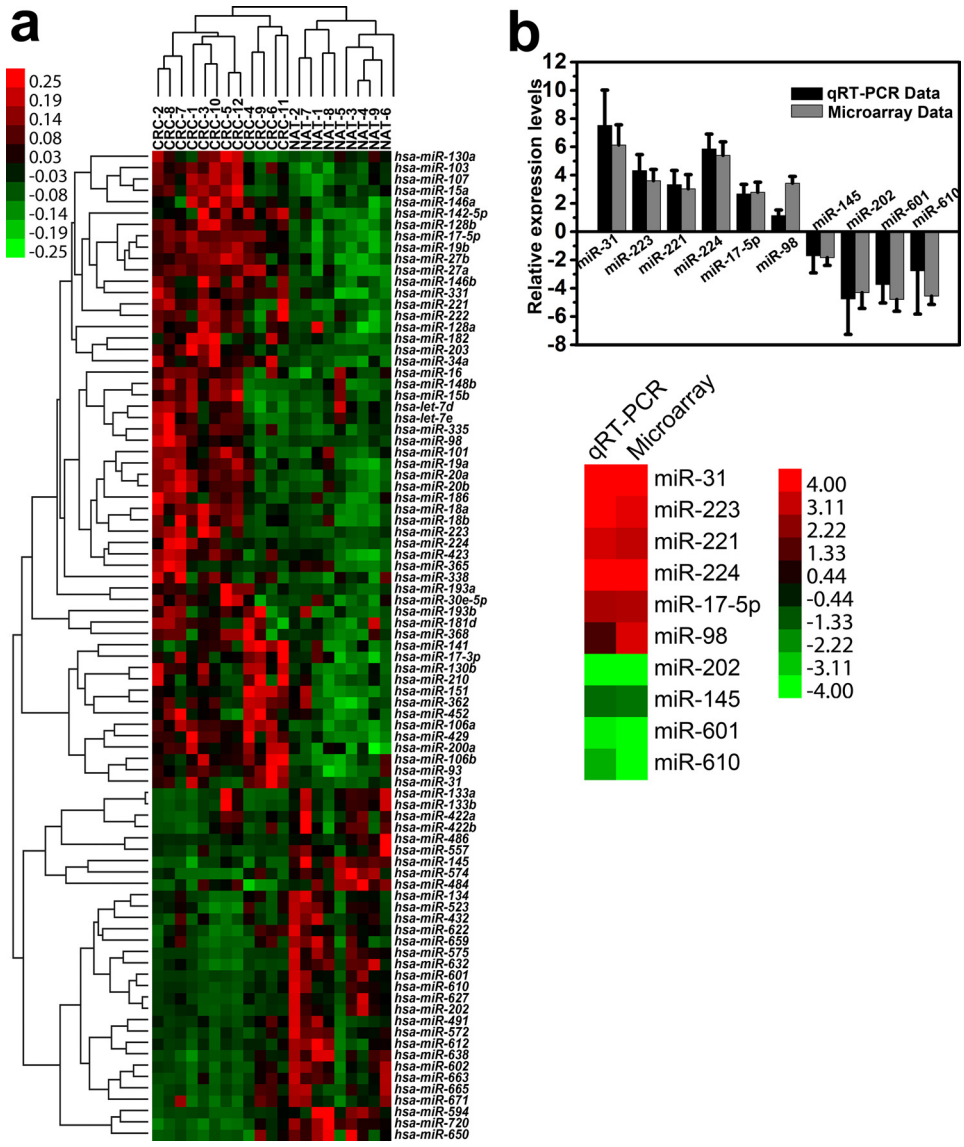

FIGURE 1. **Cluster analysis of aberrant miRNA expression in colorectal cancer according to a microarray scan and qRT-PCR validation.** *a,* dendrogram generated by cluster analysis showing the separation of CRC from NAT samples based on miRNA profiling. *b*, the miRNA expression ratios (CRC relative to NAT) from the qRT-PCR data *versus* the microarray data. $p < 0.05$ *versus* controls.

