miR-152 Is a Tumor Suppressor microRNA That Is Silenced by DNA Hypermethylation in Endometrial Cancer

Tomohiko Tsuruta1,4, Ken-ichi Kozaki1,3, Atsushi Uesugi1, Mayuko Furuta1,2, Akira Hirasawa4, Isssi Imoto1, Nobuyuki Susumu4, Daisuke Aoki4, and Johji Inazawa1,2,3

Abstract

The etiology and development of human cancers that remain little understood might be enlightened by defining tumor suppressor microRNAs (TS-miRNA). In this study, we identified TS-miRNAs silenced by aberrant DNA hypermethylation in endometrial cancer. Functional screening of 327 synthetic miRNAs in an endometrial cancer cell proliferation assay identified 103 miRNAs that inhibited cell growth. We then determined the sequence, DNA methylation status, and expression levels of these miRNAs in endometrial cancer cell lines and primary tumors. These determinations led to the identification of miR-152 as a candidate TS-miRNA gene in endometrial cancer. Epigenetic silencing documented in miR-152 was consistent with its location at 17q21.32 in intron 1 of the COPZ2 gene, which is also silenced often in endometrial cancer by DNA hypermethylation, and also with evidence that miR-152 targets the DNA methyltransferase DNMT1. Notably, restoration of miR-152 expression in endometrial cancer cell lines was sufficient to inhibit tumor cell growth in vitro and in vivo. We identified E2F3, MET, and Rictor as novel candidate targets of miR-152, suggesting how its epigenetic silencing can drive endometrial carcinogenesis. Our findings define a central role for miR-152 in endometrial cancer, and they also suggest its use in new therapeutic strategies to treat this cancer. Cancer Res; 71(20); 6450-62. ©2011 AACR.

Introduction

MicroRNAs (miRNA) are endogenous small nonprotein-coding RNAs of 19–22 nucleotides. These single-stranded RNAs are considered to play crucial roles in many normal cellular processes (1–4) and the multistep processes of carcinogenesis (5, 6). Among various mechanisms of cancer-related gene silencing in an epigenetic manner, DNA hypermethylation of CpG sites within CpG islands is known to lead to the inactivation of many tumor suppressor genes (TSG; ref. 7) and several tumor-suppressive miRNAs (TS-miRNA; ref. 8). Recently, in fact, DNA methylation–mediated downregulation of miRNAs by proximal CpG islands has been described by a number of groups, including ours (9–11), and further identification of remaining targets for methylation may clarify the specific molecular events involved in endometrial cancer progression, enabling the prevention, diagnosis, and treatment of endometrial cancer to be approached at a molecular level.

Endometrial cancer is the most common malignancy of the female genital tract, and is the fourth most common malignancy in women in the United States after breast, lung, and colon cancers. In 2009, it is estimated that 42,160 American women were diagnosed with endometrial cancer (6% of new cancer cases), and 7,780 women died of the disease (3% of all cancer deaths). Worldwide, endometrial cancer is the seventh most common cancer in women, being newly diagnosed in an estimated 226,000 women in 2007 (12). Endometrial cancer is generally considered to arise through the progressive accumulation of multiple genetic abnormalities, which may activate oncogenes and inactivate TSGs. Recently, genome-wide screenings of altered DNA methylation for exploring endometrial cancer–associated TSGs have been reported (13), though there are few reports about epigenetic alterations of miRNA genes in endometrial cancer.

Here, we identified a novel TS-miRNA frequently silenced through tumor-specific DNA methylation in endometrial cancer and its multiple targets by function-based screening with a cell proliferation assay for 327 synthetic miRNAs in HEC-1A cell line and an approach with a series of sequential analyses of DNA methylation and expression analysis in endometrial cancer cell lines and primary cases. The function-based screening makes it possible to analyze the biological effects of a large number of double-stranded RNAs (dsRNA) on cancer cells directly. In addition, this approach has already proved successful in the exploration of dsRNAs having oncogenic or tumor-suppressive effects on cancer cells (14, 15).
Consequently, a possible endometrial cancer–associated TS-miRNA, miR-152, and novel candidates for its putative targets were identified in our study. This study is the first to show clearly that the tumor-suppressive activity of miR-152 is epigenetically silenced in endometrial cancer and that its multiple targets are E2F3, MET, and Rictor.

