

# Tumor-suppressive *microRNA-143/145* cluster targets hexokinase-2 in renal cell carcinoma

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Our recent studies of microRNA (miRNA) expression signatures have indicated that the *miR-143/145* cluster is significantly downregulated in several types of cancer and represents a putative tumor-suppressive miRNA in human cancers. The aim of this study was to investigate the functional significance of the *miR-143/145* cluster in cancer cells and to identify novel molecular targets of the *miR-143/145* cluster in renal cell carcinoma (RCC). The expression levels of *miR-143* and *miR-145* were significantly downregulated in RCC tissues compared with adjacent non-cancerous tissues. A significant positive correlation was recognized between *miR-143* and *miR-145* expression. Restoration of mature *miR-143* or *miR-145* in 786-O and A498 RCC cells revealed that both mature miRNAs significantly inhibited cancer cell proliferation and invasion, suggesting that the *miR-143/145* cluster functioned as a tumor suppressor in RCC. Gene expression data and *in silico* database analysis showed that the hexokinase-2 (*HK2*) gene, which encodes a glycolytic enzyme crucial for the Warburg effect in cancer cells, was a candidate target of the *miR-143/145* cluster. Luciferase reporter assays showed that both *miR-143* and *miR-145* directly regulated *HK2*. In RCC clinical specimens, the expression of *HK2* was significantly higher in cancer tissues than in non-cancerous tissues. Silencing *HK2* suppressed RCC cell proliferation and invasion, suggesting that *HK2* has oncogenic functions in RCC. Thus, our data showed that loss of the tumor-suppressive *miR-143/145* cluster enhanced RCC cell proliferation and invasion through targeting *HK2*. (*Cancer Sci* 2013; 104: 1567–1574)

Renal cell carcinoma (RCC) is a disease in which cancer cells form in the tubules of the kidney. Globally, the incidence and mortality rates of RCC are increasing 2–3% per decade.<sup>(1)</sup> The 5-year survival rate of advanced-stage RCC is very poor (5–10%) due to recurrence or distant metastasis.<sup>(2)</sup> The latest treatment for RCC includes molecular targeted therapies, which have been developed and are being widely used for patients with metastatic or recurrent RCC. However, these types of therapies are not expected to have curative effects.<sup>(3)</sup> Therefore, to improve outcomes in patients with RCC, it is necessary to fully elucidate the molecular mechanisms of RCC.

The discovery of non-coding RNAs (ncRNAs) in the human genome was an important conceptual breakthrough in the post-genome sequencing era,<sup>(4)</sup> and it is now evident that improved understanding of ncRNAs is necessary for continued progress in cancer research. MicroRNAs (miRNAs) are endogenous small ncRNA molecules (19–22 bases in length) that regulate protein-coding gene expression by repressing translation or cleaving RNA transcripts in a sequence-specific manner.<sup>(5)</sup> Currently, 2578 human mature miRNAs are registered at miRBase (release 20, June 2013; <http://www.mirbase.org/>).

miRNAs are unique in their ability to regulate multiple protein-coding genes and are predicted to regulate more than 60% of the protein-coding genes in the human genome.<sup>(6)</sup>

A growing body of evidence suggests that miRNAs are aberrantly expressed in many human cancers and that they play significant roles in the initiation, development, and metastasis of those cancers.<sup>(7)</sup> Some highly expressed miRNAs can function as oncogenes by repressing tumor suppressors, whereas low-level miRNAs can function as tumor suppressors by negatively regulating oncogenes.<sup>(8)</sup> It is believed that normal regulatory mechanisms can be disrupted by the aberrant expression of tumor-suppressive or oncogenic miRNAs in cancer cells. Therefore, identification of aberrantly expressed miRNAs is an important first step toward elucidating miRNA-mediated oncogenic pathways.

Our laboratory and other research groups have constructed miRNA expression signatures to seek out differentially expressed miRNAs in human cancer cells.<sup>(7,9,10)</sup> According to miRNA signatures, *miR-143* and *miR-145* are the most frequently downregulated miRNAs in various types of human cancers.<sup>(11,12)</sup> Both *miR-143* and *miR-145* are known to be located close together on human chromosome 5q32, where they form a cluster.<sup>(13)</sup> Several reports have suggested that these clustered miRNAs function as tumor suppressors, targeting several oncogenic genes.<sup>(11,12)</sup> However, functional analysis of this cluster has not yet been carried out in RCC.

The aim of the present study was to investigate the functional significance of the *miR-143/145* cluster and to identify the molecular targets regulated by these miRNAs in RCC cells. Our *in silico* and gene expression analyses of the *miR-143/145* cluster showed that several oncogenes were possible targets of these miRNAs. To validate these targets, we focused on the hexokinase-2 (*HK2*) gene and investigated its functional significance in RCC. In malignant cells, the rate of glucose uptake is significantly accelerated, and oxidative phosphorylation in mitochondria is often decreased compared to normal cells. This change is termed the aerobic glycolysis effect or the Warburg effect.<sup>(14)</sup> The glycolytic enzyme *HK2* is crucial for the Warburg effect in cancer cells. It catalyzes the essentially irreversible first step of the glycolytic pathway in which glucose is phosphorylated to glucose-6-phosphate with concomitant dephosphorylation of ATP.<sup>(15)</sup>

Thus, the current elucidation of novel tumor-suppressive *miR-143/145*-mediated cancer pathways and target genes provides insights into the potential mechanisms of RCC oncogenesis and suggests novel therapeutic strategies for treatment of RCC.

