The tumor-suppressive *microRNA-1/133a* cluster targets *PDE7A* and inhibits cancer cell migration and invasion in endometrial cancer

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Abstract. In developed countries, endometrial cancer (EC) is the most common malignancy among women. Unopposed estrogen therapy, obesity, nulliparity, diabetes mellitus and arterial hypertension have been linked to an increased risk of EC. However, the molecular mechanisms of EC oncogenesis and metastasis have not yet been fully elucidated. Our recent studies of microRNA (miRNA) expression signatures revealed that the *microRNA-1/133a* (*miR-1/133a*) cluster is frequently downregulated in various types of human cancers. However, the functional role of the *miR-1/133a* cluster in EC cells is still unknown. Thus, the aim of this study was to investigate the functional significance of the *miR-1/133a* cluster and its regulated molecular targets, with an emphasis on the contributions of *miR-1/133a* to EC oncogenesis and metastasis. We found that the expression levels of *miR-1* and *miR-133a* were significantly reduced in EC tissues. Moreover, restoration of mature *miR-1* or *miR-133a* miRNAs significantly inhibited cancer cell migration and invasion, suggesting that these clustered miRNAs act as tumor suppressors. Prediction of miRNA targets revealed that phosphodiesterase 7A (*PDE7A*) was a potential target gene regulated by both *miR-1* and *miR-133a*. *PDE7A* was confirmed to be overexpressed in EC clinical specimens and silencing of *PDE7A* significantly inhibited cancer cell migration and invasion. Our data demonstrated that downregulation of the *miR-1/133a* cluster promoted cancer cell migration and invasion via overexpression of *PDE7A* in EC cells. Elucidation of the molecular networks regulated by tumor-suppressive miRNAs will provide insights into the molecular mechanisms of EC oncogenesis and metastasis.

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Introduction

In developed countries, endometrial cancer (EC) is the most common malignancy among women, accounting for ~25% of all deaths related to cancer of the female genital tract (1). Unopposed estrogen therapy, obesity, nulliparity, diabetes mellitus and arterial hypertension have been linked to an increased risk of ECs (2). ECs are clinicohistologically classified into two subgroups: type I and type II. Type I tumors, which account for ~80% of all cases, are estrogen-dependent, low-grade tumors, while type II tumors are more aggressive and exhibit invasion into the myometrium (3,4). Currently, there is a lack of effective treatments for patients with advanced stage and recurrent EC (5); thus, more effective treatment strategies based on genomic data are needed.

In the post-genome sequencing era, the discovery of non-coding RNA (ncRNA) has been a conceptual breakthrough in cancer research fields (6). For example, microRNAs (miRNAs) are small ncRNA molecules (19-22 bases in length) that function to regulate the expression of multiple protein-coding genes by repressing translation or cleaving RNA transcripts in a sequence-specific manner (7,8). Bioinformatic predictions indicate that miRNAs regulate 30-60% (or more) of the protein-coding genes in the human genome. Numerous studies have reported that various miRNAs are aberrantly expressed in many types of human cancers, affecting the development and metastasis of cancers through oncogenic or tumor-suppressive functions (9,10).

Elucidation of cancer-related miRNA networks has provided important new information about human cancers. In our previous studies, we used our miRNA expression signatures to investigate several tumor-suppressive miRNAs and their regulated cancer pathways. We recently showed that *miR-1/133a* clustered miRNAs are significantly downregulated in several cancer tissues (11-14). From our miRNA signatures, we have sequentially reported functional roles of the *miR-1/133a* cluster and the molecular targets/pathways

regulated by these miRNAs. However, the contributions of these miRNAs in EC cells have not been fully elucidated.

The aim of the present study was to investigate the functional significance of the *miR-1/133a* cluster and to identify the molecular targets regulated by these miRNAs in EC cells. We found that restoration of mature *miR-1* or *miR-133a* in EC cells significantly inhibited cell migration and invasion. Gene expression data and *in silico* analysis demonstrated that phosphodiesterase 7A (*PDE7A*), an enzyme that hydrolyzes intracellular cAMP, was a potential target of the *miR-1/133a* cluster. Elucidation of the cancer-related signaling pathways and targets regulated by the tumor-suppressive *miR-1/133a* cluster will provide new insights into the potential mechanisms of EC oncogenesis and metastasis.

Materials and methods

Clinical specimens. A total of 27 primary EC specimens were collected from patients who had undergone surgical treatment at Chiba University Hospital. Eight non-cancer endometrial specimens were obtained from patients who underwent total hysterectomy because of other gynecologic diseases (Table I). The samples were processed and stored in liquid nitrogen until RNA extraction. Our study was approved by the Bioethics Committee of Chiba University; prior written informed consent and approval was given by each patient.

Cell lines and cell culture. Hec1B cells (derived from endometrioid adenocarcinoma G1) and Hec265 cells (derived from endometrioid adenocarcinoma G2) were used in this analysis. Hec1B cells were grown in E-MEM medium supplemented with 10% fetal bovine serum. Hec265 cells were grown in E-MEM medium supplemented with 15% fetal bovine serum. All cells were cultured at 37˚C in a humidified atmosphere containing 5% CO_2.

Table I. Characteristics of endometrial cancer specimens and non-cancer specimens.