Consequently, we focused on RASA1 as the target of miR-31. RASA1 has long been considered a major member of the RAS pathway (34, 35) and also an important tumor suppressor (36). The free energy value of the hybrid between *RASA1* 3′ UTR and miR-31 was −14.75 kcal/mol, which is well within the range of real miRNA-target pairs. Moreover, there was perfect base pairing between the "seeds" (the core sequence that encompasses the first 2–8 bases of the mature miRNA) and cognate targets, and the seeds were conserved among species (Fig. 2*a*).

RASA1 was deduced to be a miR-31 target by not only computational prediction but also by validating an inverse correlation between miR-31 and the RASA1 protein level during colorectal tumorigenesis. The microarray analysis identified miR-31 as one of the most significantly up-regulated miRNAs in colorectal tumorigenesis (fold-change = 6.13, p value = 0.00). Consistently, in the qRT-PCR performed on another set of 12 control-matched CRC samples (for patient information, refer to supplemental Table S2), the overall miR-31 level was up-regulated by 7.56-fold (p value = 0.00) (Fig. 2*b*). In agreement with this observation, the overall RASA1 protein level in the CRC samples was 60.4% lower than that in NAT samples from the same patients (p value = 0.00) (Fig. 2*c*).

The correlation between miR-31 and RASA1 was further examined by evaluating the expression of RASA1 in human colorectal adenocarcinoma HT-29 cells after overexpression or knockdown of miR-31 (Fig. 3). In these experiments, miR-31 overexpression was achieved by infecting the cells with pre-miR-31-LV, whereas miR-31 knockdown was achieved by infecting the cells with anti-miR-31-LV. Control experiments were performed in the same manner but used cells infected with pre-NC-LV or anti-NC-LV. The efficiency of infection at 72 h screened by the GFP (green fluorescent protein) signal was higher than 80% (data not shown), whereas the efficiency of infection was also measured by qRT-PCR. As shown in Fig. 3*a*, the miR-31 level was increased by 10.5-fold after the cells were infected by pre-miR-31-LV compared with the pre-NC-LV,