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## Material and Methods

**Clinical specimens and tissue collection.** A total of 18 pairs of clear cell RCC and adjacent non-cancerous specimens were collected from patients who had undergone radical nephrectomies at Kagoshima University Hospital (Kagoshima, Japan). Patient characteristics and representative pathological features are shown in Table S1. The samples were processed and stored in RNAlater (Qiagen, Valencia, CA, USA) at  $-20^{\circ}\text{C}$  until RNA extraction. These specimens were staged according to the American Joint Committee on Cancer–Union Internationale Contre le Cancer TNM classification and were histologically graded.<sup>(16)</sup> Our study was approved by the Bioethics Committee of Kagoshima University; written prior informed consent and approval were given by all patients prior to enrolment in the study.

**Cell culture and RNA extraction.** In this study, we used two RCC cell lines, 786-O and A498, obtained from the ATCC (Manassas, VA, USA). These cell lines are VHL-deficient and expressed hypoxia-inducible factor (HIF)2 $\alpha$  under normoxic conditions; 786-O cells have a p53 mutation whereas A498 cells contain wild-type p53 (NCI-60 cell line database; <http://dtp.nci.nih.gov/branches/btb/ivclsp.html>).<sup>(17,18)</sup> Cells were incubated in RPMI-1640 medium supplemented with 10% FBS and maintained in a humidified incubator (5%  $\text{CO}_2$ ) at  $37^{\circ}\text{C}$ . Total RNA was extracted as previously described.<sup>(19)</sup>

**Quantitative real-time RT-PCR.** TaqMan probes and primers for HK2 (P/N: Hs00606086\_m1; Applied Biosystems, Foster City, CA, USA) were assay-on-demand gene expression products. We followed the manufacturer's protocol for PCR conditions. Stem-loop RT-PCR (TaqMan MicroRNA Assays; Assay ID: 002249 for *miR-143* and 002278 for *miR-145*; Applied Biosystems) was used to quantify miRNA expression according to earlier published conditions.<sup>(20)</sup> To normalize the data for quantification of *HK2* mRNA and the miRNAs, we used the human *GUSB* (P/N: Hs99999908\_m1; Applied Biosystems) and *U6* genes (Assay ID: 001973; Applied Biosystems), respectively, and the delta–delta Ct method was used to calculate the fold change.

**Transfection with mature miRNA, siRNA, and HK2 expression vectors.** As described elsewhere,<sup>(20)</sup> RCC cells were transfected with 10 nM mature miRNAs using Lipofectamine RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA, USA) and Opti-MEM (Invitrogen). Pre-miR and negative control miRNA (CN-002000-01-05 for 786-O, Thermo Fisher Scientific, Waltham, MA, USA; AM17111 for A498, Applied Biosystems) were used in gain-of-function experiments, whereas HK2 siRNA (HSS179238 and HSS179240, Invitrogen) and negative control siRNA (D-001810-10 for 786-O, Thermo Fisher Scientific; MISSION siRNA Universal Negative Control for A498, Sigma-Aldrich, St. Louis, MO, USA) were used in loss-of-function experiments. In this study, we selected different negative controls (miRNAs/siRNAs) for each cancer cell line to avoid off-target effects.

We evaluated the transfection efficiency of the miRNAs in RCC cells based on the downregulation of protein tyrosine kinase 9 (*PTK9*, also known as twinfilin [*TWFI*]) mRNA expression after *miR-1* transfection, as described previously, using the manufacturer's recommended protocols (Applied Biosystems).<sup>(21)</sup>

**Plasmid construction and dual-luciferase reporter assay.** According to the TargetScan database search, *HK2* has three putative *miR-143* binding sites and one *miR-145* binding site in its 3'-UTR. To check the actual binding sites of *miR-143* or *miR-145* in the *HK2*, we constructed a total of eight types of vectors (with target site, wild-type; lacking target site, deletion-type). MiRNA target sequences (three of *miR-143* sites and one of *miR-145* site) were inserted between the *XhoI*–*PmeI* restriction

sites in the 3'-UTR of the *hRluc* gene in the psiCHECK-2 vector (C8021; Promega, Madison, WI, USA). Detailed insert sequences are shown in Table S2. The RCC cells were then transfected with 15 ng vector, 10 nM miRNA, and 1  $\mu\text{L}$  Lipofectamine 2000 in 100  $\mu\text{L}$  Opti-MEM. The activities of firefly and Renilla luciferases in cell lysates were recorded, as described previously.<sup>(19)</sup>

**Cell proliferation and invasion assays.** Seventy-two hours after transfection, cell proliferation was determined with an XTT assay (Roche Applied Science, Tokyo, Japan) according to the manufacturer's instructions. Cell invasion assays were carried out using modified Boyden chambers consisting of Transwell-precoated Matrigel membrane filter inserts with 8-mm pores in 24-well tissue culture plates (BD Biosciences, Bedford, MA, USA), as described previously.<sup>(22)</sup>

**Screening of *miR-143*- and *miR-145*-regulated genes: Pathway analysis using databases.** The predicted target genes and their miRNA binding site seed regions were investigated using TargetScan (release 6.2, <http://www.targetscan.org/>). The sequences of the predicted mature miRNAs were confirmed using miRBase. To identify signaling pathways regulated by *miR-143* and *miR-145* in RCC, upregulated genes targeted by these miRNAs were analyzed in terms of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway categories using the GENECODIS program (<http://genecodis.cnb.csic.es/>).