	Sample no.
(a) Endometrial cancer	
Total no.	27
Median age, years (range)	58 (39-80)
Pathological tumor stage (UICC7th)	
1A	13 (48.1%)
1B	5 (18.5%)
2	1 (3.7%)
3A	2 (7.4%)
3B	1 (3.7%)
3C1	2 (7.4%)
3C2	2 (7.4%)
4	1 (3.7%)
Differentiation	
G1	7 (25.9%)
G2	10 (37.0%)
G3	10 (37.0%)
Lymphatic metastasis	
(+)	4 (14.8%)
(-)	20 (74.1%)
Unknown	3 (11.1%)
(b) Normal endometrium	
Total no.	8
Median age, years (range)	41 (34-76)

RNA isolation. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA concentrations were determined spectrophotometrically. RNA quality was confirmed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). First-strand cDNA was synthesized from 1 μg of total RNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Gene-specific PCR products were assayed continuously using a 7300-HT Real-Time PCR system according to the manufacturer's protocol. The initial PCR step consisted of a 10-min hold at 95˚C, followed by 40 cycles consisting of denaturation for 15 sec at 95˚C and annealing/extension for 1 min at 60˚C.

The expression levels of *miR-1* (assay ID: 002222) and *miR-133a* (assay ID: 0002246) were analyzed by TaqMan qPCR (TaqMan MicroRNA assay; Applied Biosystems) and normalized to *RNU48* (assay ID: 001006). All reactions were performed in duplicate. TaqMan probes and primers for *PDE7A* (P/N: Hs00300285_m1), *DDX3X* (P/N: Hs00606179_m1), *CORO1C* (P/N: Hs00170938_m1), *SPTBN1* (P/N: Hs00162271_m1) and *GUSB* (P/N: Hs00939627_m1; used as an internal control) were obtained from Applied Biosystems (Assay-On-Demand Gene Expression Products). All reactions were performed in triplicate and included negative control reactions that lacked cDNA. The ΔΔCt method was adopted and applied to calculate the relative quantities of target genes.

Transfections with mature miRNA and small-interfering RNA (siRNA). Cells were transfected with 10 nM mature miRNA or siRNA molecules using Lipofectamine RNAiMAX transfection reagent (Invitrogen) and Opti-MEM (Invitrogen). The following RNA species were used in this study: mature miRNA, Pre-miR™ miRNA Precursors (*hsa-miR-1*; P/N: PM10617, *hsa-miR-133a*; P/N: PM10413; Applied Biosystems), negative control miRNA (P/N: AM17111; Applied Biosystems), siRNA (Stealth siRNAs, si-*PDE7A*, P/N: HSS107737 and HSS107739; Invitrogen) and negative control siRNA (Stealth RNAi Negative Control Med GC, P/N: 12935-300; Invitrogen).

Cell proliferation, migration and invasion assays. For cell proliferation assays, cells were transfected with 10 nM miRNA or siRNA by reverse transfection and plated in 96-well plates at $3x10^3$ cells per well. After 72 h, cell proliferation was determined with XTT assays using a Cell Proliferation Kit II (Roche Applied Science, Tokyo, Japan).