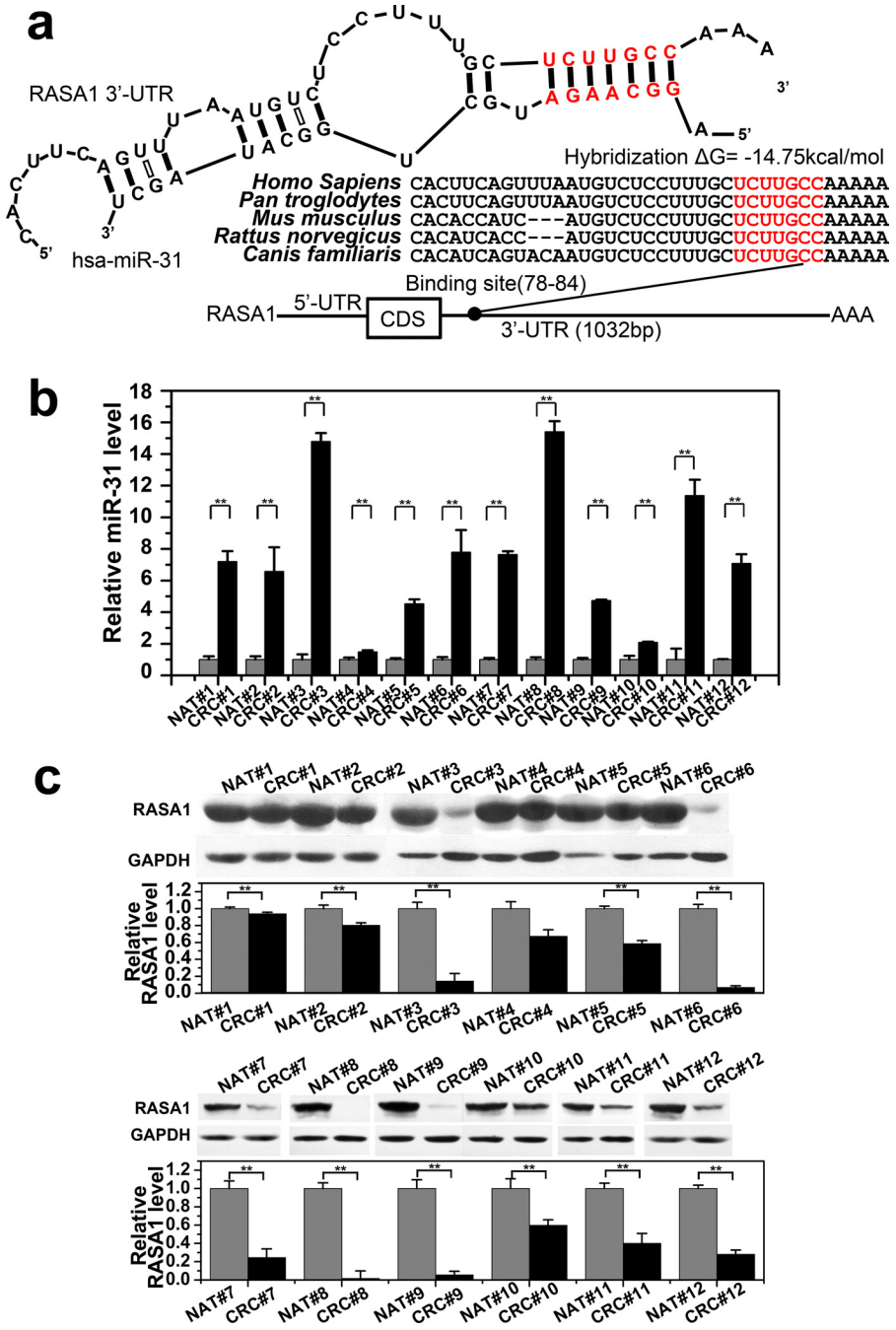

FIGURE 2. **Hybridization of the 3′ UTR of *RASA1* and miR-31 and *in vivo* inverse correlation between the miR-31 expression and RASA1 protein level.** *a*, schematic depicting the conserved binding sites for miR-31. The seed-recognizing site is marked in *red*; all nucleotides in this region were completely conserved among several species. Hypothesized duplexes formed by the interaction of the binding sites of the 3′ UTR of *RASA1* (*top*) and miR-31 (*bottom*) are illustrated, and the predicted free energy of each hybrid was indicated. The *short solid lines* between the two chains represent hydrogen bonds between adenine (A)-thymine (T) pairs or guanine (G)-cytosine (C) pairs, whereas *open lines* represent G-U pairings. *b*, relative miR-31 expression in CRC and NAT samples. **, $p < 0.05$ *versus* controls. *c*, relative RASA1 protein expression in paired CRC and NAT samples.

whereas the miR-31 level was reduced by 68.5% after cells were infected by the anti-miR-31-LV compared with the anti-NC-LV. The expression of RASA1 was significantly decreased in miR-31 overexpressed cells or enhanced in miR-31 knockdown cells (Fig. 3*b*). These results suggest that miR-31 negatively regulates RASA1.

Recently, we became convinced that miRNAs sometimes decrease the levels of a specific target mRNA by affecting its stability (37). To determine how miR-31 influences RASA1, we infected HT-29 cells with pre-miR-31-LV, pre-NC-LV, anti-miR-31-LV, or anti-NC-LV, and then evaluated the *RASA1* transcript levels 72 h after infection. In contrast with the decrease observed in the RASA1 protein level after pre-miR-31-LV transfection, the RASA1 mRNA level was not repressed by overexpression of miR-31, nor stimulated by knockdown of miR-31 (Fig. 3*c*). These results suggest that miR-31 regulates RASA1 expression via a post-transcriptional mechanism only, rather than by affecting its mRNA stability ("mRNA decay") (38).