**Western blot analysis.** Immunoblotting was carried out using diluted (1:500) monoclonal anti-HK2 antibodies (SAB2701698; Sigma-Aldrich) and anti-GAPDH antibodies (MAB374; Chemicon, Temecula, CA, USA), as described previously.<sup>(19)</sup>

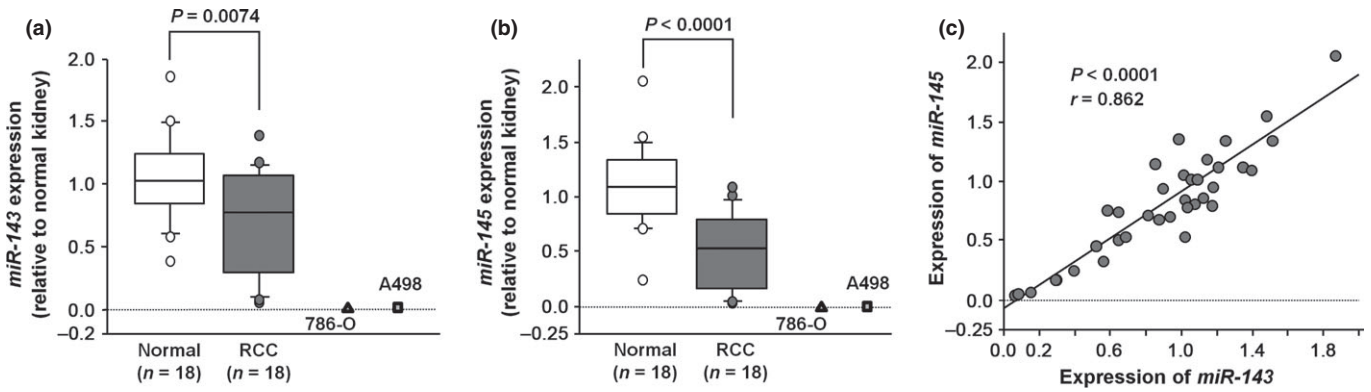
**Statistical analysis.** The relationships between two variables and the numerical values obtained by real-time RT-PCR were analyzed using Mann–Whitney *U*-tests. The relationships among three variables and numerical values were analyzed using Bonferroni-adjusted Mann–Whitney *U*-tests. Expert Stat-View analysis software (version 4; SAS Institute, Cary, NC, USA) was used in both cases.

## Results

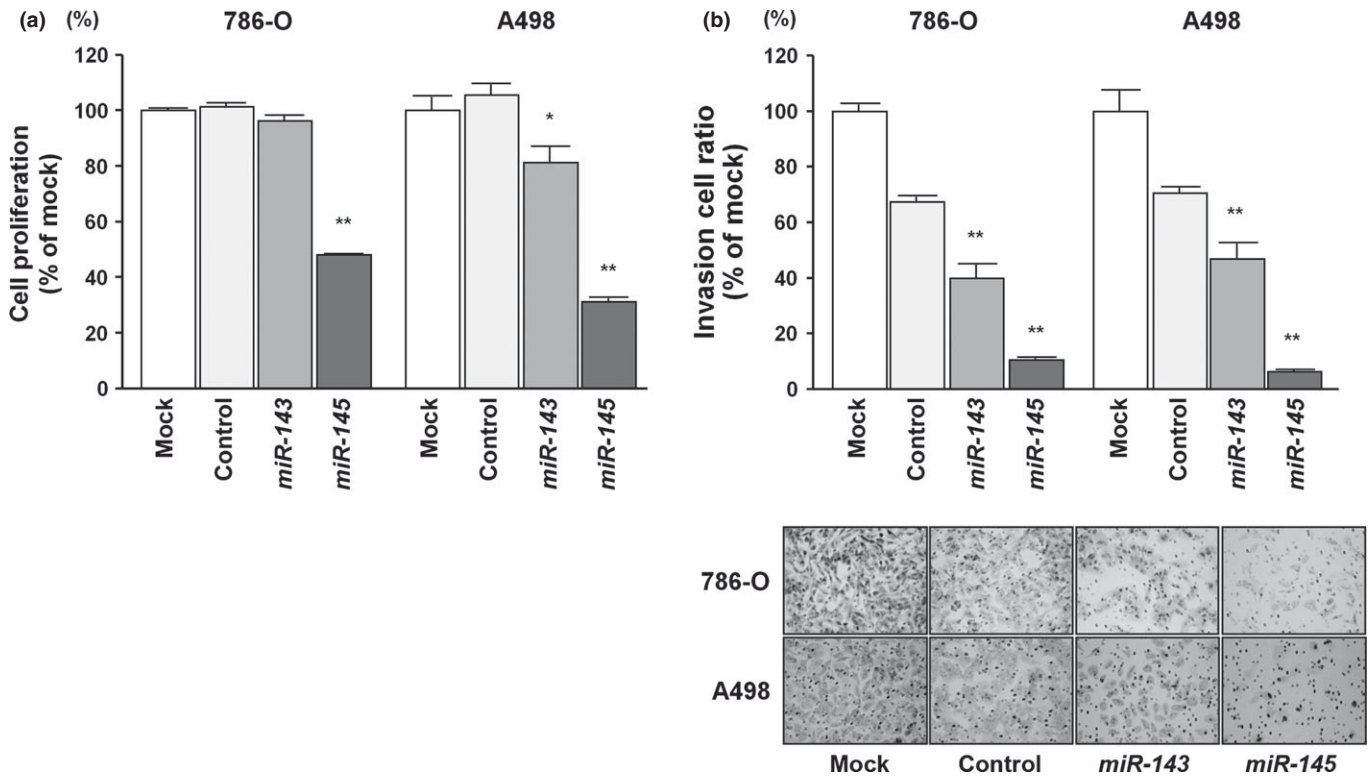
**Expression levels of *miR-143* and *miR-145* in cancer cell lines and RCC clinical specimens.** We evaluated the expression levels of *miR-143* and *miR-145* in RCC tissues ( $n = 18$ ) and adjacent non-cancerous specimens ( $n = 18$ ). Quantitative stem-loop RT-PCR showed that *miR-143* expression was significantly lower in clinical RCC specimens and RCC cell lines (786-O and A498) compared with adjacent non-cancerous tissues ( $P = 0.0074$  and  $P < 0.0001$ , respectively; Fig. 1a). Also, *miR-145* expression was significantly reduced in clinical RCC specimens and RCC cell lines ( $P < 0.0001$ ; Fig. 1b); *miR-143* expression was highly correlated with that of *miR-145* ( $r = 0.862$ ,  $P < 0.0001$ , Fig. 1c).