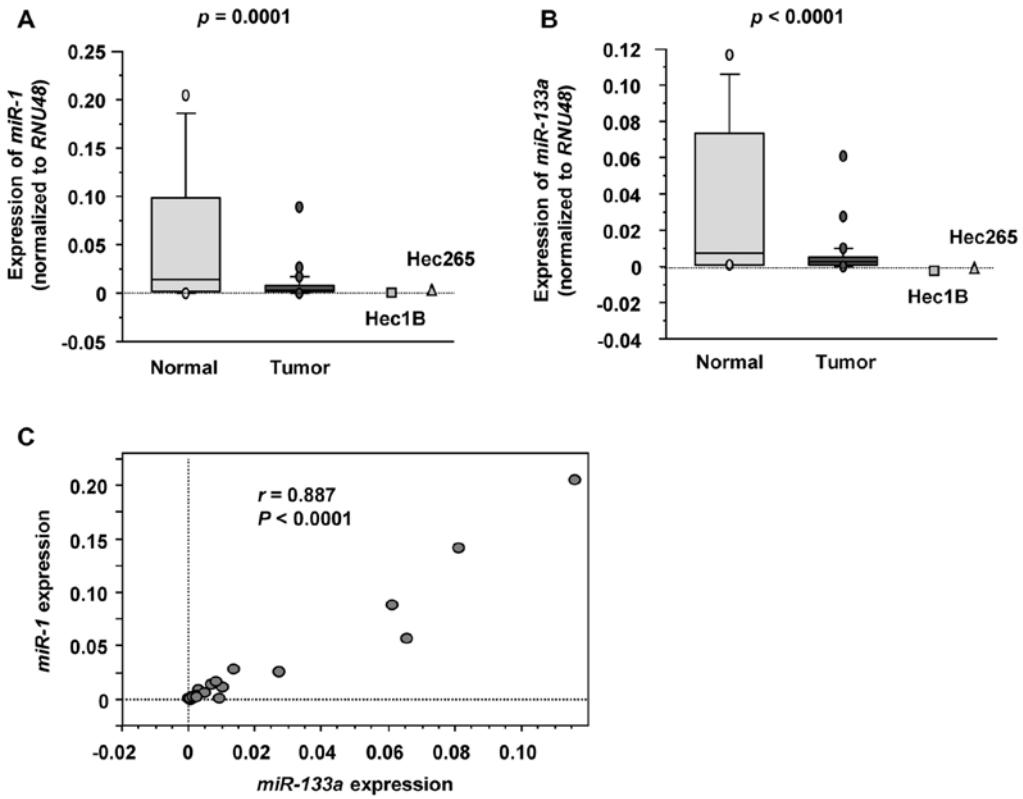

Figure 1. Expression of *miR-1* and *miR-133a* in endometrial cancer specimens, non-cancer endometrial specimens and cell lines. (A) Expression of *miR-1* and (B) *miR-133a* in endometrial cancer specimens, non-cancer endometrial specimens and cell lines as determined by qRT-PCR. *RNU48* was used as an internal control. (C) The correlation between *miR-1* and *miR-133a* expression was analyzed in endometrial cancer specimens, non-cancer endometrial specimens and cell lines.

Cell migration assay. Modified Boyden Chambers (Transwells, no. 3422; Corning, NY, USA) were used. Cells were transfected with 10 nM miRNA or siRNA by reverse transfection and plated in 10-cm dishes at $8x10^5$ cells/dish. After 48 h, $1x10^5$ cells were added to the upper chamber of each migration well and were allowed to migrate for 48 h. After gentle removal of the non-migratory cells from the filter surface of the upper chamber, the cells that migrated to the lower side were fixed and stained with Diff-Quick (no. 16920; Sysmex Corp., Japan). The number of cells migrating to the lower surface was determined microscopically by counting four areas of constant size per well. Cell invasion assays were carried out using modified Boyden chambers in 24-well tissue culture plates at $1x10^5$ cells per well (Matrigel invasion chamber, no. 354480; BD Biocoat, USA). All experiments were performed in duplicate.

Search for miR-1 and miR-133a target genes. To identify putative *miR-1*- and *miR-133a*-regulated genes, we searched the TargetScan database (http://www.targetscan.org) for genes having conserved sites for both *miR-1* and *miR-133a*. Then, we analyzed gene expression using the GEO database. Gene expression data for clinical EC specimens were entered into the GEO database (accession no. GSE17025). The procedure used for the selection of miR-1 and *miR-133a* genes is shown in Fig. 3.

Western blot analysis. Cells were harvested and lysed 72 h after transfection. Cell lysates (50 μg of protein each) were separated using Mini-PROTEAN TGX gels (Bio-Rad, Hercules, CA, USA), followed by subsequent transfer to PVDF membranes. Immunoblotting was performed with polyclonal anti-PDE7A antibodies (ab154857; Abcam, Cambridge, UK). Anti-GAPDH antibodies (ab8245; Abcam) were used as an internal control.

Plasmid construction and dual-luciferase reporter assays. Partial sequences of the *PDE7A* 3' untranslated region (3'UTR) containing the *miR-1* and *miR-133a* target sites were inserted between the *Xho*I and *Pme*I restriction sites in the 3'UTR of the *hRluc* gene in the psiCHECK-2 vector (C8021; Promega, Madison, WI, USA). Hec265 cells were then transfected with 5 ng vector or 10 nM mature miRNA.

Statistical analysis. The relationships between 2 variables and numerical values were analyzed using the Mann-Whitney U test, and the relationships between 3 variables and numerical values were analyzed using the Bonferroni-adjusted Mann-Whitney U test. Expert StatView analysis software (ver. 4; SAS Institute Inc., Cary, NC, USA) was used in both analyses. In the comparison of 3 variables, an unadjusted statistical level of significance of $P<0.05$ corresponded to the Bonferroni-adjusted level of $P<0.0083$.

Results

Expression levels of miR-1 and miR-133a in EC specimens and cell lines. To validate our previous miRNA expression

