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Short Communication

MicroRNA 96 Is a Post-Transcriptional Suppressor of Anaplastic Lymphoma Kinase Expression

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Anaplastic lymphoma kinase (ALK) constitutes a part of the oncogenic fusion proteins nucleophosmin-ALK and echinoderm microtubule–associated protein like 4–ALK, which are aberrantly expressed in a subset of T-cell anaplastic large-cell lymphoma and non–small-cell lung cancer, respectively. The expression of mutated, constitutively active ALK also occurs in a subset of neuroblastoma tumors. ALK is believed to play an important role in promoting tumor survival. Nevertheless, the mechanisms underlying the expression of ALK in cancer cells are not completely known. MicroRNA (miR) has been implicated in the regulation of the expression of both oncogenes and tumor suppressor genes. We tested the hypothesis that the expression of ALK could be regulated by miR. Three Internet-based algorithms identified miR-96 to potentially bind with the *ALK* 3′-untranslated region. Notably, miR-96 levels were markedly decreased in ALK-expressing cancer cell lines and primary human tumors compared with their normal cellular and tissue counterparts. Transfection of the cell lines with miR-96 decreased levels of the different forms of ALK protein, without significant effects on *ALK* mRNA. Furthermore, miR-96 decreased the phosphorylation of ALK target proteins, including Akt, STAT3, JNK, and type I insulin-like growth factor receptor, and it down-regulated JunB. These effects were associated with reduced proliferation, colony formation, and migration of ALK-expressing cancer cells. These data provide novel evidence that decreases in miR-96 could represent a mechanism underlying the aberrant expression of ALK in cancer cells. ***(Am J Pathol 2012, 180:1772–1780; DOI: 10.1016/j.ajpath.2012.01.008)***

Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase with structural similarities to members of the insulin receptor superfamily, which includes type I insulin-like growth factor (IGF-I), c-Met, and leukocyte tyrosine kinase receptors.[1] The physiological expression of ALK is largely limited to neural cells at an early developmental stage. In addition, ALK is aberrantly expressed as a part of the oncogenic protein nucleophosmin (NPM)–ALK in 60% of the T-cell anaplastic large-cell lymphoma cases that harbor the chromosomal translocation t(2;5)(p23;q35).[2,3] The recent discovery of the chimeric protein, echinoderm microtubule–associated protein like 4 (EML4)–ALK, that associates the inv(2)(p21p23) chromosomal abnormality, identified in 5% to 7% of non–small-cell lung cancer (NSCLC) cases, and the activating mutations of *ALK* in familial

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neuroblastoma further emphasizes the significance of ALK in human malignancies.[4–8] It is believed that ALK plays an important role in these malignancies by promoting the survival and proliferation and inhibiting the apoptosis of the neoplastic cells.[9] The oncogenic effects of ALK appear to be related to its ability to interact with and activate, through phosphorylation, several important survival-promoting transcription factors and protein kinases, including STAT3, phosphatidylinositol 3-kinase/Akt, mitogen-activated protein kinase, and IGF-I receptor (IGF-IR).[10–15] Therefore, ALK inhibitors are being tested in clinical trials that include patients with ALK-expressing (ALK^+) tumors with the hope that targeting ALK will eventually transform into an effective strategy to eradicate these aggressive types of cancer.[16–19] Intriguingly, the mechanisms that contribute to the aberrant expression of ALK in cancer cells are not completely known. Although translocations and inversions involving the *ALK* gene on chromosome 2p23 are considered to be one mechanism,[2,20] these chromosomal abnormalities are most likely not the only factor. In agreement with this idea, translocations and inversions involving *ALK* do not exist in tumors in which full-length or mutated *ALK* is aberrantly expressed.

MicroRNAs (miRNAs) form a class of small, noncoding, regulatory RNAs that bind to the 3′-untranslated region (UTR) of target mRNA, resulting in its degradation or translational inhibition. Deregulation of miRNA occurs in cancer, and certain miRNAs function as oncogenes or tumor suppressor genes.[21–24] We reasoned that one possible mechanism for the up-regulation of ALK expression in cancer cells could be mediated through deregulated expression of miRNA.

Materials and Methods

Potential Targets of miRNA

Analysis of miRNA-predicted targets was performed by using three different Internet-based algorithms: TargetScan (*http://www.targetscan.org*, last accessed March 14, 2012), miRanda (*http://www.microrna.org/microrna/home.do*, last accessed March 14, 2012), and PicTar (*http://pictar.mdc-berlin.de*, last accessed March 14, 2012).

Antibodies

Antibodies to phosphorylated ALK (pALK, Tyr1604; 3341), pSTAT3 (Tyr705; 9131), pIGF-IR (Tyr1131; 3021), Akt (9272), pAkt (Ser473; 4051), JNK (3708s), pJNK (Thr183/Tyr185; 4668), and JunB (3753) were obtained from Cell Signaling Technology (Danvers, MA). IGF-IRβ (39–6700) was obtained from Zymed Laboratories (South San Francisco, CA), ALK (M7195) was obtained from Dako (Carpinteria, CA), STAT3 (sc-8019) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and β-actin (A-2228) was obtained from Sigma (St Louis, MO).

