

MicroRNA-205 Inhibits Src-Mediated Oncogenic Pathways in Renal Cancer

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Abstract

The Src family of protein kinases (SFK) plays key roles in regulating fundamental cellular processes, including cell growth, differentiation, cell shape, migration, and survival, and specialized cell signals in various malignancies. The pleiotropic functions of SFKs in cancer make them promising targets for intervention. Here, we sought to investigate the role of microRNA-205 (miR-205) in inhibition of Src-mediated oncogenic pathways in renal cancer. We report that expression of miR-205 was significantly suppressed in renal cancer cell lines and tumors when compared with normal tissues and a nonmalignant cell line and is correlated inversely with the expression of SFKs. miR-205 significantly suppressed the luciferase activity of reporter plasmids containing the 3'-UTR (untranslated region) sequences complementary to either *Src*, *Lyn*, or *Yes*, which was abolished by mutations in these 3'-UTR regions. Overexpression of miR-205 in A498 cells reduced *Src*, *Lyn*, and *Yes* expression, both at mRNA and protein levels. Proliferation of renal cancer cells was suppressed by miR-205, mediated by the phospho-Src-regulated ERK1/2 pathway. Cell motility factor FAK (focal adhesion kinase) and STAT3 activation were also inhibited by miR-205. Transient and stable overexpression of miR-205 in A498 cells resulted in induction of G₀/G₁ cell-cycle arrest and apoptosis, as indicated by decreased levels of *cyclin D1* and *c-Myc*, suppressed cell proliferation, colony formation, migration, and invasion in renal cancer cells. miR-205 also inhibited tumor cell growth *in vivo*. This is the first study showing that miR-205 inhibits proto-oncogenic SFKs, indicating a therapeutic potential of miR-205 in the treatment of renal cancer. *Cancer Res*; 71(7); 2611–21. ©2011 AACR.

Introduction

The Src family of kinases (SFK) are prototypical modular signaling proteins and the largest family of nonreceptor protein tyrosine kinases (1–3). They have been shown to be upregulated in multiple types of human tumors, with Src activity increasing proportionally to the progressive stages of the disease (1, 4). Of the 9 family members, *c-Src*, *Fyn*, and *Yes* are widely expressed in tissues and seem to play an important role in the regulation of cell adhesion, cell growth, and differentiation (5). Among the SFKs, Src itself is most frequently implicated in human cancer, and previous studies have shown that in mouse models, Src activation is associated with progression and metastasis in pancreatic (6) and color-

ectal (7) carcinomas. In prostate cancer cells *in vitro*, inhibition of SFKs decreases proliferation (8) and, more profoundly, invasion and migration (9); the latter through selective inhibition of phosphorylation of Src substrates such as focal adhesion kinase (FAK) and Crk-associated substrate (10). In renal cancer, Src has been shown to contribute to the appearance of malignant phenotypes, particularly due to the resistance against apoptosis by Bcl-xL and angiogenesis stimulated by Src-STAT3-VEGF signaling (11). The pleiotropic effects of Src activity are due to the multiple signal pathways engaged by Src and its accompanying kinases. Src can channel phosphorylation signals through Ras/Raf/extracellular signal-regulated kinase 1/2 (ERK1/2) and in certain cells, phosphatidylinositol 3-kinase (PI3K)/AKT, pathways. Somewhat selective to SFKs is their ability to activate STAT3 and β -catenin, which leads to the activation of *c-Myc* (12, 13) and, consequently, *cyclin D1* (14, 15). Overall, these studies suggest that Src plays pleiotropic roles in cancer, often in a cell-dependent manner, and that Src is a promising target for intervention. Here, we provide the first demonstration that inhibition of SFKs can be effectively achieved by microRNA-205 (miR-205) in renal cancer.