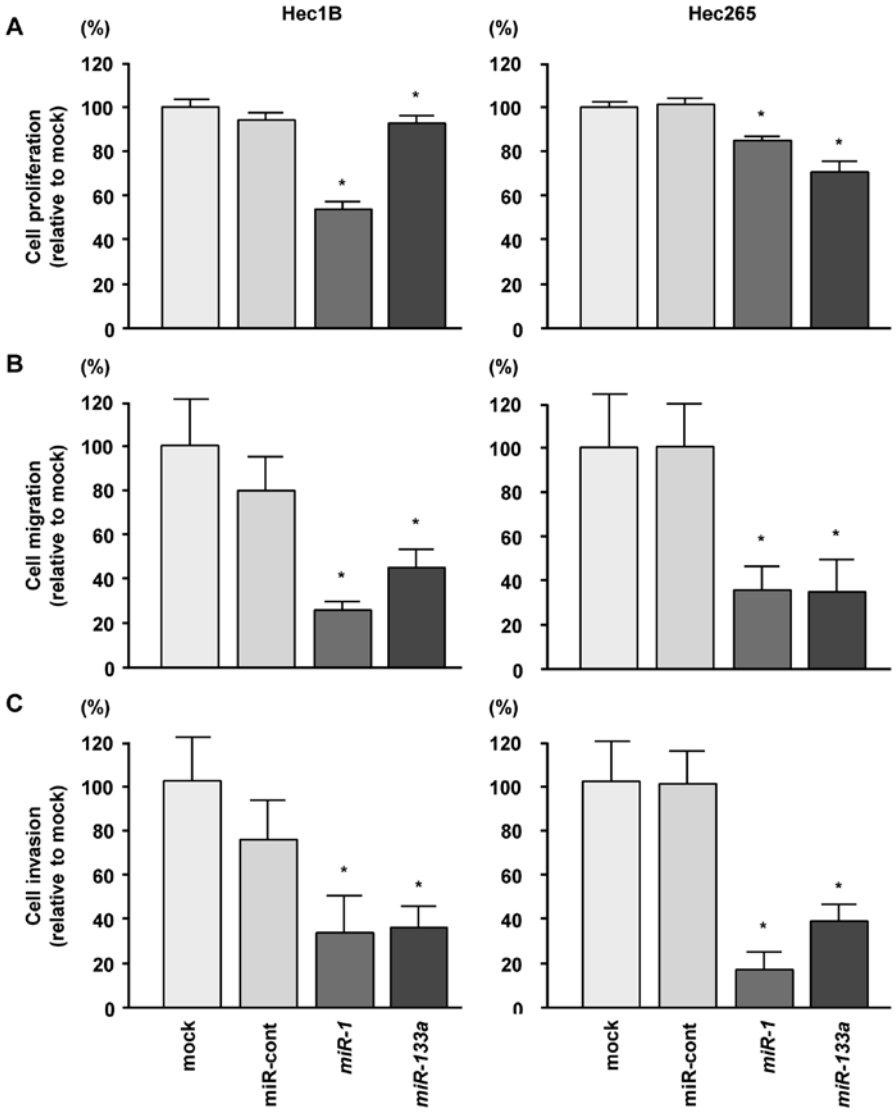

Figure 2. Effects of *miR-1* and *miR-133a* restoration on the proliferation, migration and invasion of Hec1B and Hec265 cells. (A) Proliferation activity of Hec1B and Hec265 cells as determined by XTT assays. *P<0.0083. (B) Migration activity of Hec1B and Hec265 cells as determined by migration assays. *P<0.0083. (C) Invasion activity of Hec1B and Hec265 cells as determined by Matrigel invasion assays. *P<0.0083.

signatures, we evaluated the expression levels of *miR-1* and *miR-133a* in 27 EC specimens and 8 non-cancer endometrial specimens. The backgrounds and clinicopathological characteristics of patients are summarized in Table I. Quantitative stem-loop RT-PCR demonstrated that *miR-1* and *miR-133a* expression levels were significantly lower in cancer specimens compared with non-cancer specimens (P<0.0001; Fig. 1A and B, respectively). The expression levels of *miR-1* and *miR-133a* were also reduced in 2 EC cell lines (Hec1B and Hec265). Spearman's rank test showed a positive correlation between the expression of *miR-1* and that of *miR-133a* (r=0.887, P<0.0001; Fig. 1C).

Effects of transfection with miR-1 and miR-133a on cell proliferation, migration and invasion in EC cell lines. To examine the functional roles of *miR-1* and *miR-133a*, we performed gain-of-function assays by transfecting mature miRNAs into Hec1B and Hec265 cells. XTT assays showed that cell proliferation was inhibited by transfection with *miR-1* and *miR-133a* in both Hec1B and Hec265 cells compared with mock and miRNA-control transfections (P<0.0083, Fig. 2A).

Cell migration assays demonstrated that cell migration was significantly inhibited in miRNA-transfected cells compared with mock- or miRNA-control-transfected cells (P<0.0083, Fig. 2B).

Moreover, in Matrigel invasion assays, transfection with *miR-1* and *miR-133a* significantly inhibited cell invasion as compared with mock or miRNA-control transfection (P<0.0083, Fig. 2C). These results suggested that the *miR-1/133a* cluster could represent a putative tumor suppressor in EC cells.

Identification of common targets of miR-1 and miR-133a by in silico analysis and gene expression data. To identify putative genes regulated by the *miR-1/133a* cluster (i.e., both *miR-1* and *miR-133a*), we searched the TargetScan database (Release 6.2, http://www.targetscan.org/) and analyzed

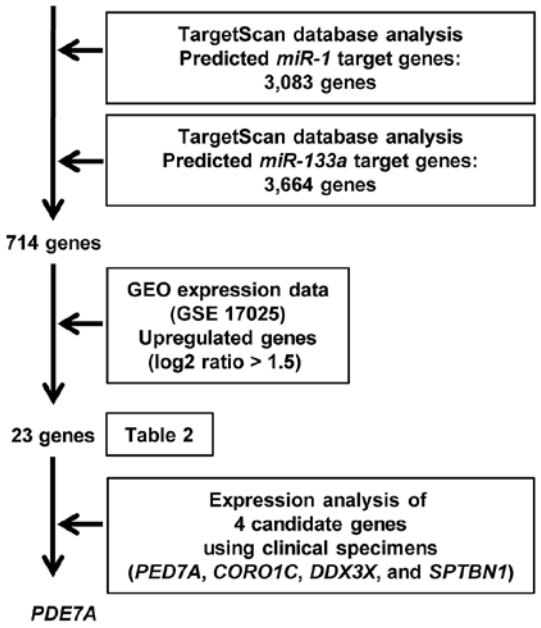

Figure 3. Identification of molecular targets regulated by *miR-1* and *miR-133a*. From analysis of data in the TargetScan database, we identified 3,083 genes with *miR-1* target sites in their 3'UTRs and 3,664 genes with *miR-133a* target sites in their 3'UTRs. Among these genes, 714 had both target sites and 23 were upregulated in endometrial cancer specimens according to expression data from the GEO database (accession no. GSE17025).

expression data of EC clinical specimens using the Gene Expression Omnibus (GEO accession no. GSE 17025). Our strategy for identification of target genes of the *miR-1/133a* cluster is shown in Fig. 3. We found that 23 genes were upregulated in EC specimens and had putative target sites for *miR-1* and *miR-133a* in their 3'UTRs. Therefore, these genes were annotated as putative targets of the *miR-1/133a* cluster (Table II). Among 23 genes, we evaluated the expression of 4 genes (*PDE7A, DDX3X, CORO1C* and *SPTBN1*) in clinical specimens. As a result, the expression of *PDE7A* mRNA was significantly higher in clinical EC specimens.