**Effects of *miR-143* and *miR-145* restoration on cell proliferation and invasion in RCC cell lines.** To investigate the functional role of the *miR-143/145* cluster, we carried out gain-of-function studies using 786-O and A498 RCC cells that had been transfected with mature *miR-143* or *miR-145*. The XTT assays indicated that cell proliferation was significantly suppressed in *miR-143* and *miR-145* transfectants compared with mock-transfected cells, with the exception of *miR-143*-transfected 786-O cells ( $P = 0.0563$  and  $P < 0.0001$ ;  $P = 0.0063$  and  $P < 0.0001$ ; Fig. 2a). Matrigel invasion assays showed that the number of invading cells was significantly decreased in miRNA transfectants compared with their counterparts ( $P < 0.0001$ ;  $P < 0.0001$ ; Fig. 2b).

It is plausible that the *miR-143/145* cluster could have an important role as tumor suppressors through downregulating several oncogenes. However, we found no synergic effects of



**Fig. 1.** (a,b) Expression levels of *miR-143* and *miR-145* in clinical renal cell carcinoma (RCC) specimens and RCC cell lines compared with clinically normal kidneys. U6 was used as the internal control. (c) Correlation between the relative expression levels of *miR-143* (x-axis) and *miR-145* (y-axis). Levels are plotted as a scatterplot.



**Fig. 2.** Effects of transfection with mature *miR-143* and *miR-145* on renal cell carcinoma cell lines. (a) Cell proliferation was determined with XTT assays in 768-O and A498 cell lines after 72 h of transfection with 10 nM *miR-143*, *miR-145*, miR-control, or mock transfection. (b) Cell invasion activity was determined by Matrigel invasion assay. \* $P < 0.01$ ; \*\* $P < 0.0001$ .

cell viability inhibition by cotransfection of mature *miR-143* and *miR-145* (Fig. S1).

**Identification of target genes and molecular pathways commonly regulated by both *miR-143* and *miR-145* in RCC.** To gain further insights into the molecular mechanisms and molecular targets regulated by the tumor-suppressive *miR-143/145* cluster in RCC, we first identified putative target genes of *miR-143* or *miR-145* by searching the TargetScan database. The database revealed 3288 target genes, with a total of 421 conserved sites and 3813 poorly conserved sites for *miR-143*. For *miR-145*, 3247 target genes were listed, with a total of 824 conserved sites and 3267 poorly conserved sites. We extracted candidate genes that were regulated by both *miR-143* and

*miR-145*. Among them, 1053 genes were listed as candidate oncogenes that were regulated by the *miR-143/145* cluster. Therefore, we investigated their expression status in RCC clinical specimens and examined gene expression profiles in the GEO database (accession numbers GSE36895 and GSE22541) to evaluate upregulated or downregulated genes in RCC specimens. Among the 1053 genes, 293 genes were significantly up-regulated in 53 RCC specimens compared to 23 non-cancerous kidney tissues. Expression data for the top 20 genes are summarized in Table 1, and the next 49 genes are shown in Table S3. Furthermore, those 293 genes were assigned KEGG annotations using singular enrichment analysis of Gene Codis<sup>(23,24)</sup> to identify molecular pathways regulated by *miR-143*

**Table 1. Top 20 highly expressed genes putatively regulated by *miR-143* and *miR-145* in clinical renal cell carcinoma specimens**

Entrez Gene ID	Symbol	Expression	Fold change	Description	P-value
3099	<i>HK2</i>	Up	26.67	Hexokinase 2	7.09E-10
29923	<i>C7orf68</i>	Up	17.60	Hypoxia inducible lipid droplet-associated	9.62E-10
285	<i>ANGPT2</i>	Up	13.14	Angiopoietin 2	7.09E-10
7903	<i>ST8SIA4</i>	Up	9.03	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 4	7.09E-10
5027	<i>P2RX7</i>	Up	8.05	Purinergic receptor P2X, ligand-gated ion channel, 7	1.56E-08
9770	<i>RASSF2</i>	Up	6.15	Ras association (RalGDS/AF-6) domain family member 2	7.99E-10
2335	<i>FN1</i>	Up	5.45	Fibronectin 1	1.95E-09
10859	<i>LILRB1</i>	Up	5.07	Leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 1	7.83E-10
3778	<i>KCNMA1</i>	Up	4.99	Potassium large conductance calcium-activated channel, subfamily M, alpha member 1	9.22E-10
8542	<i>APOL1</i>	Up	4.72	Apolipoprotein L, 1	6.95E-08
1846	<i>DUSP4</i>	Up	4.44	Dual specificity phosphatase 4	5.11E-09
56833	<i>SLAMF8</i>	Up	4.38	SLAM family member 8	3.63E-07
6507	<i>SLC1A3</i>	Up	4.30	Solute carrier family 1 (glial high affinity glutamate transporter), member 3	1.93E-08
55824	<i>PAG1</i>	Up	3.99	Phosphoprotein associated with glycosphingolipid microdomains 1	9.22E-10
84687	<i>PPP1R9B</i>	Up	3.80	Protein phosphatase 1, regulatory subunit 9B	1.82E-06
3587	<i>IL10RA</i>	Up	3.71	Interleukin 10 receptor, alpha	2.03E-08
8832	<i>CD84</i>	Up	3.65	CD84 molecule	4.00E-09
9201	<i>DCLK1</i>	Up	3.63	Doublecortin-like kinase 1	2.61E-06
963	<i>CD53</i>	Up	3.62	CD53 molecule	3.72E-09
5054	<i>SERPINE1</i>	Up	3.49	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	0.00194

**Table 2. Top seven enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways based on upregulated genes that were commonly targeted by *miR-143* and *miR-145* in renal cell carcinoma**