Materials and Methods

Cell lines and primary tumor samples
A total of 13 human endometrial cancer cell lines—HEC-1, HEC-1A, HEC-1B, HEC-50B, HEC-59, HEC-108, HEC-151, HEC-251, HEC-265, HHUA, HOOUA, Ishikawa, and SNM—were obtained from RIKEN BioResource Center, and authenticated at our laboratory by in-house BAC/PAC-based arrays for array-CGH analyses (unpublished data) in December 2009. All cell lines were maintained in Dulbecco’s modified Eagle’s medium, supplemented with streptomycin (100 μg/mL), penicillin (100 units/mL), 2 mmol/L glutamine, and 10% FBS. To analyze the restored expression of genes of interest, cells were cultured with or without 10 μmol/L of 5-aza 2’-deoxycytidine (5-aza-dCyd) for 5 days. A total of 70 frozen primary samples were obtained from endometrial cancer patients (stage I, 38 cases; stage II, 8 cases; stage III, 21 cases; and stage IV, 3 cases; Supplementary Table S1) and 6 normal endometria from patients with endometriosis or leiomyoma treated at KEIO University with written consent from each patient and after approval by the local ethics committee. The International Federation of Gynecology and Obstetrics (FIGO) classification was used.

Transfection with synthetic miRNAs and siRNAs
Endometrial cancer cells were seeded at 10,000 cells per well in 24-well plates the day before transfection. dsRNA (10 nmol/L) mimicking human mature miRNAs or control nonspecific miRNA (Ambion; Thermo Scientific Dharmacon) and 20 nmol/L of Stealth RNAi siRNA for Rictor or control nonspecific siRNA (Invitrogen) were transfected individually into endometrial cancer cells, using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. The suitable concentration of dsRNAs was decided on the basis of the result of in vitro cell proliferation assay beforehand. Representative results of those were shown in Supplementary Fig. S1A and B. The function-based screening was done by PremiR miRNA Precursor Library-Human V2 (Ambion) in duplicate. The numbers of viable cells were assessed by the colorimetric water-soluble tetrazolium salt (WST) assay (Cell counting kit-8; Dojindo Laboratories). Results were normalized to the cell numbers among control cells transfected with nonspecific miRNA. The cell cycle was evaluated by a fluorescence-activated cell sorting (FACS) analysis as described elsewhere (16).

Methylation analysis
Each gene and CpG island was searched in miRBase database (release April 17, 2011; ref. 17), UCSC Genome Browser on Human February 2009 Assembly (hg19; ref. 18), and PubMed (19). Genomic DNA was treated with sodium bisulfite and subjected to PCR, using primer sets designed to amplify regions of interest (Supplementary Table S2). The combined bisulfite restriction analysis (COBRA) and the bisulfite-sequencing analysis were carried out as described elsewhere (9).

Real-time reverse transcription PCR
Real-time reverse transcription PCR (RT-PCR) was carried out as described elsewhere (9). Relative expression levels of miRNAs and a host gene were quantified in comparison with RNU6B and GAPDH mRNA, respectively.

In vivo analysis of effects of synthetic miRNAs on tumor growth in SCID mice
Four-week-old female SCID mice were purchased from the Oriental Yeast Co. Ltd and maintained under specific pathogen-free conditions. A total of 1.0 × 10⁷ cells in 100 μL of Matrigel (BD Biosciences) were injected in the subcutaneous (s.c.) tissue of the left abdominal wall of 7-week-old SCID mice. Tumor-bearing SCID mice treated with 1 nmol of dsRNAs (Ambion) in 100 μL of AteroGene (KOKEN) were administrated into the s.c. spaces around the tumors 3 times every week from day 14. At the end of the experiment, mice were sacrificed by cervical dislocation under deep anesthesia, and the resected s.c. tumors were weighed.

miRNA target predictions, Western blotting, and luciferase activity assay
Predicted targets for candidate miRNAs and their target sites were analyzed by using miRanda (20), TargetScan (21), and PicTar (22).