PDE7A was a direct target of the miR-1/133a cluster in EC cells. Next, we performed qRT-PCR and western blotting to confirm downregulation of PDE7A mRNA and protein following restoration of *miR-1* or *miR-133a* in Hec1B and Hec265 EC cells. The mRNA and protein expression levels of PDE7A were significantly repressed in *miR-1* and *miR-133a* transfectants in comparison with mock or miR-control transfectants (P<0.0083, Fig. 4A and B).

We then performed luciferase reporter assays in EC cells to determine whether *PDE7A* was directly regulated by *miR-1* and *miR-133a*. The TargetScan database predicted that there was one binding site for *miR-1* in the 3'UTR of *PDE7A* (positions 1333-1340; Fig. 4C) and one binding site for *miR-133a*

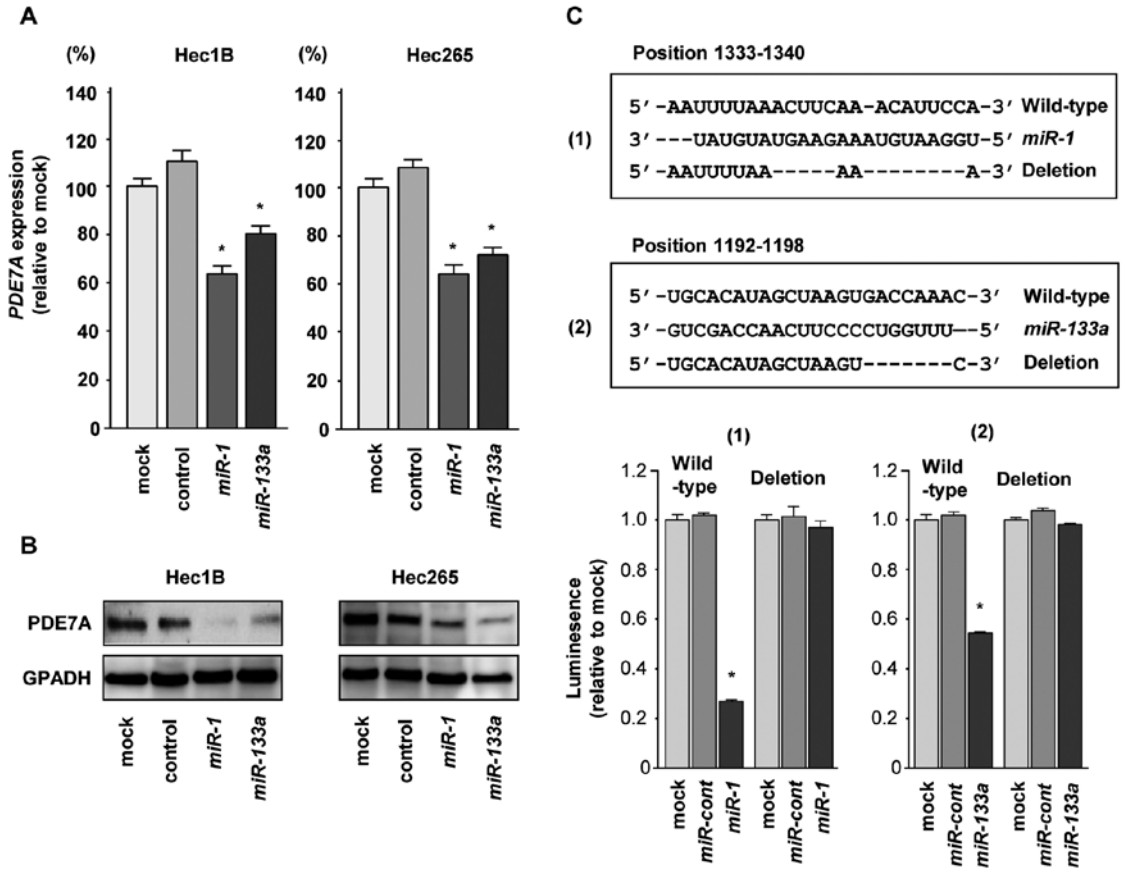

Figure 4. *miR-1* and *miR-133a* directly regulated *PDE7A* in Hec1B and Hec265 cells. (A) Expression of *PDE7A* mRNA as measured by qRT-PCR. *GUSB* was used as an internal control. *P<0.0083. (B) Expression of PDE7A protein as measured by western blot analysis. GAPDH was used as a loading control. (C) A putative *miR-1* and *miR-133a* binding site in the 3'UTR of *PDE7A* mRNA was identified using the TargetScan database. Luciferase reporter assays were performed using a vector encoding the partial sequences of the 3'UTR containing the putative *miR-1* and *miR-133a* target site. The vector (10 ng) and 10 nM *miR-1* or *miR-133a* or *miR-control* were cotransfected into Hec265 cells. Renilla luciferase activity was measured 24 h after transfection. The results were normalized to firefly luciferase values. *P<0.05.