Renal cell carcinoma (RCC) is the seventh most common cancer in the United States and was predicted to result in nearly 13,000 deaths in 2009 (16). Surgery is the first line of treatment with successful resection, often resulting in

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long-term disease-free status. Although the overall survival rate is more than 60% over 5 years (16), approximately 30% of patients who have a diagnosis of localized RCC develop metastatic recurrence (17). These patients have a very poor prognosis because of the refractory nature of RCC to current treatment regimens. Therefore, there has been much interest in the identification of biomarkers for RCC to better predict cancer development and prognosis.

miRNAs are small, noncoding RNAs that have been found to regulate expression through targeted repression of gene transcription and translation. These endogenous silencing RNAs have been shown to play important roles in development and differentiation (18, 19), cellular stress responses (20), and cancer (21). Specific subsets of miRNAs have also been shown to be dysregulated in various solid tumors (22, 23). Because of their tremendous regulatory potential and tissue- and disease-specific expression patterns (24, 25), there is increasing evidence that miRNA expression profiles could be indicative of disease risk and burden. Thus, miRNAs are being assessed as possible biomarkers to aid in the diagnosis and prognosis of different cancers (26, 27). Here, we report that miR-205 is significantly downregulated in renal cancer tissue samples and cell lines. In addition, we examined the consequences of miR-205 overexpression and identified the SFKs as direct targets of miR-205 in renal cancer.

Materials and Methods

Cell culture, plasmids, and transfection

Human RCC cell lines A498, ACHN, Caki-1, and 769-P and a nonmalignant renal cell line HK-2 were obtained from the American Type Culture Collection (ATCC) and grown according to the ATCC protocol (28). Plasmids pEZX-MT01 miRNA 3'-UTR (untranslated region) target expression clones for Src (HmiT017696-MT01), Lyn (HmiT010935-MT01), Yes (HmiT018569-MT01), Lck (CS-HmiT010565-MT01), and miRNA Target clone control vector for pEZX-MT01 (CmiT000001-MT01; GeneCopoeia), miRNAselect pEP-miR Null control vector (pEP Null), and miRNAselect pEP-hsa-miR-205 expression vector (pEP miR-205; Cell Biolabs Inc.) were purchased. TaqMan probes for hsa-miR-205 (miR-205), anti-miR-205, and negative controls pre-miR and anti-miR control (Cont-miR) were purchased from Applied Biosystems. siRNA duplexes [(Src (Human)-3 unique 27mer siRNA duplexes (SR304574)] were purchased from Origene (Origene Technologies, Inc.).

Quantitative real-time PCR

Tissue samples from radical nephrectomy were obtained from the Veterans Affairs Medical Center, San Francisco, CA. Total RNA was extracted and assayed for mature miRNAs and mRNAs, using the TaqMan MicroRNA Assays and Gene Expression Assays, respectively, in accordance with the manufacturer's instructions (Applied Biosystems). All real time reactions were run in a 7500 Fast Real Time PCR System (Applied Biosystems). Relative expression was calculated using the comparative Ct.

Flow cytometry, cell viability, migratory, clonability, and invasion assays

Fluorescence-activated cell-sorting (FACS) analysis for cell cycle and apoptosis was done 72 hours posttransfection, using nuclear stain DAPI (4',6-diamidino-2-phenylindole) for cell-cycle analysis or Annexin V-FITC/7-AAD Kit (Beckman Coulter, Inc.) for apoptosis analysis, according to the manufacturer's protocol. Cell viability was determined at 24, 48, and 72 hours by using the CellTiter 96 AQueous One Solution Cell Proliferation Assay Kit (Promega), according to the manufacturer's protocol. For colony formation assay, cells were seeded at low density (1,000 cells/plate or 200 cells/plate) and allowed to grow till visible colonies appeared. Then, cells were stained with Giemsa, and colonies were counted. An artificial "wound" was created on a confluent cell monolayer, and photographs were taken after 24 hours for migration assay. Also, a cytoselect 24-well cell migration and invasion assay kit (Cell Biolabs, Inc.) was used for migration and invasion assays, according to manufacturer's protocol.

Immunoblotting

Immunoblotting was done as described previously (29). Briefly, protein was isolated from 70% to 80% confluent cultured cells, using the M-PER Mammalian Protein Extraction Reagent (Pierce Biotechnology), following the manufacturer's directions. Equal amounts of protein were resolved on 4% to 20% SDS polyacrylamide gels and transferred to nitrocellulose membrane. The resulting blots were blocked with 5% nonfat dry milk and probed with antibodies. All antibodies were obtained from Cell Signaling Technology, Inc., except *c-Myc* and *cyclin D1*, which were purchased from BD Pharmingen (BD Biosciences). Blots were visualized using enhanced chemiluminescence (Pierce Biotechnology).