No. of genes	Annotations	KEGG entry number	P-value	Genes
6	RNA degradation	3018	0.00142926	<i>PABPC1, DCP2, XRN1, NAA38, DIS3, CNOT4</i>
9	Endocytosis	4144	0.00193920	<i>MDM2, IL2RA, AP2B1, ARAP2, ADRBK2, ARRB1, EEA1, CBL, SMURF1</i>
9	Tuberculosis	5152	0.00234740	<i>APAF1, MAPK1, CAMK2D, HLA-DOA, TLR6, HLA-DPB1, IL10RA, MAPK14, EEA1</i>
5	Shigellosis	5131	0.00589653	<i>MAPK1, DIAPH1, ELMO1, MAPK14, CD44</i>
5	Long-term potentiation	4720	0.00656683	<i>MAPK1, CAMK2D, NRAS, PRKX, RPS6KA3</i>
5	p53 signaling pathway	4115	0.00735070	<i>APAF1, MDM2, TNFRSF10B, CCND2, SERPINE1</i>
9	MAPK signaling pathway	4010	0.00829911	<i>MAPK1, NRAS, DUSP4, ELK4, TAOK1, PRKX, MAPK14, RPS6KA3, ARRB1</i>

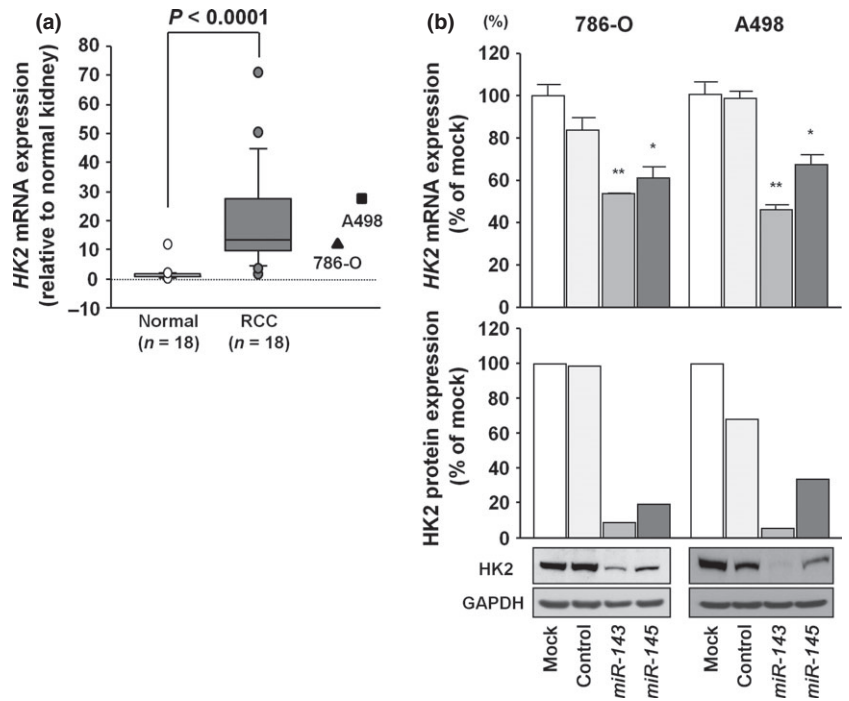
and *miR-145* in RCC. Seven signaling pathways were identified in this analysis (Table 2).

**Expression of *HK2* mRNA in RCC cell lines and clinical RCC specimens.** To validate the functional significance of *miR-143*- and *miR-145*-regulated genes, we focused on *HK2* because it was the most highly upregulated in RCC specimens and had putative target sites for these miRNAs. Quantitative real-time RT-PCR analysis showed that *HK2* mRNA expression was significantly higher in clinical RCC specimens than in adjacent non-cancerous tissues ( $13.311 \pm 2.381$  and  $1.000 \pm 0.302$ , respectively,  $P < 0.0001$ ; Fig. 3a). Also, *HK2* mRNA expression in 786-O and A498 cells was higher than that in normal kidney tissues (relative to the normal,  $16.121 \pm 4.885$  and  $38.591 \pm 3.998$ , respectively,  $P < 0.0001$ ; Fig. 3a). No significant relationship was found between the patients' clinicopathological characteristics and *HK2* mRNA expression (data not shown).

The correlations between *HK2* mRNA expression and *miR-143* or *miR-145* expression were investigated in clinical specimens. A significant inverse correlation was recognized between *HK2* and *miR-145* expression ( $P = 0.0085$ ,  $r = -0.445$ ; Fig. S2). In contrast, no correlation was recognized between *HK2* and *miR-143* expression.

***HK2* as a target of post-transcriptional repression by *miR-143/145* cluster.** Next, we carried out quantitative real-time RT-PCR and Western blot analysis to investigate whether *HK2* mRNA and protein were downregulated by restoration of *miR-143* or *miR-145*. Importantly, *HK2* mRNA and protein levels were significantly repressed in *miR-143* or *miR-145* transfectants in comparison with mock-transfected cells or miRNA-control transfectants (Fig. 3b). Thus, we concluded that *HK2* was regulated by *miR-143* and *miR-145* in RCC cells.

To determine whether the 3'-UTR of *HK2* contained an actual binding site for *miR-143* and *miR-145*, we carried out luciferase reporter assays using several vectors containing the partial sequences of 3'-UTR of *HK2* mRNA, including one of the specific binding sites (for *miR-143*: position 143–149, position 671–677, or position 2291–2298; for *miR-145*: position 2170–2176) or each mutant vector. For *miR-143*, we found that the luminescence intensity was significantly reduced only in the vector including position 2291–2298 ( $P < 0.001$ ; Fig. 4). In contrast, the luminescence intensity was not decreased when the seed sequence of each target site was deleted from the vectors. For *miR-145*, the luminescence intensity was significantly reduced by transfection of the wild-type vector (position 2170–2176), whereas transfection with



**Fig. 3.** (a) Expression levels of hexokinase-2 (*HK2*) mRNA in clinical renal cell carcinoma specimens and cell lines compared with clinically normal kidneys; *GUSB* was used as the internal control. (b, upper) *HK2* mRNA expression after 24 h of transfection with 10 nM *miR-143* or *miR-145*. (b, lower) *HK2* protein expression after 96 h of transfection with 10 nM *miR-143* or *miR-145*. GAPDH was used as a loading control. \* $P < 0.01$ ; \*\* $P < 0.0001$ .

the deletion-type vector blocked this decrease in luminescence (Fig. 4).