Cell Lines and Human Tissue Specimens

The NPM-ALK–expressing (NPM-ALK^+) T-cell lymphoma cell lines Karpas 299, SUP-M2, SU-DHL-1, SR-786, and DEL were obtained from DSMZ (Braunschweig, Germany). The NSCLC cell line H2228, which expresses EML4-ALK; the neuroblastoma cell line SH-SY5Y, which expresses ALK with the somatic activating mutation F1174L (ALK^{F1174L}); and the human embryonic kidney cell line 293T were obtained from American Type Culture Collection (Manassas, VA). Control human cells used in the study included normal human T lymphocytes (Stemcell Technologies, Vancouver, BC, Canada), the human lung epithelial cells BEAS-2B (American Type Culture Collection), and the human brain neural cells HCN-1A (American Type Culture Collection). Cell lines were maintained in RPMI 1640 medium (Karpas 299, SUP-M2, SU-DHL-1, SR-786, DEL, and H2228), minimal essential medium (SH-SY5Y), or Dulbecco's modified Eagle's medium (HCN-1A and 293T), supplemented with 10% fetal bovine serum (Sigma), 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in humidified air with 5% CO_2. The BEAS-2B cells were maintained in bronchial epithelial cell growth medium, with all supplements as provided by the manufacturer (Lonza, Allendale, NJ).

The expression of miR-96 was also analyzed in archival formalin-fixed, paraffin-embedded (FFPE) tissue sections from five patients with ALK^+ T-cell lymphoma and four patients with EML4-ALK^+ NSCLC. The expression of ALK was established based on immunohistochemical staining in all tumors. In addition, EML4-ALK expression was confirmed by fluorescence *in situ* hybridization analysis. Three reactive lymph nodes and two normal lung tissues were used as controls. Approval of the institutional ethical committee was secured before the initiation of these studies.

Transfection of miRNA and Plasmids

The RNA mimic for miR-96 and the control mimic miRNA (cel-miR-67; developed from *Caenorhabditis elegans* with no sequence identity with human miRNA) were obtained from Dharmacon (Lafayette, CO). Electroporation and the Nucleofector V solution were used (A-030 program; Amaxa Biosystems, Basel, Switzerland) to transfect the NPM-ALK^+ T-cell lymphoma cell lines with miRNA or plasmids. Transfection of H2228, SH-SY5Y, and 293T cells was performed by using DharmaFECT Duo transfection reagent (Dharmacon) or Lipofectamin 2000 (Invitrogen, Carlsbad, CA).

Plasmids, Cloning, and Luciferase Assay

To induce the expression of wild-type (WT) NPM-ALK protein, portions of *NPM-ALK* were amplified by PCR using the following primers: *NPM-ALK*-Forward: 5′-GCGAAGCTTATGGAAGATTCGATGGACAT-3′ (bearing HindIII cassette), and *NPM-ALK*-Reverse: 5′-AGTCTCGAGTCAGGGCCCAGGCTGGTTCATG-3′ (bearing XhoI cassette). The PCR product subsequently was digested

with HindIII/XhoI and subcloned into pCDNA4.0 vector (Invitrogen). To induce the expression of NPM-ALK protein with the 3′-UTR region containing the miR-96 binding site, two sets of primers were used in 3 PCR reactions. In the first reaction, *NPM-ALK*-Forward (F1): 5′-GCGAAGCTTATGGAAGATTCGATGGACAT-3′ (bearing HindIII cassette) and *NPM-ALK*-3′-UTR-Reverse (R1): 5′-GACCGAGCTCAGGGCCCAGGCT-3′ were used, and in the second reaction, *NPM-ALK*-3′-UTR-Forward (F2): 5′-AGCCTGGGCCCTGAGCTCGGTC-3′; *NPM-ALK*-3′-UTR-Reverse (R2): 5′-GTGCTCGAGAGTCATTACAAATAACTCC-3′ (bearing XhoI cassette) were used. Finally, in the third PCR reaction, F1 and R2 primers were used to generate the final product that contains NPM-ALK with 3′-UTR. The PCR product was subsequently digested with HindIII/XhoI and subcloned into pCDNA4.0 vector. Positive clones were confirmed by sequencing.