Luciferase assays

The Src, Lyn, Yes, Lck, and control vectors were purchased from GeneCopoeia and named as Src-3'-UTR, Lyn-3'-UTR, Yes-3'-UTR, Lck-3'-UTR, and empty vector, respectively. Mutated 3'-UTR sequences of Src, Lyn, and Yes complementary to miR-205 were cloned and named Src-Mut, Lyn-Mut, and Yes-Mut, respectively. For reporter assays, cells were transiently transfected with wild-type or mutant reporter plasmid and miR-205 or control-miR. Firefly luciferase activities were measured by using the Dual Luciferase Assay (Promega) 24 hours after transfection and the results were normalized with *Renilla* luciferase. Each reporter plasmid was transfected at least 3 times (on different days), and each sample was assayed in triplicate.

Stable cell generation and *in vivo* study

A498 cells were transfected with pEP Null vector and pEP miR-205 vector (Cell Biolabs) and selected with puromycin (1 μ g/mL). pEP miR-205 vector was labeled with red fluorescent protein. After transfection, cells were observed under a microscope to check for red fluorescence and then selected with a

cell sorter (BD FACSAria II; BD Biosciences). The sorted cells were grown in puromycin and real-time quantitative PCR (qRT-PCR) was done to check the expression of miR-205. For *in vivo* studies, 5×10^6 cells were injected into nude mice subcutaneously and tumor growth was followed for 28 days. We also looked at the antitumor effects of miR-205 by local administration in established tumors. Each mouse was injected with 7.5×10^6 cancer cells. Once palpable tumors developed (average volume = 80 mm^3), $6.25 \mu\text{g}$ of synthetic miRNA complexed with $1.6 \mu\text{L}$ siPORT Amine transfection reagent (Ambion) in $50 \mu\text{L}$ PBS was delivered 7 times intratumorally in 3-day intervals. Tumor growth was followed for 21 days from first injection. All animal care was in accordance with the institutional guidelines.

Statistical analysis

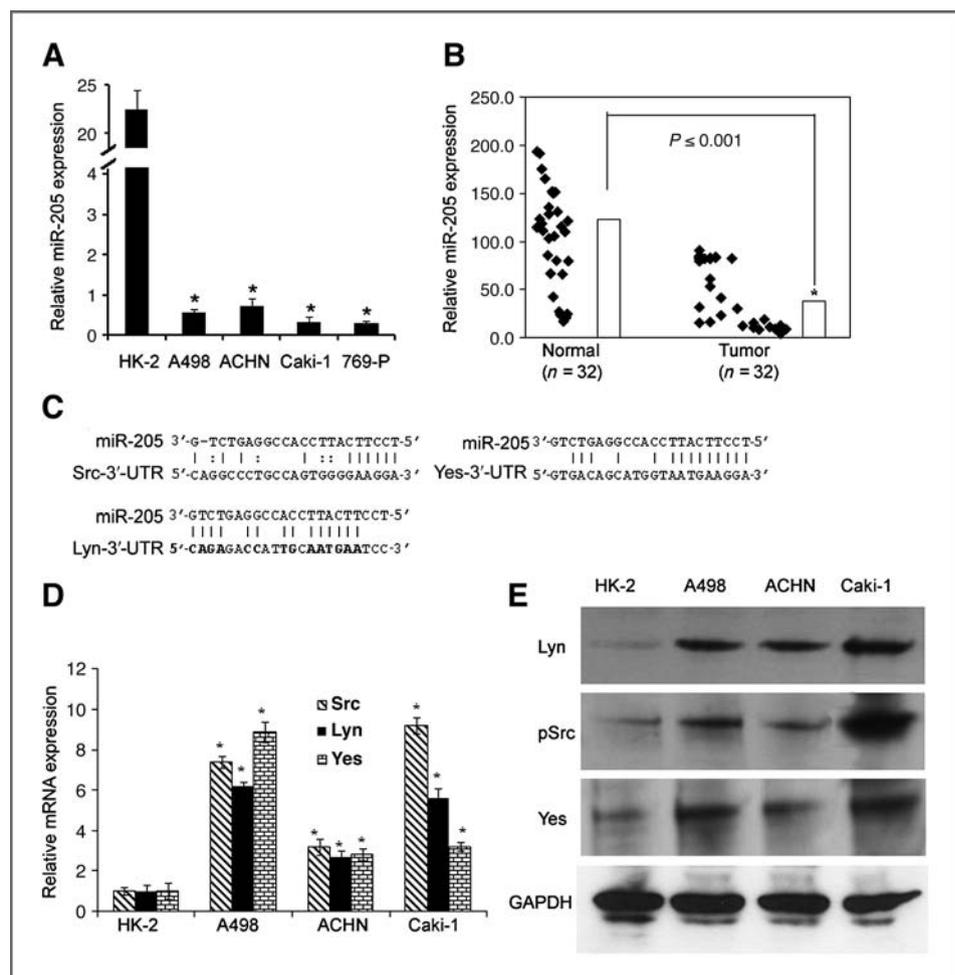
All quantified data represent an average of at least triplicate samples or as indicated. Error bars represent SD of the mean. Statistical significance was determined by the Student's *t* test and 2-tailed *P* values less than 0.05 were considered significant.