Table II. Putative target genes regulated by the *miR-1/133a* cluster in endometrial cancer cells.

Entrez Gene ID	Gene symbol	Gene name	Expression (log2 ratio)	P-value	Putative target site			
					miR-1		*miR-133a*	
					Conserved	Poorly	Conserved	Poorly
5150	PDE7A	Phosphodiesterase 7A	1.79	1.2E-07	1		1	
6711	SPTBN1	Spectrin, β, non-erythrocytic 1	1.71	1.6E-03	1		1	2
23603	CORO1C	Coronin, actin binding protein, 1C	1.67	2.0E-06	2		1	
1654	DDX3X	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked	1.51	1.9E-03	1		1	
377	ARF3	ADP-ribosylation factor 3	2.62	2.5E-05	1	2		1
10888	GPR83	G protein-coupled receptor 83	2.07	2.5E-05	1			1
6373	CXCL11	Chemokine (C-X-C motif) ligand 11	2.04	6.6E-04	1			1
7267	TTC3	Tetratricopeptide repeat domain 3	1.99	2.0E-03	1			1
7705	ZNF146	Zinc finger protein 146	1.66	3.4E-04	1			1
4804	NGFR	Nerve growth factor receptor	1.58	3.8E-03	1			1
93685	ENTPD7	Ectonucleoside triphosphate diphosphohydrolase 7	1.75	5.7E-07	1	1		1
2321	FLT1	fms-related tyrosine kinase 1	2.95	7.2E-06		1	1	
55143	CDCA8	Cell division cycle associated 8	2.67	5.5E-07		1	1	
6789	STK4	Serine/threonine kinase 4	2.32	3.4E-06		1	1	
5451	POU2F1	POU class 2 homeobox 1	1.87	1.3E-02		1	2	1
2043	EPHA4	EPH receptor A4	3.01	1.5E-04		1		1
7545	ZIC1	Zic family member 1	2.99	4.9E-02		1		1
57823	SLAMF7	SLAM family member 7	2.07	1.9E-03		1		1
57522	SRGAP1	SLIT-ROBO Rho GTPase activating protein 1	1.99	1.2E-06		1		1
85645	EPT1	Ethanolaminephosphotransferase 1 (CDP-ethanolamine-specific)	1.99	2.5E-09		1		2
13869	Erbb4	v-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian)	1.69	3.0E-02		1		1
72555	Shisa9	Shisa homolog 9 (*Xenopus laevis*)	1.56	7.0E-03		1		1
10640	EXOC5	Exocyst complex component 5	1.51	1.8E-02		1		1

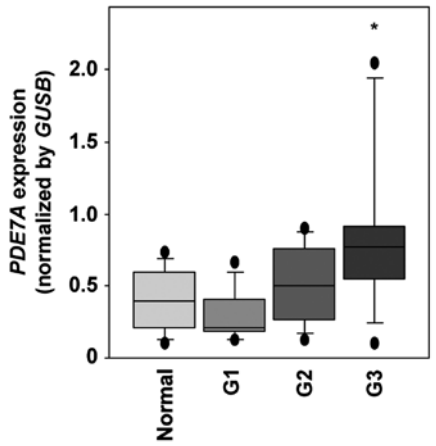

Figure 5. Expression of *PDE7A* in endometrial cancer specimens. Expression of *PDE7A* in cancer and non-cancer specimens as determined by qRT-PCR. *GUSB* was used as an internal control. *P<0.0083.

in the 3'UTR of *PDE7A* (positions 1192-1198; Fig. 4C). We then used vectors encoding the partial wild-type sequence of the 3'UTR of *PDE7A* mRNA, including the predicted *miR-1* or *miR-133a* target sites. We found that the luminescence intensity was significantly reduced by cotransfection with *miR-1* or *miR-133a* and the vector carrying the wild-type 3'UTR of *PDE7A*. In contrast, transfection with the mutant vector, in which the sequence within positions 1333-1340 or 1192-1198 had been changed, blocked the decrease in luminescence (P<0.0001, Fig. 4C). These data suggested that *miR-1* and *miR-133a* bound directly to specific sites in the 3'UTR of *PDE7A* mRNA.

Expression levels of PDE7A in EC clinical specimens. Twenty-seven EC and 8 normal endometrium specimens were subjected to *PDE7A* mRNA expression analysis in this study. qRT-PCR analysis showed that the expression of *PDE7A* mRNA was