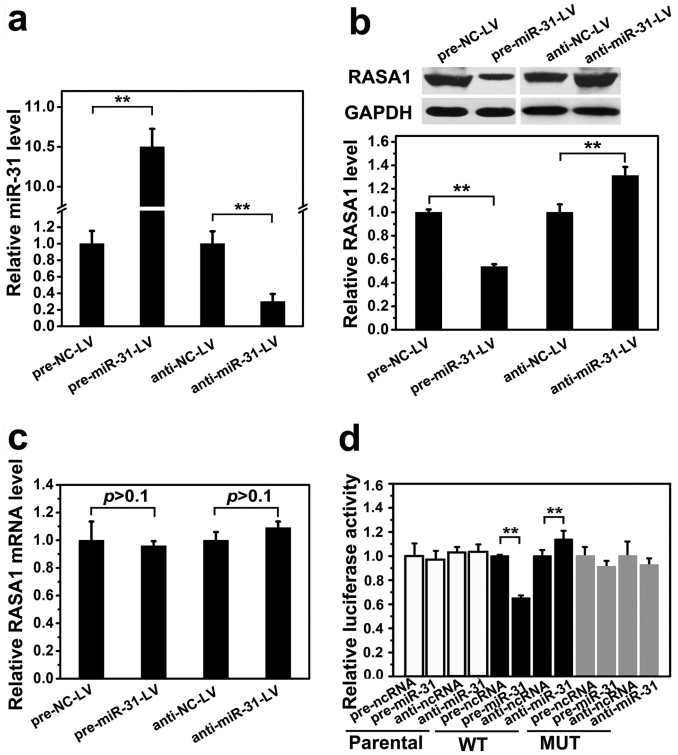

FIGURE 3. ***In vitro* identification of RASA1 as a target of miR-31.** *a,* relative miR-31 level after infection with pre-miR-31-LV or anti-miR-31-LV in HT-29 cells. One representative experiment of three is shown, with the average values of triplicate wells. *b,* relative RASA1 protein expression after infection with pre-miR-31-LV or anti-miR-31-LV in HT-29 cells. One representative experiment of three is shown. *c,* relative RASA1 mRNA levels after infection with pre-miR-31-LV or anti-miR-31-LV in HT-29 cells. One representative experiment of three is shown. *d,* relative luciferase activity after transfection with pre-miR-31 or anti-miR-31 in Caco-2 cells. One representative experiment of three is shown. **, $p < 0.05$ *versus* controls.

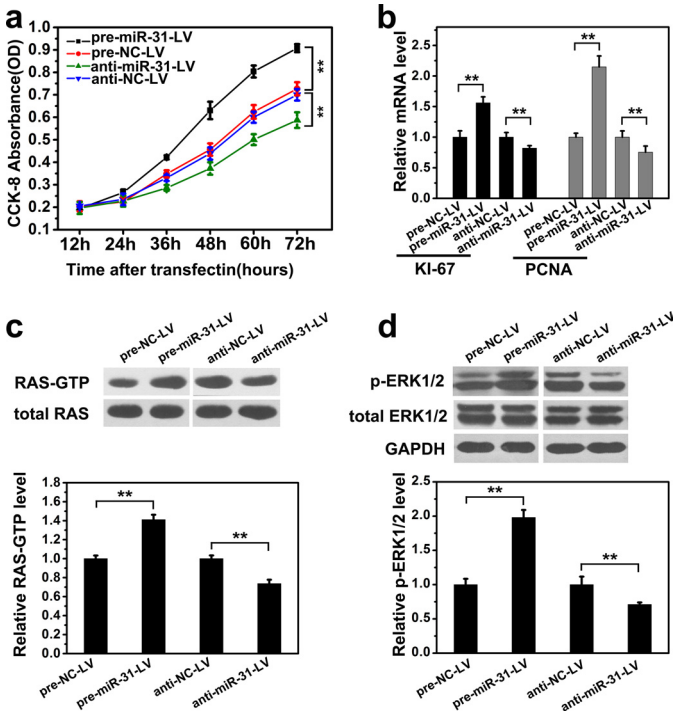

FIGURE 4. **The biological effect of miR-31 in the RAS-MAPK pathway in HT-29 cells.** *a,* the growth-promoting function of miR-31. One representative experiment of three is shown. *b, PCNA* and *KI-67* levels assessed by qRT-PCR after overexpression or knockdown of miR-31. One representative experiment of three is shown. *c,* the relative level of GTP-bound Ras (RAS-GTP) detected by Western blotting after overexpression or knockdown of miR-31. One representative experiment of three is shown. *d,* phospho-ERK1/2 and total ERK1/2 levels assessed by Western blotting analysis after overexpression or knockdown of miR-31. One representative experiment of three is shown. **, $p < 0.05$ *versus* controls.