For the luciferase assay experiments, human *ALK* 3′-UTR (454 bp) was amplified from genomic DNA using the following primers: *ALK*-3′-UTR-Forward: 5′-AGTTCTAGAATGAACCAGCCTGGGCCCTGAGCTCG-3′ (bearing XbaI cassette), *ALK*-3′-UTR-Reverse: 5′-AGTTCTAGAAGTCATTACAAATAACTCCTTTATTTC-3′ (bearing XbaI cassette), cloned in XbaI site of pGL3-control vector (Promega, Madison, WI), and designated as pGL3-control-WT-*ALK*-3′-UTR. Also, *ALK* mutated at 3′-UTR (original sequence: 5′-GTCACGTTTTGTTTTGTGCCAAC-3′; sequence after mutation: 5′-GTCACGTTTTGTTTTGTAAC-3′) was generated by using site directed mutagenesis and a commercially available kit (200523, Agilent Technologies, Santa Clara, CA). Primers used for PCR amplification of mutated *ALK* 3′-UTR were: Forward: 5′-TAGTCAGTTGACGAAGATCTGGTCAAGAACTAATTAAATGTTTCATT-3′; and Reverse: 5′-AATGAAACATTTAATTAGTTCTTGACCAGATCTTCGTCAACTGACTA-3′. The 293T cells were transfected with empty vector (pGL3-control vector, Promega), WT *ALK* (pGL3-WT-*ALK*-3′-UTR) or mutated (MUT) *ALK* (pGL3-MUT-*ALK*-3′-UTR) for 48 hours. Renilla luciferase activity was measured by using DualGlo Luciferase assay system (Promega).

qPCR Analysis

Total RNA was extracted according to the manufacturer's instructions (Qiagen, Valencia, CA). The relative level of miRNA was determined by quantitative real-time PCR (qPCR) using specific primers according to the TaqMan miRNA assay protocol (Applied Biosystems, Foster City, CA). To measure miRNA levels in FFPE tissue sections prepared from tissue specimens, the High Pure FFPE RNA Micro Kit (Roche Applied Science, Indianapolis, IN) was used, according to the manufacturer's protocol. After isolation of total RNA, complementary DNA was synthesized by using the TaqMan miRNA RT-PCR Kit (Applied Biosystems). The miRNAs were amplified individually and in duplicate. The relative level of individual miRNA with reference to the small nucleolar RNA RNU48 (Applied Biosystems) was calculated using the ΔC_T method. For *ALK* cDNA synthesis, 300 ng of total RNA was reverse transcribed using random primers and SuperScript II Reverse Transcriptase (Invitrogen). *NPM-ALK*, *EML4-ALK*, and *ALK* mRNAs were quantified by the TaqMan qPCR kit (Applied Biosystems) and normalized to the ribosomal RNA 18S. Each sample in the PCR assay included 12.5 μL of TaqMan Universal Master Mix No Amperase UNG (2×), 1.15 μL of 20 times Assays-on-Demand Gene Expression Assay Mix, 10.35 μL of nuclease-free H_2O, and 1.0 μL of cDNA diluted in RNase-free water, in a final volume of 25 μL. All qPCR tests were performed by using the Applied Biosystems 7500 Sequence Detection System. The PCR thermal cycling conditions were as follows: 10 minutes at 95°C for AmpliTaq Gold activation and 45 cycles for the melting (95°C, 15 seconds) and annealing/extension (60°C, 1 minute) steps. The C_T data were determined using default threshold settings.

Measurement of Cell Proliferation by BrdU Assay

Cell lines were transfected with control mimic or miR-96, and cell proliferation was measured using the 5-bromo-2′-deoxyuridine (BrdU) assay kit (ExAlpha, Shirley, MA), according to the manufacturer's instructions. Briefly, 2×10^5 cells/mL were plated into a 96-well plate. The BrdU label (1:500 dilution) was added, and the plate was incubated for 24 hours at 37°C. Cells were then fixed for 30 minutes at room temperature. The anti-BrdU antibody was added for 1 hour after washing, followed by peroxidase goat anti-mouse IgG conjugate (1:2000 dilution) for 30 minutes. Thereafter, the 3,3′,5,5′-tetramethylbenzidine peroxidase substrate was added and incubated for 30 minutes at room temperature in the dark. The plate was read at 450 nm using an ELISA plate reader after adding the stop solution. Experiments were performed in triplicate.

Methylcellulose Colony Assay

The methylcellulose medium was prepared by mixing 1.0% methylcellulose stock (Methocult H4230; Stemcell Technologies) with RPMI 1640 medium, prepared as previously described, in a 1:5 ratio. Then, 3.5 mL of methylcellulose was added to 15-mL tubes. Cells were resuspended to eliminate clumping and then added in a 1:10 (v/v) ratio to the methylcellulose. Tubes were tightly capped, and the mix was gently inverted several times. Then, 500 μL of the mix was divided into humidified 6-well plates in triplicate. Plates were placed in a humidified incubator at 37°C in 5% CO_2 for 5 days and then *p*-iodonitrotetrazolium violet was added for 48 hours. Colonies were visualized using Alexfluor (Invitrogen).