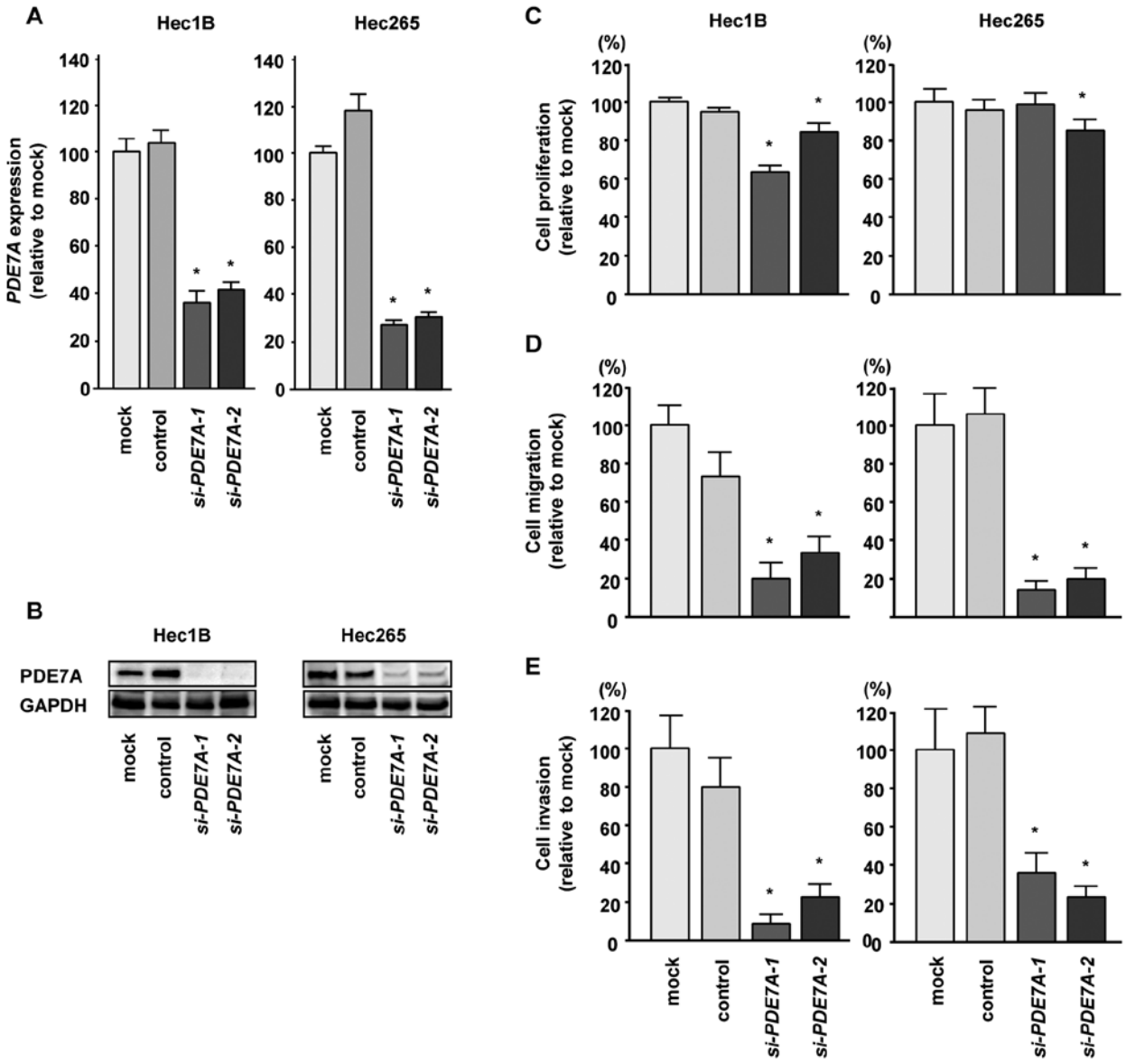

Figure 6. Effects of *PDE7A* knockdown by si-*PDE7A* transfection in Hec1B and Hec265 cells. (A) Expression of *PDE7A* mRNA as revealed by qRT-PCR. *GUSB* was used as an internal control. *P<0.0083. (B) Expression of PDE7A protein as revealed by western blot analysis. GAPDH was used as a loading control. (C) Cell proliferation activity in Hec1B and Hec265 cells as measured by XTT assays. *P<0.0083. (D) Cell migration activity in Hec1B and Hec265 cells. *P<0.0083. (E) Cell invasion activity in Hec1B and Hec265 cells. *P<0.0083.

significantly higher in clinical EC (differentiation G3) specimens than in normal specimens (P=0.0022, Fig. 5).

Downregulation of PDE7A expression in EC cells affected cell proliferation, migration and invasion activities. To investigate the functional role of *PDE7A*, we performed loss-of-function studies using *si-PDE7A* transfectants. First, we evaluated the knockdown efficiency of *si-PDE7A* transfection in Hec1B and Hec265 EC cells. Western blotting and qRT-PCR indicated that *si-PDE7A* effectively downregulated PDE7A expression in EC cells (P<0.0083, Fig. 6A and B).

Next, we analyzed the functional effects of *PDE7A* knockdown in EC cells. XTT assays demonstrated that cell proliferation was significantly inhibited in *si-PDE7A* transfectants in comparison with mock or si-control transfectants (P<0.005, Fig. 6C). Moreover, cell migration assays revealed significant inhibition of cell migration in *si-PDE7A* transfectants in comparison with mock or si-control transfectants (P<0.0001, Fig. 6D). Similarly, Matrigel invasion assays revealed that the number of invading cells was significantly decreased when EC cells were transfected with *si-PDE7A* (P<0.0001, Fig. 6E). These findings suggested that *PDE7A* acted as an oncogene in EC cells.