The protein levels of predicted targets in transfectants were analyzed by Western blotting, using anti-Akt, anti-phospho-Akt (Ser-473), anti-MET, anti-PCNA, anti-Rictor (Cell Signaling Technology), anti-E2F3 (Abcam), anti-DNMT1 (Santa Cruz Biotechnology) rabbit polyclonal antibodies, and anti-β-actin monoclonal antibodies (Sigma).

Luciferase constructs were made by ligating oligonucleotides containing the 3’-UTR target sites downstream of luciferase gene in pMIR-REPORT luciferase vector (Ambion). Luciferase activities were measured as described elsewhere (9).

Statistical analysis
Differences between subgroups were tested with the Mann–Whitney U test.

Results

Function-based screening of TS-miRNAs in endometrial cancer cell lines
To identify TS-miRNAs silenced by DNA hypermethylation in endometrial cancer, we first conducted function-based screening, in which the proliferation-inhibitory effect was made an index, using HEC-1A cell line and 327 synthetic miRNAs at 10 nmol/L. The strategy and partial results of this study are shown in Figure 1A. Relative cell growth ratios in Figure 1B and Supplementary Table S3 indicate effects of each miRNA 5 days after transfection. In this first screening,
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Figure 1. HEC-1A cell line. A, strategy for identifying epigenetically silenced TS-miRNAs in endometrial cancer. B, results of function-based screening of TS-miRNAs in HEC-1A cells, using Pre-miR miRNA Precursor Library-Human V2 (Ambion). The closed arrow at the bottom indicates 327 miRNAs examined in this screening, and the closed arrow at the top indicates 103 miRNAs which showed marked growth inhibitory effects (growth ratio > 60%); Supplementary Table S2). C, summary of DNA methylation status of CpG islands around 32 mature sequences of miRNAs located at 37 loci in 13 endometrial cancer cell lines and 6 normal endometria determined by COBRA. PCR products used for COBRA were digested with BstU1, TagI, or HfII (Fig. 2A; Table 1; Supplementary Table S3), and electrophoresed (Fig. 2B; Supplementary Fig. S2B; data not shown). Black, gray, white, and slant boxes indicate complete, partial, no digestion, and not determined, respectively. EC, endometrial cancer.
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<th>Locus</th>
<th>Host gene</th>
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NOTE: These data were searched in miRBase database (release April 17, 2011), UCSC Genome Browser on Human February 2009 Assembly (hg19), and PubMed. Abbreviation: ND, not determined.
103 miRNAs, including known TS-miRNAs such as miR-124 (23), miR-126 (24), miR-137 (9), and miR-491 (14), showed remarkable inhibitory effects on cell growth (relative growth ratio < 0.5). Then we enrolled miRNAs with CpG islands in the 5' upstream region and excluded miRNAs previously reported to show a correlation between DNA hypermethylation and tumor-suppressive activity. Consequently, 32 miRNAs emerged as candidates for new TS-miRNAs silenced by DNA hypermethylation in endometrial cancer cell lines (Table 1).