Results

miR-205 is downregulated in renal carcinoma, and its expression is inversely correlated with that of SFKs

Preliminary microarray data revealed that miR-205 was highly downregulated in renal cancer cell lines compared with the nonmalignant HK-2 cell line (data not shown). We validated the microarray data by miRNA qRT-PCR (miR qRT-PCR) analysis and results confirmed that miR-205 was downregulated in all the cancer cell lines (Fig. 1A). To examine the clinical relevance of miR-205, its expression was analyzed in carcinoma and normal renal tissue samples. Patients and tumor characteristics are summarized in Supplementary Table S1. Almost all carcinoma samples showed significant downregulation of miR-205 expression with respect to the normal samples, and an overall lower relative average expression was observed in carcinoma than in normal samples (Fig. 1B). These results suggest a potential tumor suppressor role for miR-205 in renal carcinoma. To identify the potential targets of miR-205, we used different algorithms that predict the mRNA targets of a miRNA: miRanda (30), miRNA target

Figure 1. miR-205 expression is downregulated in renal cancer and inversely correlated with expression of *Src*, *Lyn*, and *Yes*. A, qRT-PCR analysis of miR-205 expression levels in renal cancer and nonmalignant cell lines. B, miR-205 expression in a cohort of renal cancer and normal tissue samples. C, the miR-205 seed sequence is complementary to the 3'-UTR of *Src*, *Lyn*, and *Yes*. D and E, *Src*, *Lyn*, and *Yes* mRNA and protein expression in human renal cancer and nonmalignant cell lines. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; *, $P < 0.05$.