Discussion

The 5-year survival rate of patients with stage I EC is >90%; however, that in patients with stages III or IV EC is much lower, ranging from 40 to 80% (3,4). Previous studies have demonstrated that mutation of K-ras or PTEN is common in low-grade EC, while high-grade EC is associated with P53 mutation (15). These data suggest that differences in expression of cancer-related genes have substantial effects on disease progression. However, EC is a complex disease and cannot be explained only by mutations in these few genes; thus, elucidation of the involvement of other unknown genetic abnormalities and signaling pathways, including ncRNAs, is critical.

miRNAs are unique in their ability to regulate multiple protein-coding genes. Recent bioinformatic predictions have shown that miRNAs regulate >30-60% of the protein-coding genes in the human genome (9,10). Accumulating evidence has suggested that aberrantly expressed miRNAs disrupt tightly regulated RNA networks in cancer cells. These events are believed to initiate cancer cell development and metastasis. Therefore, identification of key miRNAs and the networks regulated by these miRNAs will provide new insights into the potential mechanisms of cancer initiation, development and metastasis. Recent studies have reported the differential expression of miRNAs in EC cells; for examples, *miR-205, miR-210, miR-429* and *miR-449* are upregulated in EC tissues, whereas *let-7e, miR-30c, miR-204* and *miR-221* are downregulated in EC tissues (16). Upregulation of *miR-205* is significantly correlated with disease survival in EC, and thus, *miR-205* is considered a potential prognostic marker in EC. Interestingly, *miR-205* directly regulates the expression of *PTEN* and inhibits apoptosis in EC cells (17).

To identify novel miRNA-mediated RNA networks in cancer cells, we have constructed miRNA expression signatures in several types of cancers and investigated the roles of miRNAs in oncogenesis and metastasis using differentially expressed miRNAs (12,18,19). These miRNA signatures have revealed that the *miR-1/133a* cluster is frequently downregulated in several types of cancers, including head and neck squamous cell carcinoma, prostate cancer, bladder cancer and lung cancer (11-14). Our present study demonstrated that *miR-1* and *miR-133a* were significantly downregulated in EC specimens and cell lines. Moreover, restoration of these miRNAs significantly inhibited cancer cell migration and invasion, suggesting that this miRNA cluster may function as a tumor suppressor in EC cells, similar to its function in other cancers (20-22). A full understanding of the targets in EC cells that are regulated by the *miR-1/133a* cluster may contribute to our knowledge on EC oncogenesis and metastasis. Recently, we established a strategy for identification of pathways and genes regulated by tumor-suppressive miRNAs (14,22). In the present study, we used this strategy and found 23 putative candidate genes potentially regulated by the *miR-1/133a* cluster in EC cells. This is the first report demonstrating that *PDE7A* is directly regulated by the *miR-1/133a* cluster in EC cells.

PDEs are enzymes that regulate the cellular levels of the secondary messengers cAMP and cGMP by controlling their rates of degradation. PDEs can be categorized into 11 families (PDE1-11), which are structurally related but functionally distinct (23). PDE7, including isoforms PDE7A and PDE7B, is a high-affinity cAMP-specific PDE (24-26). Three variant forms of PDE7A have been annotated: PDE7A1 and PDE7A2 are N-terminal variants, and PDE7A3 is a C-terminal variant (25,27). The expression of PDE7A is elevated in pro-inflammatory and immune cells, supporting the role of PDE7A as a therapeutic target for inflammation disorders (28). A recent study indicated that the PDE7A-specific inhibitor ASB16165 suppresses keratinocyte proliferation on TPA-induced skin inflammation (29).

Many tumor cells exhibit significantly decreased cAMP levels as a consequence of overexpression of PDEs in chronic lymphocytic leukemia (CLL) and malignant carcinoma cells (30-32). PDE7A is overexpressed in CLL, and stimulation of the cAMP signaling pathway has been shown to induce apoptosis and augment the effects of glucocorticoids in inducing apoptosis in CLL cells (33). Increasing intracellular concentrations of cAMP may arrest growth, induce apoptosis and attenuate cancer cell migration in various cancers (34-37). The effects of cAMP are mediated by two ubiquitously expressed intracellular cAMP receptors, protein kinase A (PKA) and exchange protein directly activated by cAMP (Epac) (38). The cAMP/PKA signaling pathway may have an important role in tumor migration. Indeed, activation of the cAMP/PKA pathway inhibits cancer cell migration in various cancers by targeting matrix metalloproteinase (MMP)2, actin, integrin, MMP9 and MMP4 (39-42). Interestingly, PDE7A contains a PKA pseudosubstrate site within 2 repeated sequences at the N-terminal region of PDE7A. The PDE7A1 N-terminal repeat region inhibits the C subunit of PKA (C) activity and suppresses C-dependent, cAMP-independent, physiological responses. These observations demonstrate that PDE7A1 can inhibit cAMP signaling via direct binding to C (43).

In conclusion, downregulation of the *miR-1/133a* cluster was a frequent event in EC. Moreover, the tumor-suppressive

miR-1/133a cluster directly regulated PDE7A, a high-affinity cAMP-specific enzyme. Restoration of *miR-1/miR-133a* or silencing of PDE7A inhibited cancer cell migration and invasion, suggesting that the *miR-1/miR-133a*-PDE7A pathway contributes to the metastasis of EC. Identification of molecular targets regulated by tumor-suppressive miRNAs will provide insights into the potential mechanisms of EC oncogenesis and metastasis, facilitating the development of novel therapeutic strategies for the treatment of this disease.

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