**Methylation and expression analyses of candidates in endometrial cancer cell lines and primary cases**

Next, we explored DNA methylation status of CpG islands around 32 mature sequences of miRNAs located at 37 loci in a panel of 13 endometrial cancer cell lines and 6 normal endometria determined by COBRA (Fig. 1C). As multiple copies of some mature miRNAs, as listed in Table 1, are transcribed from different loci, the number of mature forms of miRNAs is smaller than the number of genomic loci. On the basis of a comparison of mean methylation frequencies in each region examined, frequent DNA hypermethylation (>60% of endometrial cancer lines) was found in only 3 of 32 miRNAs, that is, miR-9 (96.9%), miR-152 (87.2%), and miR-149 (61.5%), although a few normal endometria were found to have hypermethylated CpG islands on/around these miRNAs (Fig. 2A; Supplementary Fig. S2A and B). Notably, DNA hypermethylation within region 1 in miR-9-1 (1q22), region 1 and 3 in/around miR-9-3 (15q26.1), and region 3 in miR-152 (17q21.32) was observed in all endometrial cancer cell lines. Therefore, we focused on these 3 miRNAs. We next determined the correlation between the DNA methylation status of these miRNAs and their expression patterns in 13 endometrial cancer cell lines and 6 normal endometria. Frequencies of translational downregulation of miR-9, miR-152, and miR-149 in endometrial cancer cell lines (<0.5-fold expression) were 61.5% (8 of 13), 100% (13 of 13), and 15.4% (2 of 13), respectively (Fig. 2A; Supplementary Fig. S2C and D), whereas 3 miRNAs were detected in all normal endometria. Furthermore, the expressions of miR-9 and miR-152 were also restored by demethylation with 10 μmol/L 5-aza-dCyd at a high frequency in endometrial cancer cell lines lacking their expressions (Fig. 2A; Supplementary Fig. S2A and B), strongly suggesting that DNA methylation around CpG islands on miR-9 and miR-152 suppressed their expressions in endometrial cancer cell lines. Additionally, as shown in Table 2, the downregulation of miR-9, miR-152, and miR-149 expression correlated with DNA hypermethylation in 61.5% (8 of 13), 100% (13 of 13), and 0% (0 of 9) of endometrial cancer cell lines, respectively. These findings indicate that miR-152 is the only miRNA showing a complete consistency in the correlation between DNA methylation status and expression pattern in a panel of endometrial cancer cell lines.

To determine the correlation between DNA methylation status and expression patterns of miR-152 in 70 primary endometrial cancer tumors, we carried out COBRA and real-time RT-PCR analysis, respectively. In these analyses, aberrant DNA methylation in region 3 containing pre-miR-152
and deregulation of its expression were detected in 92.9% (65 of 70) and 97.1% (68 of 70) of endometrial cancer cases, respectively, and consistency in DNA hypermethylation of region 3 and silencing of \textit{miR-152} expression was found in 100.0% (65 of 65) of endometrial cancer cases (Fig. 2B), whereas such consistencies found in \textit{miR-152} were not observed in \textit{miR-9} (Table 2; Supplementary Fig. S3A and B).

Moreover, both tumor-specific DNA hypermethylation and downregulated expression of \textit{miR-152} were observed in 66.7% (2 of 3) of paired samples from primary endometrial cancer tumors and their corresponding normal endometria (Fig. 2C). The DNA hypermethylation of this CpG island was also confirmed by bisulfite sequencing of selected positive cell lines and cases (Fig. 2D). These results suggest that \textit{miR-152} is the most likely TS-miRNA frequently silenced through tumor-specific hypermethylation in endometrial cancer. Thus, hereafter we focused on \textit{miR-152} as a prime candidate.

Tumor-suppressive effects of ectopic \textit{miR-152} expression on tumor cell growth \textit{in vitro} and \textit{in vivo}

To confirm the effects of \textit{miR-152} on tumor cell growth, we introduced the dsRNA mimicking mature \textit{miR-152} into...
endometrial cancer cell lines, HEC-1 and HEC-1A, lacking its expression, in vitro and in vivo. Although miR-152 overexpression significantly inhibited cell growth in vitro, potential dose-dependent effects of this dsRNA on cell proliferation in vitro were not observed in these cell lines (Supplementary Fig. S1A). On the contrary, expression levels of miR-152 were remarkably increased in endometrial cancer cells transfected with this dsRNA (<0.005 nmol/L) as compared with untreated endometrial cancer cells and normal endometria (Supplementary Fig. S1B). Then 2 synthetic miRNAs purchased from Ambion and Thermo Scientific Dharmacon were used in consideration of off-target effects of dsRNAs. Consistent with the results of function-based screening, restoring miR-152 expression significantly reduced cell proliferation in all endometrial cancer cell lines tested (Fig. 3A), confirming the tumor-suppressive function of miR-152; note that a large number of miR-152 transfectants were rounded and floating compared with their control counterparts (Fig. 3A). Because such apoptotic changes were more remarkable in HEC-1 than HEC-1A under the phase-contrast microscope at 5 days after transfection, we carried out FACS analysis and Western blot analysis for caspase-mediated apoptosis, using endometrial cancer cell lines 72 hours after the transfection. In FACS analysis, over-expression of miR-152 induced the accumulation of cells in sub-G1 phase or G2–M phase to HEC-1 or HEC-1A cell lines, respectively (Fig. 3A). The results of Western blotting, moreover, showed that the ectopic expression of miR-152 remarkably increased levels of caspase-3, cleaved caspase-3, and cleaved PARP in HEC-1 cells, whereas only caspase-3 was increased in HEC-1A (Fig. 3B). These findings suggest miR-152 to be associated with cell cycle arrest at both the G1–S and G2–M checkpoints in endometrial cancer cells, leading us to further investigate the potential of dsRNA mimicking miR-152 as a therapeutic agent. Therefore, we examined whether miR-152 could suppress tumor growth in vivo, and consequently significant reduction of s.c. tumors of HEC-1 cells could be observed by treatment with miR-152 (Fig. 3C).