**Effects of *HK2* knockdown on cell proliferation and invasion in RCC cells.** Next, we investigated the oncogenic role of *HK2* in RCC cells. The expression levels of *HK2* mRNA and protein were markedly decreased in si-*HK2*-1 and si-*HK2*-2 transfectants in comparison with mock-transfected cells and si-control transfectants ( $P < 0.0001$ ; Fig. 5a). This result showed that the two siRNAs were useful for loss-of-function assays in this study. Thus, to examine the functional role of *HK2*, we carried out loss-of-function studies using si-*HK2*-transfected 786-O and A498 cells. The XTT assays indicated significant inhibition of cell proliferation in the two si-*HK2* transfectants in comparison with mock-transfected cells and si-control transfectants ( $P < 0.0001$ ; Fig. 5b, upper). Matrigel invasion assays showed that the number of invading cells was significantly decreased in the two si-*HK2* transfectants compared with controls ( $P < 0.0001$ ; Fig. 5b, lower).

## Discussion

Accumulating evidence of abnormally expressed miRNAs in human cancers has confirmed the existence of miRNA-mediated functions and has shown that differentially expressed miRNAs contribute to cancer initiation, development, and metastasis.<sup>(7)</sup> In cancer cells, aberrant expression of miRNAs causes destruction of tightly regulated miRNA protein-coding RNA networks. Therefore, studies of differentially expressed miRNAs in cancer cells provide important information regarding the molecular mechanisms underlying oncogenesis and metastasis. To better understand human oncogenesis, we have identified tumor-suppressive miRNAs, miRNA-mediated molecular pathways, and target genes based on miRNA expression signatures in several types of cancers.<sup>9,10,19</sup>

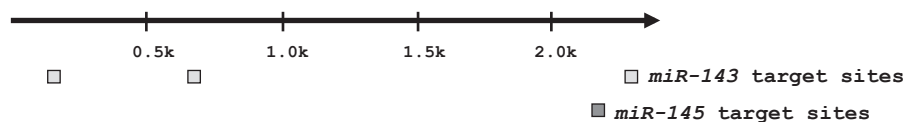
Analysis of downregulated miRNAs in miRNA signatures has revealed that several miRNAs form clusters in the human genome.<sup>(7)</sup> Indeed, 247 human miRNAs have been found to be located in 64 clusters at inter-miRNA distances of <5000 bp in the human genome.<sup>(25)</sup> The *miR-15a/16* cluster acts as a tumor

suppressor by targeting multiple oncogenes, including *BCL2*, *MCL1*, *CCND1*, and *WNT3A*.<sup>(26)</sup> We previously reported that *miR-1-1/133a-2* and *miR-1-2/133a-1* formed clusters in different chromosomal loci in the human genome (i.e., 20q13.33 and 18q11.2, respectively).<sup>(9,27)</sup> These clusters are frequently expressed at reduced levels in cancer cells and function as tumor suppressors in human cancers, including RCC, where they target several oncogenes.<sup>(21)</sup> As shown in our previous reports, although the seed sequences of *miR-1* and *miR-133a* are different, the *miR-1/miR-133a* cluster regulates common oncogenic genes, such as transgelin-2 and purine nucleoside phosphorylase, in cancer cells.<sup>(9,21,27)</sup> Thus, investigating downregulated clustered miRNAs helps to elucidate important molecular mechanisms in human oncogenesis.

Studies of miRNA signatures have shown that the *miR-143/145* cluster is frequently downregulated in several types of cancers.<sup>(12,28)</sup> Also, many reports have indicated that these miRNAs function as tumor suppressors.<sup>(11,12)</sup> In this study, we confirmed that the *miR-143/145* cluster was significantly reduced in RCC and functioned as a tumor suppressor, similar to its role in other cancers. MiRNAs are unique in their ability to regulate multiple protein-coding genes. Bioinformatic predictions have indicated that miRNAs may regulate more than 60% of the protein-coding genes in the human genome.<sup>(6)</sup> Therefore, if these clustered miRNAs function as tumor suppressors, it is important to determine the molecular pathways and target genes that these miRNAs regulate. Unfortunately, in this study we could not find more effective cell viability inhibition through cotransfection of mature *miR-143* and *miR-145* at the same time (Fig. S1). Further examinations are necessary to elucidate the interaction between the miRNAs.

In the current study, we used the TargetScan database and GEO expression data to search for oncogenes regulated by the *miR-143/145* cluster in RCC. A total of 293 genes were listed as oncogenic genes putatively regulated by the *miR-143/145* cluster. Furthermore, a total of 293 targets were categorized into known molecular pathways using KEGG pathways, seven of which, including the p53 signaling pathway, were selected. In the p53 signaling pathway, five genes (*ARAF1*, *MDM2*,