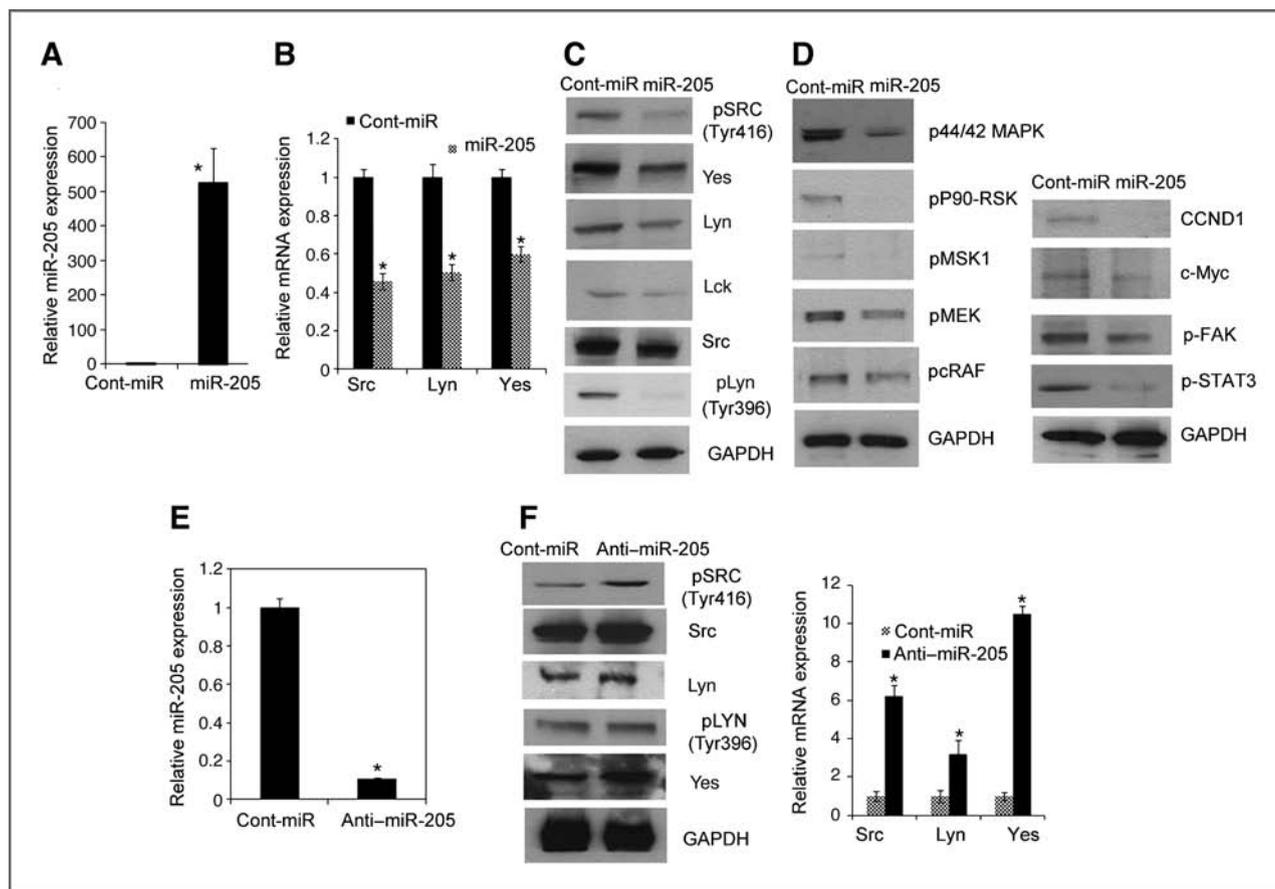


Figure 3. miR-205 suppresses *Src*, *Lyn*, and *Yes* expression and regulates the ERK1/2 pathway. **A**, relative miR-205 expression level. **B** and **C**, qRT-PCR and Western blot analysis showing decreased *Src*, *Lyn*, and *Yes* expression. **D**, Western analysis showing a decrease in the ERK1/2 pathway, *cyclin D1*, and *c-Myc* and a decrease in phospho-STAT3 and FAK in miR-205-transfected A498 cells. **E**, relative miR-205 expression in anti-miR-205 transiently transfected A498 cells. **F**, Western blot and qRT-PCR analysis showing that *Src*, *Lyn*, and *Yes* expression was rescued in cells transfected with anti-miR-205. MAPK, mitogen activated protein kinase; MEK, MAP/ERK kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

been found to inhibit cancer cell proliferation (8), invasion, and migration (9); the later through selective inhibition of phosphorylation of Src substrates such as FAK and Crk-associated substrate (10). To determine whether these effectors are affected by miR-205-mediated suppression of *Src*, A498 cells were transfected with miR-205 or Cont-miR. Western blot analysis showed reduced levels of the members of the phospho-ERK1/2 pathway, phospho-STAT3, phospho-FAK, *c-Myc*, and *cyclin D1* (Fig. 3D) in cells with suppressed phospho-Src expression following miR-205 overexpression. We next inhibited the endogenous expression of miR-205 in A498 cells by transfecting anti-miR-205 (Fig. 3E), an inhibitory oligonucleotide designed specifically to bind and sequester the mature miR-205 sequence to see whether the expression of target genes is rescued by inhibiting miR-205. Indeed, the expression of all the 3 genes was restored at both the protein and mRNA levels (Fig. 3F) in anti-miR-205-transfected cells. These data indicate that miR-205 targets *Src*, which, in turn, results in suppression of the ERK pathway and the genes involved in migration/invasion and proliferation.