Cell Migration Assay

Untreated cells or cells transfected with negative control mimic or miR-96 for 48 hours were washed with PBS and resuspended in serum-free medium. Thereafter, 0.6 mL of serum-free medium, with or without 500 ng/mL IGF-I (R&D Systems, Minneapolis, MN), was added to the bot-

Figure 1. A: Sequence alignment shows potential binding sites between miR-96 seed sequence and *ALK* 3′-UTR. **B:** The qPCR analysis demonstrates that the endogenous expression of miR-96 is markedly decreased in the NPM-ALK$^+$ T-cell lymphoma cell lines Karpas 299, SUP-M2, SU-DHL-1, SR-786, and DEL. In contrast, a remarkably higher level of miR-96 is seen in the normal human T lymphocytes ($P < 0.0001$). **C:** The NSCLC cell line, H2228, and the neuroblastoma cell line, SH-SY5Y, which express EML4-ALK and ALK^{F1174L} proteins, respectively, express negligible amounts of miR-96 compared with the control cell lines: the normal human lung epithelial cells, BEAS-2B, and the normal human neural brain cells, HCN-1A ($P < 0.0001$ and $P = 0.0007$), respectively. **D** and **E:** The levels of *ALK* mRNA are markedly increased in the ALK$^+$ cancer cell lines when compared with normal human T-lymphocyte and the HCN-1A (**D**) and the BEAS-2B (**E**) cell lines. $^*P < 0.05$, $^\dagger P < 0.01$, $^\ddagger P < 0.0001$ compared with the corresponding normal cellular counterparts. **F:** The qPCR analysis shows that the expression of miR-96 is significantly decreased in five human primary ALK$^+$ T-cell lymphoma tumors compared with three reactive lymph nodes. $P = 0.0003$. LN, lymph node; Pt, patient. **G:** miR-96 is markedly decreased in four EML4-ALK$^+$ NSCLC primary tumors compared with two normal human lung tissues. $P = 0.007$. The results represent the mean ± SE of three different experiments, performed in triplicate, with consistent results.

tom compartment of a 24-well Transwell plate (Fisher Scientific, Pittsburgh, PA), followed by the addition of 0.1 mL of transfected cells into the top compartment. Plates were incubated for 4 hours at 37°C. Cells migrating through the membrane into the lower chamber were counted using a particle counter and size analyzer (Coulter, Indianapolis, IN).

Western Blot Analysis

Cell lysates were collected using standard techniques, as previously described.[15,25] The lysis buffer contained 25 mmol/L HEPES (pH 7.7), 400 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 2 mmol/L EDTA, 0.5% Triton X-100, 0.1 mmol/L phenylmethylsulfonyl fluoride, 3 mmol/L dithiothreitol, phosphatase inhibitor cocktail (20 mmol/L β-glycerol phosphate and 1 mmol/L Na_3VO_4; Roche Applied Science), and protease inhibitor cocktail (10 μg/mL leupeptin, 2 μg/mL pepstatin, 50 μg/mL antipain, 1× benzamidine, 2 μg/mL aprotinin, and 20 μg/mL chymostatin; Roche Applied Science). For Western blot analysis, 50 to 80 μg of total proteins was electrophoresed on 8% SDS-PAGE. The proteins were transferred to nitrocellulose membranes, probed with specific primary antibodies, and then probed with the appropriate secondary antibodies. Proteins were detected using a chemiluminescence-based kit (GE Healthcare, Piscataway, NJ).

Statistical Analysis

The Student's *t*-test for paired data was used. $P < 0.05$ was considered statistically significant.

Results

Potential Binding Sites between miR-96 and ALK 3′-UTR

We used three different miRNA-target prediction algorithms, and all of them identified miR-96 as an miRNA that can potentially bind with *ALK* 3′-UTR. The binding sites between miR-96 and *ALK* 3′-UTR are shown in Figure 1A.

miR-96 is Markedly Decreased in Cancer Cell Lines and Primary Human Tumors that Aberrantly Express the Different Forms of ALK Protein

We used qPCR analysis to measure the levels of miR-96 in human ALK$^+$ cancer cell lines. Compared with normal human T lymphocytes, the level of miR-96 was markedly reduced in the T-cell lymphoma cell lines that express NPM-ALK (Figure 1B). miR-96 was also decreased in the NSCLC cell line H2228, which expresses EML4-ALK, compared with the normal human lung epithelium cell line BEAS-2B, and in the neuroblastoma cell line SH-SY5Y, which expresses ALK^{F1174L}, compared with normal human neural brain cells HCN-1A (Figure 1C). Conversely, the ALK$^+$ cancer cell lines expressed much higher levels of *ALK* mRNA compared with the normal cellular counterparts (Figure 1, D and E). Similar to the cell lines, miR-96 was remarkably decreased in ALK$^+$ T-cell lymphoma tumors compared with reactive lymph nodes (Figure 1F). Furthermore, miR-96 was significantly decreased in EML4-ALK$^+$ NSCLC tumors compared with normal lung tissues (Figure 1G).

Interactions between miR-96 and ALK 3′-UTR

To examine whether miR-96 interacts with *ALK* 3′-UTR, we used a luciferase assay analysis in 293T cells that showed that miR-96 suppresses the luciferase activity driven by *ALK* 3′-UTR, not the luciferase activity driven by *ALK* mutated at 3′-UTR (Figure 2A). Furthermore, 293T cells transfected with *NPM-ALK* plasmid associated with its 3′-UTR or, alternatively, with *NPM-ALK* plasmid that lacks the 3′-UTR, were cotransfected with miR-96. Thereafter, Western blot studies showed that miR-96 induced a marked decrease in the *NPM-ALK*-3′-UTR construct, not in the *NPM-ALK* construct that lacked 3′-UTR (Figure 2B).