Human *HK2* (NM\_000189) 3'-UTR length:2413



Position 143-149 of 3'-UTR

5' -UGGGUUUUGUCUCUGCAUCUCAU-3'	WT
3' --CUCGAUGUCACGAAGUAGAGU--5'	<i>miR-143</i>
5' -UGGGUUUUGUCUCUG-----AU-3'	Deletion

Position 671-677 of 3'-UTR

5' -UUGGGCCAGCUGUCGHCACUCCAG-3'	WT
3' --CUCGAUGUCACGAAGUAGAGU--5'	<i>miR-143</i>
5' -UUGGGCCAGCUGUCG-----AG-3'	Deletion

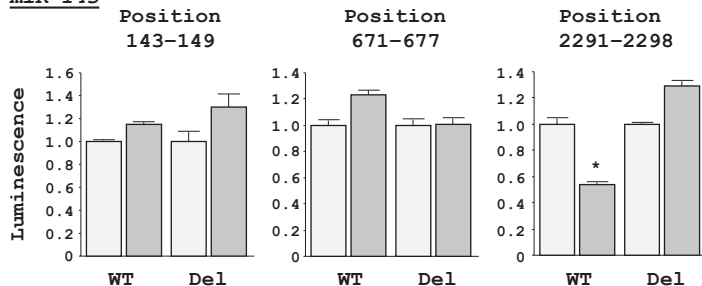
Position 2291-2298 of 3'-UTR

5' -GUGAUGAAUAGCGAAUUCUCA-3'	WT
3' --CUCGAUGUCACGAAGUAGAGU--5'	<i>miR-143</i>
5' -GUGAUGAAUAGCGAA-----A-3'	Deletion

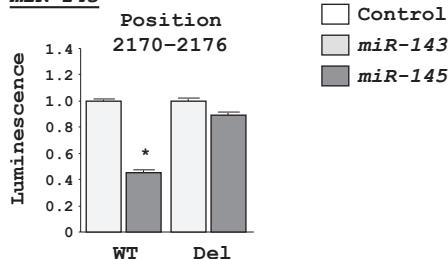
Position 2170-2176 of 3'-UTR

5' --UUUCCAAAAGGAACACUGGAAA-3'	WT
3' -UCCCUAAGGACCCUUUUGACCG---5'	<i>miR-145</i>
5' --UUUCCAAAAGGAAC-----AA-3'	Deletion

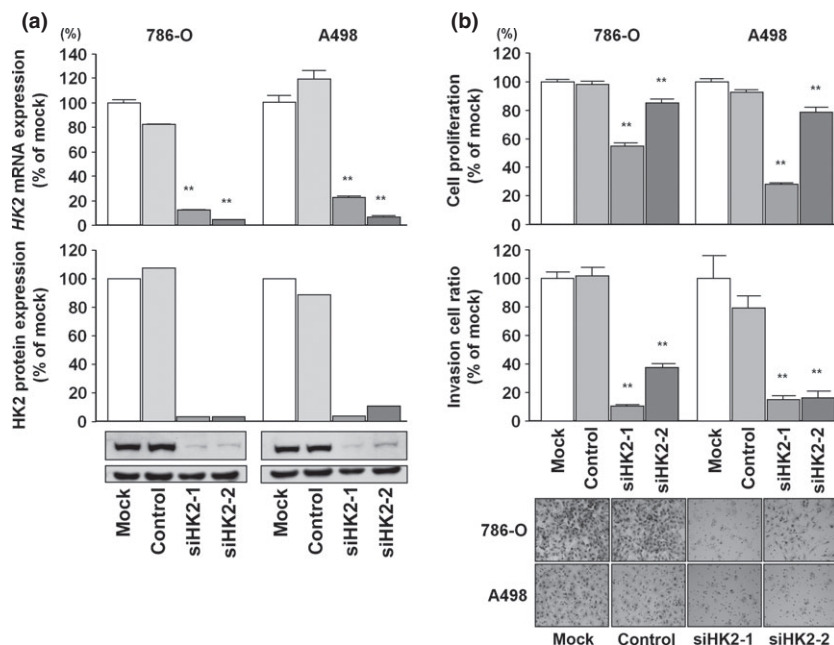
*miR-143*



*miR-145*



**Fig. 4.** Binding sites of *miR-143* and *miR-145* in the 3'-UTR of hexokinase-2 (*HK2*) mRNA. Results of luciferase reporter assays using six vectors encoding putative *miR-143*-target sites: position 143-149 (+); position 671-677 (+); position 2291-2298 (+); and each mutated vector, in which the specific sites targeted by *miR-143* were deleted (Del). Assays also used two vectors encoding the putative *miR-145* target sites: position 2170-2176 (+); and the position-deleted vector. Renilla luciferase values were normalized to firefly luciferase values. \* $P < 0.001$ .



**Fig. 5.** Effects of hexokinase-2 (*HK2*) silencing by si-*HK2* in 786-O and A498 renal cell carcinoma cell lines. (a, upper) *HK2* mRNA expression after 24 h of transfection with 10 nM si-*HK2*. (a, lower) *HK2* protein expression after 72 h of transfection with 10 nM si-*HK2*. GAPDH was used as a loading control. (b, upper) Cell proliferation was determined with XTT assays in 786-O and A498 cells after 72 h of transfection with 10 nM si-*HK2* or siRNA-control, or after mock transfection. (b, lower) Cell invasion activity was determined with the Matrigel invasion assay after 48 h of transfection with si-*HK2*. \*\* $P < 0.0001$ .

*TNFRSF10B*, *CCND2*, and *SERPINE1*) were upregulated in RCC clinical specimens and had putative *miR-143* and *miR-145* binding sites. Among them, p53 E3 ubiquitin protein ligase homolog, double minute 2 protein (MDM2) and serpin peptidase inhibitor, clade E, member 1 (*SERPINE1*)/plasminogen activator inhibitor-1 have been reported to be directly regulated by the *miR-143/145* cluster in human cancers.<sup>(11,12)</sup> These findings support the accuracy of our list of oncogenes targeted by the *miR-143/145* cluster in this study.