miR-205 induces apoptosis, cell-cycle arrest, impairs cell viability, migratory, clonability, and invasive properties of renal cancer cells

Because *Src* has been reported to be involved in cancer cell proliferation (8), invasion, and migration (9), we sought to determine whether downregulation of *Src* by miR-205 has effect on the cell cycle, viability, migratory, or invasion properties of A498 cancer cells. A significant decrease in cell proliferation was observed over time in A498 cells expressing miR-205 (Fig. 4A) as compared with cells expressing Cont-miR. The miR-205-transfected cells also had low colony formation ability, as both the size and number of foci in miR-205-expressing cells were suppressed when compared with Cont-miR-expressing cells (Fig. 4B). To determine whether miR-205 affects renal cancer cell migration or invasiveness, wound healing, migration, and invasion assays were conducted. miR-205-overexpressing A498 cells were less proficient than Cont-miR-transfected cells in closing an artificial wound created over a confluent monolayer (Fig. 4C). Less absorbance was observed at 560 nm with miR-205-transfected

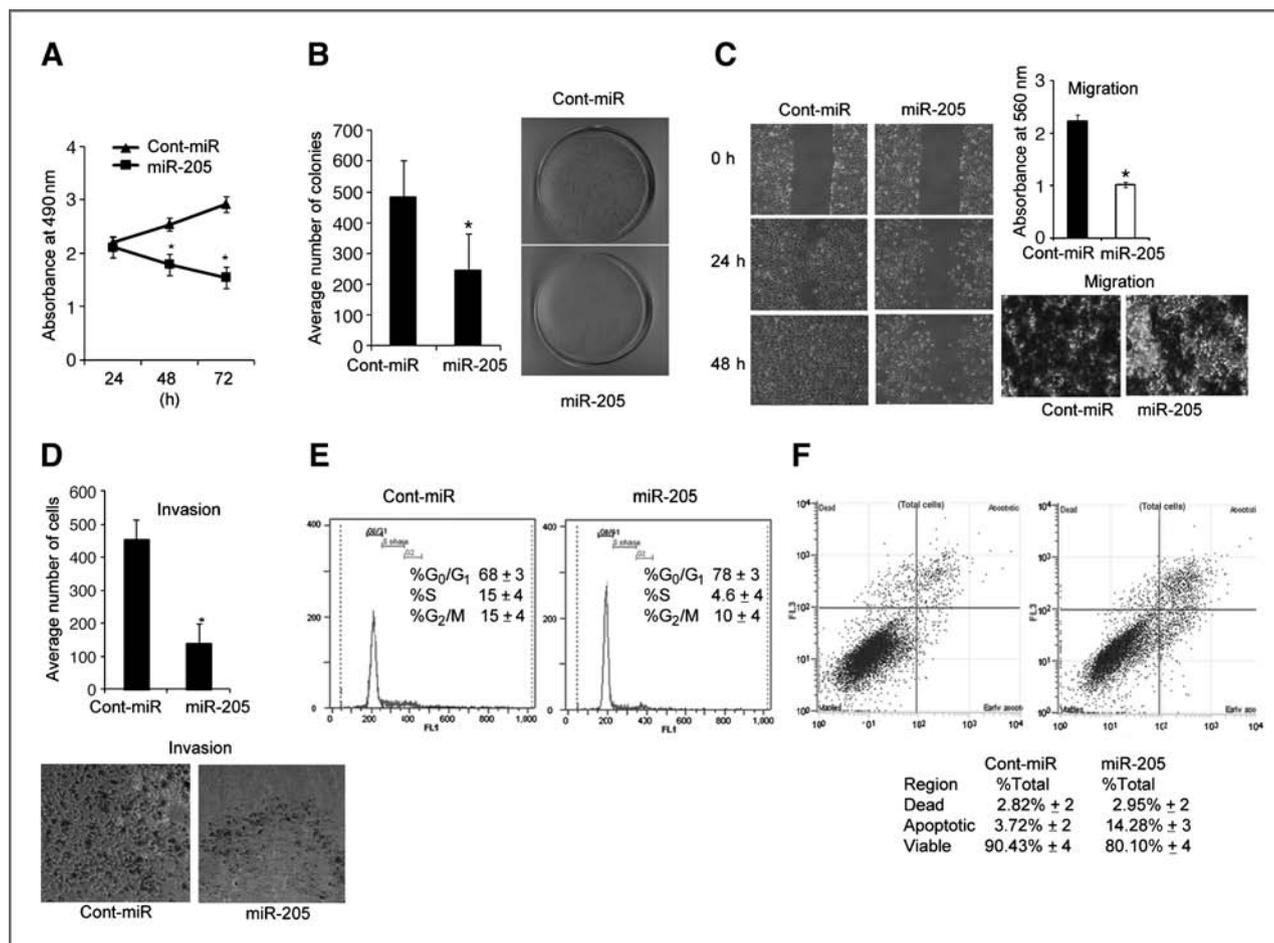


Figure 4. Transient transfection of miR-205 inhibits renal cancer cell proliferation, colony formation, migration, and invasion and induces apoptosis and cell-cycle arrest in A498 cells. **A**, proliferation of A498 cells after miR-205 transfection was significantly reduced compared with Cont-miR. **B**, miR-205 overexpression significantly inhibits colony formation of A498 cells. **C**, wound healing and migration assays of A498 cells transfected with miR-205. **D**, invasion assay shows a significant decrease in the number of invading A498 cells transfected with miR-205. **E**, cell-cycle analysis showing an increase in the G₀/G₁ phase of A498 cells overexpressing miR-205. **F**, apoptosis assay showing induction of apoptosis by miR-205. *, $P < 0.05$.