Transfection of miR-96 Decreases Levels of the Different Forms of ALK Protein, and Down-Regulation of the ALK Proteins Enhances the Expression of miR-96

Transfection of Karpas 299 and SUP-M2 with miR-96 induced a substantial decrease in NPM-ALK protein levels at 48 hours after transfection (Figure 3A). Furthermore, miR-96 also efficiently decreased the levels of EML4-ALK and ALK^{F1174L} proteins in the H2228 and SH-SY5Y cell lines, respectively (Figure 3A). Transfection of

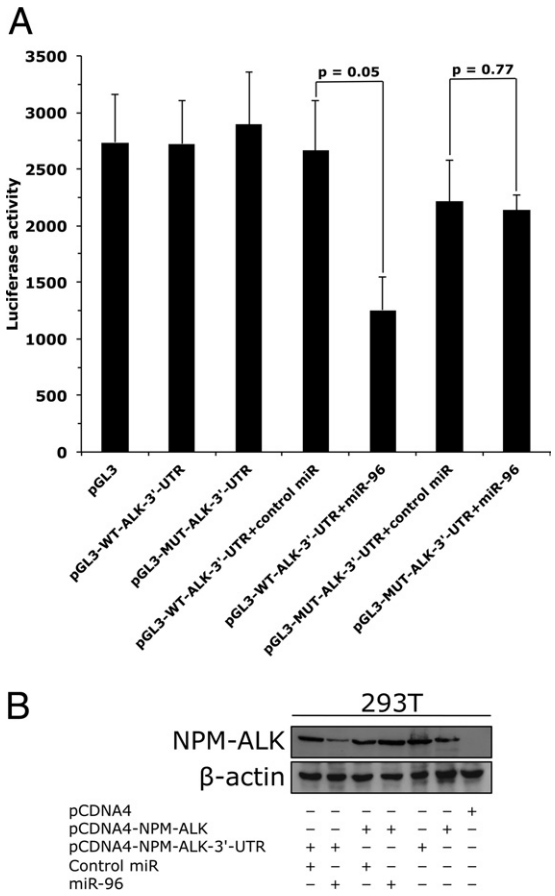

Figure 2. **A:** The 293T cells were cotransfected with WT *ALK* or *ALK* mutated at 3′-UTR (MUT *ALK*) simultaneously with miR-96 or control mimic cel-miR-67. After 48 hours, firefly and Renilla luciferase activities were measured using a dual-luciferase assay. The results represent firefly luciferase normalized to Renilla luciferase. Although miR-96 induced a marked decrease in luciferase activity of *ALK* with intact 3′-UTR ($P = 0.05$), it failed to induce a similar effect when the *ALK* 3′-UTR was mutated at its binding site with miR-96 ($P = 0.77$). Luciferase activity measured in control 293T cells transfected with empty vector pGL3, WT *ALK*, or *ALK* mutated at 3′-UTR alone is also depicted. The mean ± SE values of three different experiments, performed in triplicate, are shown. **B:** *NPM-ALK* with 3′-UTR (pCDNA4-*NPM-ALK*-3′-UTR) or *NPM-ALK* lacking its 3′-UTR (pCDNA4-*NPM-ALK*) was cotransfected into 293T cells with miR-96 or the control miRNA cel-miR-67, as indicated. After 48 hours, NPM-ALK protein levels were determined by using Western blot analysis. miR-96 induced a significant decrease in NPM-ALK transfected with its 3′-UTR, with no effect on the levels of NPM-ALK that lacks the 3′-UTR. Baseline expression levels of the empty vector pCDNA4, pCDNA4-*NPM-ALK*, and pCDNA4-*NPM-ALK*-3′-UTR are illustrated. β-Actin shows equal protein loading.

miR-96 did not induce significant change in *ALK* mRNA (data not shown). The decrease in the different forms of ALK protein was associated with a remarkable down-regulation of their phosphorylated forms, which was also associated with a marked decrease in the phosphorylation levels of ALK oncogenic target proteins, including Akt, STAT3, JNK, and IGF-IR. Similar changes were not detected in the basal levels of Akt, STAT3, JNK, or IGF-IR (Figure 3A). The decrease in ALK was also associated with a marked decrease in JunB protein (Figure 3A). These experiments were also performed in other NPM-ALK$^+$ T-cell lymphoma cell lines, with similar results (data not shown). When the ALK proteins were efficiently down-regulated by small-interfering RNA (siRNA; Figure 3B), we detected a significant increase