Table 2. Frequencies of endometrial cancer cell lines and primary cases, in which DNA hypermethylation accorded with downregulation of miRNA expression

<table>
<thead>
<tr>
<th>miRNA gene</th>
<th>Region in COBRA</th>
<th>endometrial cancer cell lines</th>
<th>endometrial cancer cases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Methylation frequency (%)</td>
<td>Consistency of methylation with downregulation (%)</td>
<td>Methylation frequency (%)</td>
</tr>
<tr>
<td>miR-9-1</td>
<td>1 100.0 (13/13)</td>
<td>61.5 (8/13)</td>
<td>60.0 (42/70)</td>
</tr>
<tr>
<td>miR-9-3</td>
<td>1 100.0 (13/13)</td>
<td>61.5 (8/13)</td>
<td>84.3 (59/70)</td>
</tr>
<tr>
<td>miR-9-3</td>
<td>3 100.0 (13/13)</td>
<td>61.5 (8/13)</td>
<td>35.7 (25/70)</td>
</tr>
<tr>
<td>miR-152</td>
<td>3 100.0 (13/13)</td>
<td>100.0 (13/13)</td>
<td>92.9 (65/70)</td>
</tr>
<tr>
<td>miR-149</td>
<td>1 69.2 (9/13)</td>
<td>0.0 (0/9)</td>
<td>ND</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.

∗Frequency of cell lines or primary cases, in which DNA hypermethylation were detected by COBRA (Figs. 1C, 2B and E; Supplementary Figs. S2 and S3).

†Frequency of cell lines or primary cases, in which downregulation was consistent with DNA hypermethylation (Fig. 2C and F; Supplementary Figs. S2 and S3).

Coexpression of intronic miRNA, miR-152, and its host gene, COPZ2

Interestingly, the miR-152 gene is located at 17q21.32 and within intron 1 of the coatomer protein complex, subunit zeta 2 (COPZ2) gene (Fig. 2A). In real-time PCR analysis using endometrial cancer cell lines and primary cases, the expression pattern of these 2 transcripts seemed to be similar (Fig. 2A and B); note that the frequencies of downregulation of COPZ2 in endometrial cancer cell lines and primary cases (<0.5-fold expression) were 100% (13 of 13) and 73.7% (53 of 70), respectively. The correlation between the DNA methylation status of COPZ2 in region 3 in COBRA and its expression patterns was 100% (13 of 13) in endometrial cancer cell lines and 75.4% (49 of 65) in endometrial cancer cases. To investigate the relationship between DNA hypermethylation in this CpG island and the downregulation of miR-152 and COPZ2 expression, we treated endometrial cancer cell lines with 5-aza-dCyd. The expression of intronic miR-152 gene and the host gene, COPZ2, was restored to a similar pattern in endometrial cancer cell lines 5 days after treatment with 5-aza-dCyd (concordance rate at 2-fold increased restoration between downregulation and restoration of COPZ2 expression = 69.2%, 9 of 13; Fig. 2A), strongly suggesting that DNA methylation around CpG islands on miR-152 also suppressed the expression of COPZ2 in cell lines. To determine whether COPZ2, as well as miR-152, had a potent tumor-suppressive function, we also carried out colony-formation assays using the full coding sequence of COPZ2 with Myc-tags cloned into a mammalian expression vector (pCMV-3Tag4A-COPZ2) and 2 endometrial cancer cell lines lacking the expression of COPZ2 (Supplementary Fig. S4A). However, the number of large colonies produced by COPZ2-transfected HEC-1 cells significantly decreased compared with those produced by empty vector-transfected counterparts 2 weeks after the transfection and subsequent selection of drug-resistant colonies, such findings were not observed in COPZ2-transfected HEC-1A cells (Supplementary Fig. S4B). Further detailed analyses will be necessary to prove that COPZ2 is a TSG.
Screening of predicted targets for miR-152 in endometrial cancer cells