Luciferase binding assays were conducted using a pMIR-REPORT^{TM} luciferase plasmid to determine whether miR-31 suppressed RASA1 through direct binding to its 3′ UTR. Wild-type *RASA1* 3′ UTRs and mutants whose seed sequence TCTTGCC was replaced by AGAACGG were independently cloned into pMIR-REPORT, which contains a firefly luciferase gene, and transfected into Caco2 cells along with a control plasmid (β-gal). As expected, the overexpression of miR-31 resulted in a 35% reduction of firefly luciferase reporter activity (normalized to β-gal activity) compared with cells transfected with pre-ncRNA, whereas inhibition of miR-31 resulted in a 14% increase in reporter activity compared with the control cells (Fig. 3*d*). Mutation of the miR-31-binding site abolished these effects. These results unequivocally demonstrate that miR-31 directly recognizes the 3′ UTR of the *RASA1* transcript. Thus, up-regulation of miR-31 enhances suppression of RASA1 and induces tumorigenesis, supporting an oncogenic role for miR-31.

Role of miR-31 in Tumorigenesis by Targeting the RAS Pathway—To test the downstream biological consequences of the miR-31-driven inhibition of RASA1 expression, HT-29 cells were infected with pre-miR-31-LV, pre-NC-LV, anti-miR-31-LV, or anti-NC-LV, and the cell proliferation rate was determined using a CCK-8 assay. As shown in Fig. 4*a*, HT-29 cells treated with pre-miR-31-LV had a significant increase in cell proliferation rate compared with cells infected with pre-NC-LV. By contrast, knockdown of miR-31 resulted in a decrease in cell viability. These results indicate that miR-31, which targets RASA1, plays a role in the RAS pathway and accelerates cell proliferation. In support of this idea, the cells infected with pre-miR-31-LV showed an increase in cell proliferation at 72 h, as indicated by the induction of the proliferation biomarkers *KI-67* and *PCNA*, whereas knockdown of miR-31 had the opposite effect on cell proliferation (Fig. 4*b*).

We next analyzed the molecular biological consequences of miR-31-driven inhibition of *RASA1* gene expression in HT-29 cells. Previous reports have shown that the inhibition of RASA1 can trigger the RAS-MAPK signaling pathway by activating the GTPase activity of RAS, consequently increasing cell proliferation, suppressing apoptosis, deregulating the cell cycle, and eventually resulting in a malignant transformation that leads to human CRC (39). Therefore, RAS activation was then examined using an Active GTPase Pull-down and Detection Kit at 72 h after HT-29 cells were infected with pre-miR-31-LV, pre-NC-LV, anti-miR-31-LV, or anti-NC-LV. This assay relies on measurement of the level of the RAS-GTP form, which increases as the GTPase activity of RAS decreases. As shown in Fig. 4*c*, the level of GTP-bound RAS was significantly increased by treatment of pre-miR-31-LV and inhibited by anti-miR-31-LV; there was no significant change in the total RAS. We next examined ERK1/2 phosphorylation, which is a