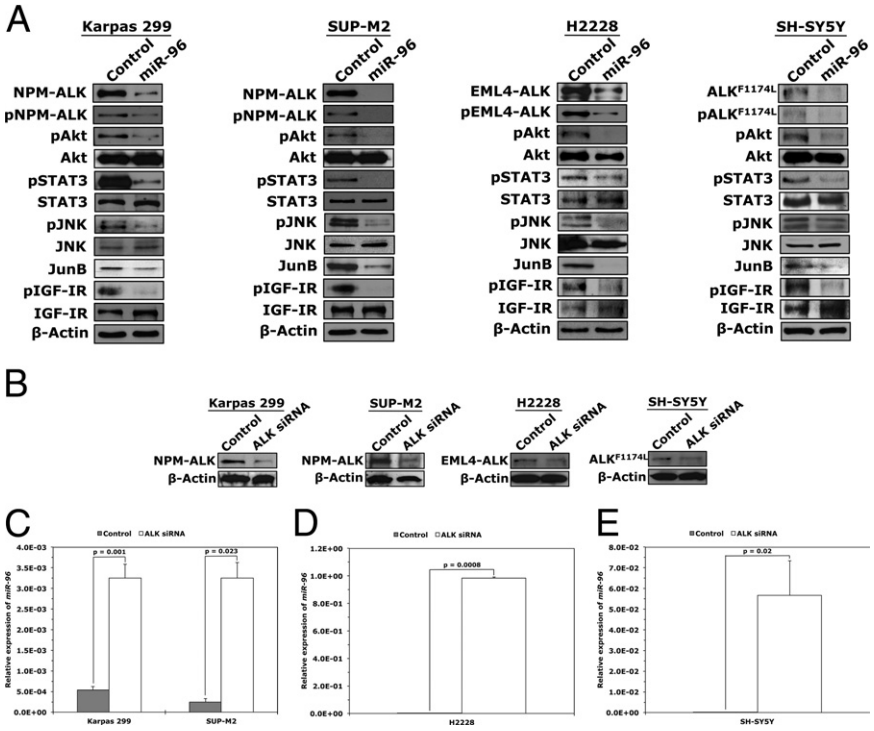

Figure 3. A: A Western blot shows that miR-96 down-regulates the expression of NPM-ALK in Karpas 299 and SUP-M2, EML4-ALK in H2228, and ALK^{F1174L} in SH-SY5Y cell lines. Control cells were transfected with cel-miR-67. The decrease in the different forms of ALK protein was associated with a marked decrease in the corresponding phosphorylated ALK protein, which was also associated with a pronounced down-regulation of pAkt, pSTAT3, pJNK, JunB, and pIGF-IR. Changes were not seen in the basal levels of Akt, STAT3, JNK, or IGF-IR proteins. β-Actin shows equal protein loading. The Western blot studies were repeated four times with consistent results. **B:** A Western blot shows that siRNA induced a marked decrease in ALK protein levels in Karpas 299, SUP-M2, H2228, and SH-SY5Y cell lines. β-Actin shows equal protein loading. **C:** The decrease of NPM-ALK by siRNA in Karpas 299 and SUP-M2 cell lines was associated with a significant increase in miR-96 expression ($P = 0.001$ and $P = 0.023$, respectively, compared with cells transfected with scrambled siRNA). **D** and **E:** The decrease in EML4-ALK in H2228 (**D**) and ALK^{F1174L} in SH-SY5Y (**E**) cell lines by ALK siRNA was also associated with a marked increase in miR-96 levels ($P = 0.0008$ and $P = 0.02$, respectively). The mean ± SE values of three different experiments, performed in triplicate, are shown.

in miR-96 levels in the ALK$^+$ cancer cell lines, suggesting that ALK may induce reciprocal inhibitory effects on the expression of miR-96 (Figure 3, C–E).

Transfection of miR-96 Decreases Proliferation, Colony Formation, and Migration of ALK$^+$ Cancer Cells

Next, we used a BrdU assay to test the effect of miR-96 on the proliferation of the cancer cell lines that express the different forms of ALK protein. When compared with cells transfected with the negative control cel-miR-67, Karpas 299, SUP-M2, H2228, and SH-SY5Y cell lines transfected with miR-96 demonstrated significant decreases in their proliferation (Figure 4A). In addition, Karpas 299 and DEL cell lines transfected with miR-96 demonstrated a marked decrease in their ability to form colonies in methylcellulose (Figure 4B). Moreover, transfection of the ALK$^+$ cancer cell lines with miR-96 abrogated their migration in response to IGF-I stimulation (Figure 4C).

Discussion

The regulatory mechanisms that lead to up-regulation or down-regulation of oncogenic or anti-oncogenic proteins, respectively, are not completely known. For instance, ALK is a tyrosine kinase with a well-described oncogenic potential.[9] By excluding the early stage of human development, during which ALK is primarily expressed in neuronal cells, the expression of ALK is aberrant and essentially limited to cancerous cells. Although chromosomal abnormalities involving the *ALK* gene, such as t(2;5)(p23;q35) and inv(2)(p21p23), seen in some cases of T-cell lymphoma and NSCLC, respectively, contribute to the aberrant expression of ALK,[2,20] these abnormalities do not exist in all types of ALK$^+$ cancer cells. Therefore, it is reasonable to assume that other mechanisms may contribute to the aberrant expression of ALK.