To explore oncogenic targets of miR-152 in endometrial cancer cells, we searched for potential genes involved in tumorigenesis, using algorithms such as miRanda, TargetScan, and Pictar, and identified DNA methyltransferase 1 (DNMT1), E2F transcription factor 3 (E2F3), MET, and rapamycin-insensitive companion of mTOR (Rictor). DNMT1 had
already been described as a target of miR-152 (25). We first carried out Western blot analysis of these predicted targets in HEC-1 and HEC-1A cells 48 hours after the transfection of 2 kinds of dsRNAs mimicking miR-152 (Fig. 4A), and observed that protein levels were reduced in the transfectants compared with their control counterparts, although the reductions were not remarkable. To further determine whether the predicted target sites for miR-152 in the 3'UTR of the mRNAs of DNMT1, E2F3, MET, and Rictor (Fig. 4B) were responsible for the translational regulation by dsRNA, we next carried out luciferase assays with vectors containing these 3'UTR target sites downstream of the luciferase reporter gene. The reporter construct containing the miR-152 binding site within the 3'-UTR of DNMT1 was used as a positive control. In endometrial cancer cells transfected with dsRNAs mimicking miR-152 purchased from Ambion, a statistically significant reduction in luciferase activity was observed in DNMT1, MET, and Rictor, but

![Diagram A](Image)

Figure 4. Screening of predicted targets of miR-152. A, the results of Western blotting of predicted targets for miR-152 in endometrial cancer cell lines 48 hours after cotransfection of miR-REPORT luciferase vectors containing wild type (Wt) or mutated (Mut) 3'-UTR target sites of DNMT1, E2F3, MET, and Rictor for miR-152, dsRNA mimicking miR-152 or control nonspecific miRNA (Ambion), and pRL-CMV internal control vector using Lipofectamine 2000 (Invitrogen). *, P < 0.05 versus vehicle-treated cells, statistical analysis with the Mann–Whitney U test. Right, putative binding site of miR-152 in the 3'-UTR region of their targets. These target sites were analyzed by miRand (20), TargetScan (21), and PicTar (22). C, comparison of Rictor protein expression levels and AKT activation in endometrial cancer cell lines by Western blot analysis. Western blot analysis for the phosphorylation of AKT at Ser-473 in endometrial cancer cell lines 48 hours after the transfection of 2 kinds of dsRNA mimicking miR-152 or control nonspecific miRNA (left) and 2 kinds of Rictor-specific siRNA (si-Rictor #1 and si-Rictor #2; Sigma) or control nonspecific siRNA (right). To determine the increased phosphorylation of AKT, total AKT levels in the same samples were evaluated. The quantification of each protein band was done by using LAS-3000 with MultiGauge software (Fujifilm). D, effects of downregulation of Rictor protein expression on cell proliferation in endometrial cancer cell lines. Growth curves (top) and phase-contrast micrographs (bottom) of HEC-1 and HEC-1A cells in which 20 nmol/L of si-Rictor #1, si-Rictor #2, or nonspecific siRNA was transfected. The numbers of viable cells after transfection were assessed by WST assay. Each data point represents the mean of triplicate determinations (bars, SD) in these experiments. *, P < 0.05; **, P < 0.01, statistical analysis with the Mann–Whitney U test. Phase-contrast micrographs show endometrial cancer cells 5 days after transfection.
not in their mutant constructs (Fig. 4B), although synthetic miR-152 purchased from Thermo Scientific Dharmacon markedly decreased the luciferase activities in all of 4 targets, including E2F3 (Supplementary Fig. S5). On the contrary, expressions of DNMT1, E2F3, MET, and Rictor were upregulated in 47.1% (33 of 70), 28.6% (20 of 70), 1.5% (1 of 70), and 20% (14 of 70) of endometrial cancer cases, respectively (Supplementary Fig. S6). Taken together, these findings suggested that DNMT1 and Rictor might be the most likely targets for miR-152 in endometrial cancer.