Based on recent miRNA studies, novel cancer pathways mediated by *miR-145* have been identified. The tumor suppressor p53 induces the expression of *miR-145* by interaction with a potential p53 response element in the *miR-145* promoter region.<sup>(29)</sup> In the current study, we used two RCC cell lines, 786-O and A498, which show very low expression levels of *miR-143/145*. The NCI-60 cell line database (<http://dtp.nci.nih.gov/branches/btb/iv-clsp.html>) indicates that 786-O has a p53 mutation whereas A498 contains wild-type p53. However, p53 phosphorylation was not actually induced in either wild-type 786-O or A498 cells.<sup>(18)</sup> These facts might be the reason why *miR-143/145* expressions were strongly repressed in 786-O and A498.

Moreover, *c-Myc*, a well-known oncogene that plays an important role in human cancers, is directly repressed by *miR-145*.<sup>(29)</sup> Thus, tumor-suppressive *miR-145*, a new member of the p53 regulatory network, contributes to the direct linkage between p53 and *c-Myc* in human cancer regulatory networks. Therefore, elucidation of the *miR-143/145* cluster-mediated molecular network provides important information to the cancer research field.

Several key molecules of human cancer such *c-Myc*, HIF1a, and p53 are found to be master regulators of metabolism. Recent studies indicate the interplay between miRNAs and oncogenes / tumor suppressor genes (*c-Myc* and *p53*), through key metabolic enzyme effectors, which could facilitate the Warburg effect in cancer cells.<sup>(30,31)</sup> For example, p53-inducible *miR-34a* inhibited key metabolic enzymes such as HK2, HK1, glucose-6-phosphate isomerase, and pyruvate dehydrogenase kinase-1.<sup>(32)</sup> Therefore, silencing of the p53 expression induces glycolysis in cancer cells and might be promoted to cancer cell proliferation, invasion, and resistance of apoptosis. In this study, we focused on the *HK2* gene and investigated the putative targets of the *miR-143/145* cluster and its functional role in RCC. Although there was no significant relationship between *HK2* expression and clinicopathological parameters, including tumor stage, tumor grade, or patient prognosis, due to the small size of the cohort, we found significantly elevated expression of *HK2* mRNA in clinical RCC specimens in comparison with adjacent normal tissues. Furthermore, we showed that *HK2* was directly regulated by *miR-143* and *miR-145*. Although similar results have been reported in other cancers, such as colorectal cancer and head and neck squamous cell carcinoma, in which *HK2* was revealed to be regulated by only *miR-143*,<sup>(33,34)</sup> this is the first report showing that *HK2* was directly regulated by *miR-143* and *miR-145*.

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Cancer cells have a characteristic metabolic phenotype, termed aerobic glycolysis or the Warburg effect, whereby there is increased metabolism of glucose to lactate in the presence of sufficient oxygen.<sup>(14)</sup> The glycolytic enzyme HK2 is crucial for the Warburg effect in cancer cells.<sup>(15)</sup> Thus, we also investigated the functional role of HK2 in RCC cells. Silencing of HK2 in RCC cells significantly inhibited cancer cell proliferation and invasion, suggesting that *HK2* functioned as an oncogene in RCC. These data indicated that the tumor-suppressive *miR-143/145*-HK2 pathway may represent a new target in the development of therapies to treat RCC. Glycolytic enzymes including HK1, HK2, glucose transporter protein type 1 (GLUT1), and GLUT3 appear to have other functions, such as resistance to apoptosis and invasiveness, that directly contribute to human oncogenesis.<sup>(35)</sup> Several reports indicated that the binding capacity and activity of HK2 to mitochondria were significantly elevated in cancer cells and this phenomenon might be controlled to prevent apoptosis.<sup>(36)</sup> In a clinical study, over-expression of HK2 was associated with brain metastasis of breast cancer.<sup>(37)</sup> The detailed molecular mechanisms that over-expression of *HK2* causes in cancer cell invasion and metastasis are still unclear and it is a future research theme. Interestingly, we also found that GLUT1 was overexpressed in RCC cells and that GLUT1 was directly regulated by tumor-suppressive *miR-1291*.<sup>(38)</sup> A facilitator of the transport of glucose across the plasma membranes of mammalian cells,<sup>(39)</sup> GLUT1 is also responsible for the low level of basal glucose uptake required to sustain respiration in all cells.<sup>(39)</sup> Our results (and other studies) indicated that tumor suppressive miRNA contributed to the regulation of glucose metabolism in cancer cells.

A unique aspect of miRNA biogenesis is that one miRNA regulates many protein-coding genes. At present, it is not clear how an miRNA or a cluster of miRNAs, especially the *miR-143/145* cluster, is downregulated. In fact, the nature of the network of miRNAs and protein-coding genes in the human genome remains to be clarified. Further examinations are necessary to develop a better understanding of underlying cancer mechanisms.

In conclusion, the *miR-143/145* cluster was significantly downregulated in RCC clinical specimens and appeared to function as a tumor suppressor through regulation of oncogenic HK2 in RCC. Elucidation of the cancer pathways and target genes regulated by the tumor-suppressive *miR-143/145* cluster could provide new information on potential therapeutic targets in the treatment of RCC.

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## Disclosure Statement

The authors declare no conflict of interest.

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## Supporting Information

Additional supporting information may be found in the online version of this article:

**Fig. S1.** Effects of *miR-143* and *miR-145* transfection on 786-O and A498 renal cell carcinoma cells. Suppression effects of 786-O and A498 cell proliferation after transfection with *miR-143*, *miR-145*, and cotransfection of *miR-143* and *miR-145* as determined by XTT assay. \**P* < 0.0005; \*\**P* < 0.0001.

**Fig. S2.** Inverse correlations between hexokinase-2 (*HK2*) mRNA and *miR-143* or *miR-145* expression in RCC clinical specimens.

**Table S1.** Patient characteristics.

**Table S2.** Insert sequences of wild-type and deletion vectors for luciferase assay.

**Table S3.** Last 49 highly expressed genes regulated by the putative targets *miR-143* and *miR-145* in clinical renal cell carcinoma specimens.