In the present study, we sought to explore whether ALK oncogenic tyrosine kinase is regulated by miRNA. To predict miRNA that may regulate ALK expression, we used a consensus approach by using three miRNA-target prediction algorithms. The three algorithms simultaneously identified miR-96 as an miRNA that contained a potential binding site compatible with *ALK* 3′-UTR. Because this 3′-UTR region also exists in *NPM-ALK* and *EML4-ALK*, we measured endogenous levels of miR-96 in several cell lines that express different forms of ALK protein. miR-96 expression levels in the NPM-ALK$^+$ T-cell lymphoma cell lines Karpas 299, SUP-M2, SU-DHL-1, SR-786, and DEL were remarkably lower than the normal human T lymphocytes. Similarly, the expression of miR-96 was markedly decreased in the NSCLC cell line H2228, which expresses EML4-ALK, and the neuroblastoma cell line SH-SY5Y, which expresses ALK^{F1174L}, compared with the normal human epithelial lung cells BEAS-B2 and the normal human neural brain cells HCN-1A, respectively.

The interactions between miR-96 and *ALK* 3′-UTR were demonstrated when miR-96 down-regulated the luciferase activity of WT *ALK* and, in contrast, it failed to induce a similar effect on an *ALK* construct with mutated 3′-UTR. To further confirm the specific nature of the interactions between miR-96 and *ALK* 3′-UTR, we inserted *NPM-ALK* containing or lacking the 3′-UTR into expression vector and cotransfected these constructs with miR-96 or control miRNA into 293T cells. Western blot studies

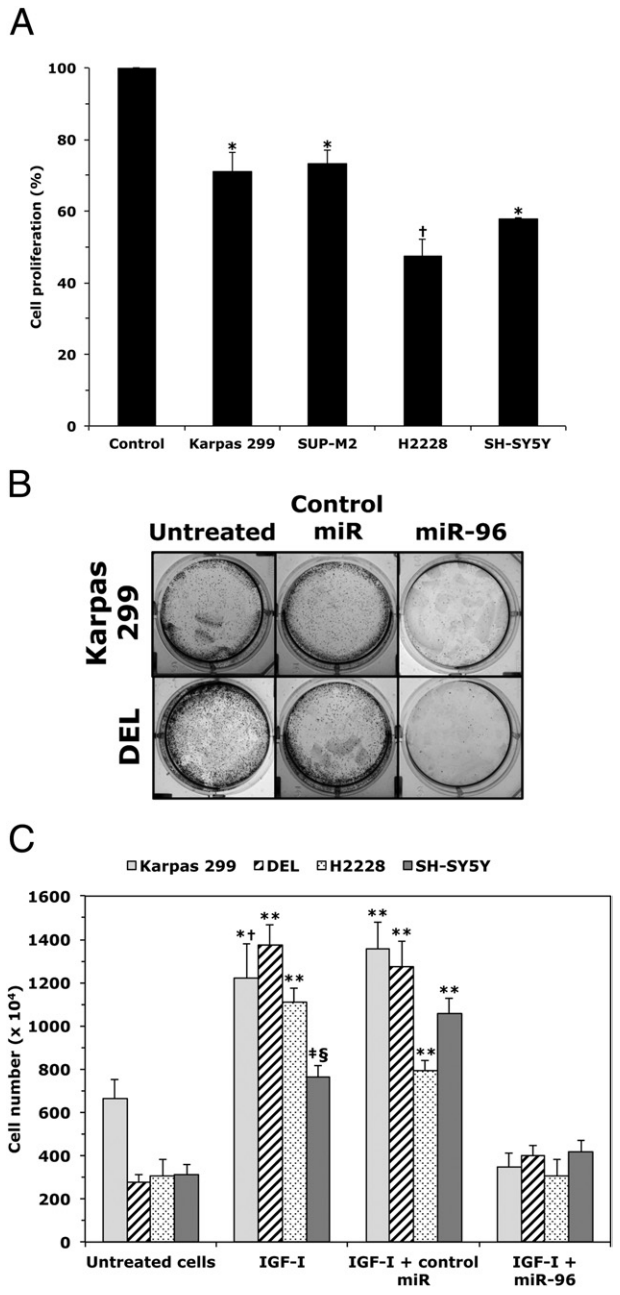

Figure 4. A: The BrdU assay demonstrates that miR-96 decreases the proliferation of the different ALK^+ neoplastic cell lines at 48 hours after transfection. The proliferation of cells transfected with miR-96 is shown compared with the proliferation of cells transfected with the negative control cel-miR-67. The results represent the mean ± SE of three different experiments, performed in triplicate, with consistent findings. $^*P < 0.05$, $^\dagger P < 0.01$ compared with control cells. **B:** Although transfection of miR-96 abrogates Karpas 299 and DEL cell growth in methylcellulose, cel-miR-67 failed to induce similar effects. The experiment was repeated three times, with similar results. **C:** miR-96 significantly decreased IGF-I–stimulated migration of the ALK^+ cancer cell lines. $^*P < 0.001$ versus IGF-I + miR-96; $^\dagger P < 0.05$ versus untreated cells; $^{**}P < 0.001$ versus untreated cells and IGF-I + miR-96; $^\ddagger P < 0.01$ versus IGF-I + miR-96; $^\S P < 0.001$ versus untreated cells. The results represent the mean ± SE of three different experiments, performed in triplicate.

showed that only the NPM-ALK construct that included the 3′-UTR was down-regulated by miR-96, further demonstrating the presence of an miR-96–specific target region within the 3′-UTR of *ALK*. These results support the direct interactions between miR-96 and *ALK* 3′-UTR.