Rictor, together with the mTOR kinase, forms mTOR complex 2 (mTORC2), and the Rictor–mTOR complex directly regulates the phosphorylation of Akt at Ser-473, resulting in cell growth (26). To investigate whether Rictor participates in the growth inhibitory effects of miR-152 in endometrial cancer cells, we analyzed the phosphorylation of Akt and proliferation in HEC-1 and HEC-1A cells transfected with or without 2 dsRNAs mimicking miR-152 or 2 specific siRNAs for Rictor. Levels of Rictor protein and phosphorylation of Akt at Ser-473 were weakly decreased in HEC-1A cells 48 hours after the transfection of miR-152 compared with those in the control transfectants, whereas levels of phosphorylated Akt showed no reduction in HEC-1 cells (Fig. 4C). The transient transfection of specific siRNA for Rictor clearly decreased levels of the transcripts (data not shown) and protein of Rictor (Fig. 4C) and inhibited cell growth (Fig. 4D) in both cell lines. Forty-eight hours after the knockdown of Rictor, similar to the results for miR-152, a marked reduction in the phosphorylation of Akt was detected in HEC-1A cells, but not in HEC-1 cells (Fig. 4C), suggesting Akt to be significantly activated and phosphorylated by another pathway, for example, phosphatidylinositol 3-kinase (PI3K)-Akt signaling, different from TOR-Akt signaling for the silencing of miR-152 through DNA hypermethylation in HEC-1A cells.

Discussion

In this study, we carried out function-based screening by using 327 dsRNA mimicking mature human miRNAs as the first screening of TS-miRNAs having remarkable inhibitory effects on the growth of endometrial cancer cell lines although expression-based or DNA methylation–based screening had been successfully performed in our previous studies in oral squamous cell carcinoma (9) and hepatocellular carcinoma (10). Actually, several known TS-miRNAs, such as miR-124 (10, 23), miR-126 (27), miR-137 (9), and miR-491 (14), were identified through similar screening processes, strongly suggesting the function-based approach presented here to be a powerful tool for exploring dsRNAs having tumor-suppressive effects, including TS-miRNAs and siRNAs, as therapeutic agents for several types of cancer cells. The tumor-suppressive function of a candidate miRNA eventually identified in our study was reexamined by using 2 kinds of dsRNA purchased from independent companies to take account of off-target effects by dsRNA. Although such effects have been known to complicate the interpretation of phenotypic effects in gene-silencing experiments using siRNAs (28), dsRNAs mimicking miRNAs can potentially cause these unwanted actions. These unpredictable target-independent effects should be addressed during data interpretation in all dsRNA-based studies related with functional genomics, drug target discovery and validation, and dsRNA therapeutics. However, dsRNAs including miRNAs and siRNAs have commonly been used so far at 1.0 to 50.0 nmol/L in dsRNA-based studies, remaining the possibility that overexpressions above their physiologic conditions may lead to biologically toxic effects.