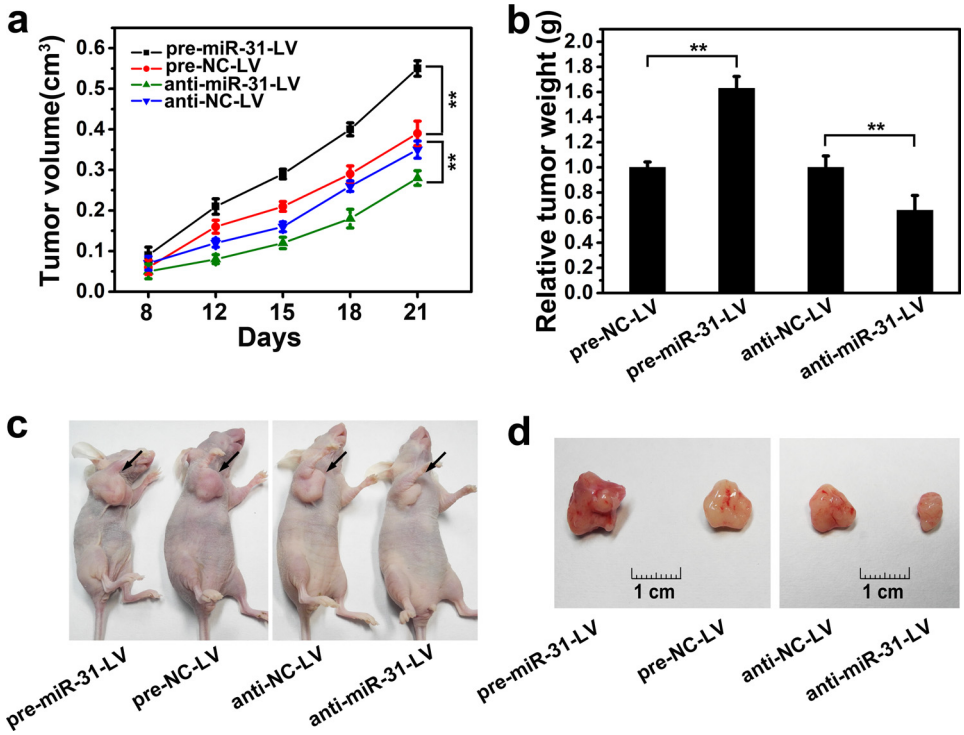

FIGURE 5. **The biological role of miR-31 in tumorigenesis by targeting the RAS pathway.** *a*, the volume of xenograft tumors in nude mouse derived from subcutaneous implantation of HT-29 cells ($n = 7$ per group). *b*, the relative tumor weight at 21 days post-implantation. *c*, photograph of nude mice bearing xenograft tumors at 21 days post-implantation. *d*, photograph of the excised tumors at 21 days post-implantation. **, $p < 0.05$ *versus* controls.

characteristic downstream signature of RAS pathway activation. At 72 h after overexpression or knockdown of miR-31, the ratio of phospho-ERK1/2 to total ERK1/2 was significantly increased by treatment with pre-miR-31-LV and significantly inhibited by treatment with anti-miR-31-LV (Fig. 4*d*). These results suggest that inhibition of RASA1 expression by miR-31 increases GTP binding by RAS, thus activating the RAS pathway and inducing the constitutive phosphorylation of ERK1/2. Therefore, modulation of the RASA1 protein level by miR-31 partially explains why down-regulation of miR-31 promotes cell proliferation.

To further examine the *in vivo* biological role of miR-31, we conducted the experiment of tumorigenicity in nude mice using HT-29 cells, in which miR-31 was successfully overexpressed or knocked down by lentivirus infection. After 4 groups of nude mice (7 mice/group) were subcutaneously implanted with the infected tumor cells, we observed that the size of tumors in the pre-miR-31-LV group was significantly larger than those in the pre-NC-LV group at each time point, whereas the size of tumors in the anti-miR-31-LV group had an opposite trend (Fig. 5*a*). The excised tumors from the pre-miR-31-LV group were 62.5% heavier in weight than those from the pre-NC-LV group at 21 days post-implantation, whereas the tumors from the anti-miR-31-LV group weighted 30.8% less than those from the anti-NC-LV group (Fig. 5*b*). As shown in Fig. 5, *c* and *d*, the tumor size in the pre-miR-31-LV group is much larger than that of the control, whereas the tumor size in the anti-miR-31-LV group had an opposite trend. These results are consistent with that of the cell proliferation assay, which firmly validates the role of miR-31 in tumorigenesis by targeting the RAS pathway.

As a conclusion, in studying the potential mechanisms of miRNA action in colorectal cancer, we identified RASA1 as a target of miR-31 by bioinformatics analysis and binding assays, confirming that it is indeed down-regulated by miR-31. A biological association between miR-31 and RASA1 was uncovered. We confirmed that as a result of RASA1 suppression by miR-31, there were increases in GTP-bound RAS, phosphorylation of ERK1/2, levels of pro-proliferative factors including *PCNA* and *KI-67*, and eventually cell proliferation, which led to tumorigenesis.