Next, we questioned whether miR-96 is involved in the regulation of ALK expression. Karpas 299, SUP-M2, H2228, and SH-SY5Y cell lines were transfected with miR-96 or a negative control miRNA. miR-96 induced marked down-regulation of NPM-ALK, EML4-ALK, and ALK^{F1174L} proteins. In contrast, similar changes were not observed in *NPM-ALK, EML4-ALK,* or ALK^{F1174L} mRNA after transfection of miR-96 (data not shown). These data suggest that miR-96 induces negative regulation of ALK expression post-transcriptionally. When the different forms of ALK protein were down-regulated by siRNA, there was a marked increase in miR-96 levels, indicating that ALK appears to sustain its own expression by exerting a reciprocal negative feedback loop that hinders the expression of miR-96. This is an intriguing finding, and further studies are required to characterize the mechanisms by which ALK induces this effect.

The different activated forms of ALK protein are believed to play a central role in driving the oncogenic signaling and inducing cellular transformation through the phosphorylation/activation of important survival proteins, including Akt, STAT3, and JNK, and up-regulating the expression of JunB.[3,4,9–14] Consistent with these observations, down-regulation of NPM-ALK, EML4-ALK, or ALK^{F1174L} by miR-96 substantially decreased their phosphorylated/activated forms, which was simultaneously associated with decreased pSTAT3, pAkt, and pJNK levels and decreased JunB levels. We have recently shown that NPM-ALK can also activate/phosphorylate IGF-IR oncogenic tyrosine kinase, which enhances the oncogenic effects of NPM-ALK.[15] In the present study, when we used miR-96, we found not only that the down-regulation of NPM-ALK but also the down-regulation of EML4-ALK and ALK^{F1174L} can induce a marked decrease in pIGF-IR levels, providing novel evidence that the different forms of ALK protein are effectively capable of phosphorylating IGF-IR tyrosine kinase, in a manner similar to their effects on Akt, STAT3, and JNK. Notably, the basal levels of Akt, STAT3, JNK, and IGF-IR remained unchanged after transfection of miR-96, indicating that miR-96 had no direct effect on these proteins and that the decrease in their phosphorylation was secondary to the decrease in ALK/pALK levels. However, transfection of miR-96 was also associated with a decrease in JunB protein expression. Thus, we questioned whether JunB protein is a direct target of miR-96. Nevertheless, searching the three Internet-based algorithms did not lead to the identification of potential binding sites between miR-96 and *JunB* 3′-UTR. Therefore, it is most likely that the decrease in JunB levels after transfection of miR-96 was secondary to the decrease in ALK protein.[14] It is important to emphasize that the decrease in pAkt, pSTAT3, pJNK, pIGF-IR, and JunB after down-regulating ALK by miR-96 was associated with a significant decrease in cell viability, growth in methylcellulose, and IGF-I–stimulated migration of ALK^+ cancer cell lines. The inhibitory effects of miR-96 on IGF-I–stimulated migration further support that the IGF-I/IGF-IR signaling axis is functionally active in these tumors and that this axis is interacting with ALK.[15]

Mutations in the seed region of miR-96 were previously implicated in autosomal dominant, progressive hearing

loss.[26] These mutations may induce marked reduction in miR-96 potential to target mRNA. The previously reported roles of miR-96 in cancer appear to be cancer cell type dependent. For example, miR-96 decreased FOXO1 protein levels and, consequently, contributed to the survival of breast cancer cells.[27] Alternatively, a more recent study[28] showed that miR-96 induces tumor suppression of pancreatic cancer cells by decreasing the expression of KRAS. The latter findings are in line with our proposed model in which miR-96 down-regulates the expression of ALK and, therefore, decreases the proliferation, colony formation, and migration of ALK$^+$ cells. Similar to our findings in miR-96, a recent study[29] showed that the expression of abelson murine leukemia viral oncogene homolog 1 (ABL1) and breakpoint cluster region-abelson murine leukemia viral oncogene homolog 1 (BCR-ABL1), two kinases with some biological features that are comparable to ALK and NPM-ALK, was attributed to decreased miR-203 levels.

ALK has been recently highlighted as a potential therapeutic target, and ALK small-molecule inhibitors are being tested in clinical trials that include patients with ALK$^+$ malignant tumors.[16–19] Although these inhibitors may prove to be beneficial, the recent identification of mutations or amplifications of ALK that demonstrate significant resistance to ALK inhibitors stresses the importance of identifying alternative and, perhaps, more effective approaches to target ALK.[30,31] Our findings illustrate miR-96 as a novel mechanism for the suppression of ALK expression and suggest that transfection of miR-96 could represent an effective strategy to down-regulate ALK and to eradicate ALK$^+$ tumor cells.[32–34]

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