We have considered that the hypermethylation of CpG islands on/around miRNA genes is a good marker to explore novel epigenetically silenced TS-miRNAs, similar to classic TSGs in several types of human cancers, and have already reported miR-137, miR-193a, miR-124, and miR-203 as TS-miRNAs (9, 10). This study showed that the second screening, combining DNA methylation and expression analyses in endometrial cancer cell lines and primary cases, resulted in the identification of miR-152 as a prime candidate for a TS-miRNA silenced by DNA hypermethylation in endometrial cancer. Because tumor-suppressive effects of miR-152 have not been described to date, we carried out detailed analyses of miR-152. Recently, in MLL-rearranged acute lymphoblastic leukemia, aberrant DNA methylation of miR-152 CpG islands was reported to correlate with a poor clinical outcome (29). Altered expression of miR-152 was shown in gastrointestinal cancer (30) and cholangiocarcinoma (25). Our data showed that DNA hypermethylation and downregulation of miR-152 were frequently observed in endometrial cancer cell lines and primary tumors, and that treatment with 5-aza-dCyd restored the expression of miR-152 in endometrial cancer cell lines lacking it. In addition, the correlation between DNA methylation status around miR-152 and expression pattern was 100% (13 of 13) in a panel of 13 endometrial cancer cell lines and 100% (65 of 65) in endometrial cancer cases, suggesting that DNA hypermethylation around the CpG island of miR-152 seemed to deregulate its expression in endometrial cancer. Moreover, we successfully showed for the first time that dsRNA mimicking miR-152, in its administration with atelo-collagen for the drug delivery in mice, suppressed in vivo growth of an endometrial cancer cell line. The finding led us to consider the possibility that miR-152 might have some benefit as therapeutic agents. This miRNA is located at 17p21.32 in intron 1 of the host gene COPZ2. COPZ2 was described as a coat protein (COP) and to form part of a COP1 complex which transports vesicles from the Golgi apparatus to endoplasmic reticulum (31). In addition, a recent study showed that COP1 was required for early endosomal maturation in autophagy, whereas the biological function of COPZ2 remains uncharacterized (32). Our data showed that DNA hypermethylation–mediated silencing of miR-152 and COPZ2 was a relatively frequent molecular event in endometrial cancer and inhibited cell growth in endometrial cancer cell lines, suggesting the epigenetic silencing of these genes to contribute to endometrial carcinogenesis, although further experiments will be needed to confirm that the coexpression has tumor suppressor effects in this disease.

In this study, we successfully identified possible direct targets of miR-152 other than DNMT1 (25), such as E2F3,
MET, and Rictor (Supplementary Fig. S7). E2F3 is a transcriptional repressor of the E2F family, and its potential oncogenic capacity was also shown recently (33). MET is already well known as an oncogene and the cell-surface receptor for hepatocyte growth factor (34). These previous studies as well as our own results suggest that the altered expression of these targets caused by the downregulation of miR-152 expression through CpG island hypermethylation may contribute to the tumorigenesis through the activation of target oncogenic pathways in endometrial cancer. Rictor is a component of the mTOR-containing complex, mTORC2, which directly regulates the phosphorylation of Akt at Ser-473 (26), and has already been reported the correlation with the molecular mechanisms in cancer cells (35, 36). These results including our findings support the hypothesis that the silencing of miR-152 through CpG methylation may contribute to carcinogenesis and cancer progression at least partly through the activation of mTORC2-Akt signaling in endometrial cancer. However, knockdown of Rictor was reported to induce G1 arrest in the breast cancer cell line MCF7 and prostate cancer cell line PC3 (37), and inhibition of the Akt-mTOR pathway was shown to induce G2/M arrest and autophagy in the breast cancer cell line MDA-MB-231 (38). On the contrary, we showed here that ectopic miR-152 expression reduced cell proliferation and induced the accumulation of cells into sub-G1 phase or G2/M phase in HEC-1 or HEC-1A cell lines, respectively. Cell cycle progression of miR-152 transfectants may be affected by its targets other than Rictor, considering that miRNAs regulate target gene expression through imperfect pairing with target messenger RNAs. Although Akt signaling pathways were also described to play a key role in both G1–S and G2/M transitions (39), these differences in effects on cell cycle progression remain largely unknown.

In conclusion, we describe here the identification of a TS-miRNA, miR-152, frequently silenced by DNA hypermethylation in endometrial cancer, using function-based screening and a series of sequential analyses. Moreover, we identified E2F3, MET, and Rictor as potential targets of miR-152, suggesting that the epigenetic silencing of miR-152 and consequent activation of its multiple targets may contribute to endometrial carcinogenesis, and that miR-152 and targeting E2F3, MET, and Rictor may provide a novel treatment strategy for endometrial cancer, although further studies in vivo will be needed to confirm that dsRNA mimicking miR-152 can work as a TS-miRNA in this disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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miR-152 Is a Tumor Suppressor microRNA That Is Silenced by DNA Hypermethylation in Endometrial Cancer

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