DISCUSSION

The aberrant expression of miR-31, which targets RASA1, exerts profound influence into the cell signaling network in colorectal cancer, because RASA1 and its downstream proteins play essential roles in control of cellular growth and differentiation (35). The RAS protein cycles between the inactive GDP-bound and the active GTP-bound state, and the GTPase activity of normal RAS p21 is stimulated by RASA1 (40). RASA1 acts as a suppressor of RAS function by enhancing the weak intrinsic GTPase activity of RAS proteins (41), thus resulting in an increase in the inactive GDP-bound form of RAS, thereby leading to aberrant intracellular signaling through the RAF-MEK-ERK and PI3K-Akt pathways (35, 36, 42). Because the RAS-RAF-MEK-ERK pathway regulates normal cell cycle control, down-regulation of RASA1 causes an increase in cell proliferation. Aberrant signaling through the PI3K-AKT pathway activates a cascade of anti-apoptotic and pro-survival signals (43). In addition, RASA1 is suggested to play a probable role in angiogenesis and tumor progression (44). These downstream effects further support the hypotheses that miR-31

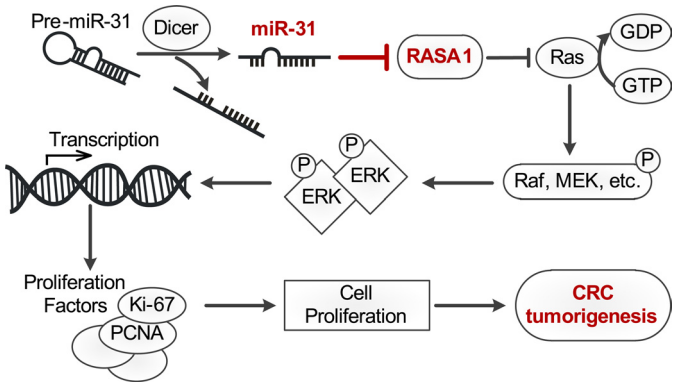

FIGURE 6. **Schematic of the entire signaling pathway described, including the interaction between miR-31 and RASA1, along with a brief description of the portion of the RAS-MAPK pathway mentioned in the article.**

functions as an oncogene in colorectal cancer and that up-regulation of miR-31 has the potential to drive tumor progression (Fig. 6).

That up to 54% of the total reported CRC cases and cell lines lack mutations in the RAS protein (45–48) implies there are other mechanisms leading to increased cell proliferation in colorectal cancer. Because RAS can be activated by either an activating mutation in the *RAS* gene or by mutations in genes that signal through RAS, and because our experiments were performed in the RAS GTPase wild-type colon cancer cell line HT-29, the miR-31 regulation of wild-type RAS protein could be an important factor leading to the aberrant activation of RAS signaling in colorectal cancer. This discovery adds to our understanding of aberrant RAS signaling activation in CRC cases that contain wild-type RAS protein.

The cellular influence of miR-31 through the regulation of the gene expression network implies possible therapeutic prospects. Since the initial discovery of a functional RNA interference (RNAi) system in mammals, significant efforts have been devoted to the development of therapeutics that utilize this system (49). Researchers are now focused on linking miRNAs with their targets, and with the RNAi theory to form a new therapeutic system. It has recently been shown that a novel class of chemically engineered oligonucleotides, termed "antagomirs," can effectively silence endogenous miRNAs *in vivo* (50). Other modified oligonucleotides, such as locked nucleic acid-modified oligonucleotides, can inhibit endogenous miRNAs, leading to up-regulation of the cognate target protein (31). These studies demonstrate powerful methods to silence miRNAs, which can be applied to abolish aberrant expression of miRNAs; such approaches have been already successfully applied in a murine liver cancer model and in chronic hepatitis C infection (51, 52).

However, a word of caution should be raised about unwanted effects given the multiplicity of miRNA targets (47, 53). This will be an important concern in the future when the goal is the restoration of the loss or gain of miRNA expression. The network of miRNAs and their targets is extremely complicated, as a single miRNA normally targets hundreds of targets, and a single protein can also be targeted by multiple miRNAs. Not until we have had a clearer overview of the complete network of miRNA-protein interactions can a wider variety of new therapies based on miRNAs be developed. A better understanding of the network of genes and cellular pathways regulated by miRNAs will undoubtedly enable us to understand tumor pathogenesis and therapy. To accomplish this, identifying the genome-wide targets of these miRNAs is essential.

Provided that tumor sizes were significantly reduced by inhibition of miR-31 in the nude mouse tumor model, it is indicated that the function of miR-31 targeting RASA1 in human colorectal tumorigenesis may shed light on new strategies for future colorectal tumor therapy, and we hope that miRNA-based therapeutics will become a reality in the near future.

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