cells than Cont-miR-transfected cells in the migration assay (Fig. 4C). miR-205 overexpression also significantly reduced the invasiveness of A498 cells (Fig. 4D). FACS analysis revealed that reexpression of miR-205 leads to a significant increase (10% ± 3%) in the number of cells in the G₀/G₁ phase of the cell cycle whereas the S-phase population decreased from 15% ± 4% to 5% ± 3%, suggesting that miR-205 causes a G₀/G₁ arrest in miR-205-transfected A498 cells compared with a nonspecific miRNA control (Cont-miR; Fig. 4E). FACS analysis for apoptosis was conducted using Annexin V-FITC-7-AAD dye. The percentage of total apoptotic cells (early apoptotic + apoptotic) was significantly increased (14% ± 3%) in response to miR-205 transfection compared with Cont-miR (4% ± 2%), with a corresponding 10% ± 4% decrease in the viable cell population (Fig. 4F). All the functional assays were confirmed in the 769-P cell line, which is from the same tumor type as A498 cells, and the results were consistent (Supplementary Fig. S2). These results indicate that suppression of phospho-Src by miR-205 inhibits renal cell proliferation, invasion, and

migration by inhibiting phosphorylation of FAK, a Src substrate, *c-Myc*, a Src target gene (39), and cyclin D1, the rate limiting factor for cellular proliferation (40, 41).

Src inhibition by siRNA mimics miR-205 reconstitution in renal cancer and attenuation of miR-205 in nonmalignant cells increases proliferation, migration, and invasion

Phenocopy experiments inhibiting Src expression by siRNA were also conducted (Fig. 5). We initially tested 3 siRNAs to achieve 80% to 90% Src gene knockdown and confirmed the results at the mRNA and protein levels (Fig. 5A). Then we selected one siRNA (S-1) for further experiments. Our results showed that siRNA inhibition of Src caused decreased cell viability (Fig. 5B), migratory, and invasive (Fig. 5C) capability of A498 cancer cells. We also observed a G₀/G₁ cell-cycle arrest (14%), whereas there was a decrease of 11% in S-phase cell population (Fig. 5D). Almost 5% of the cells were in the apoptotic fraction in Src siRNA-transfected cells compared

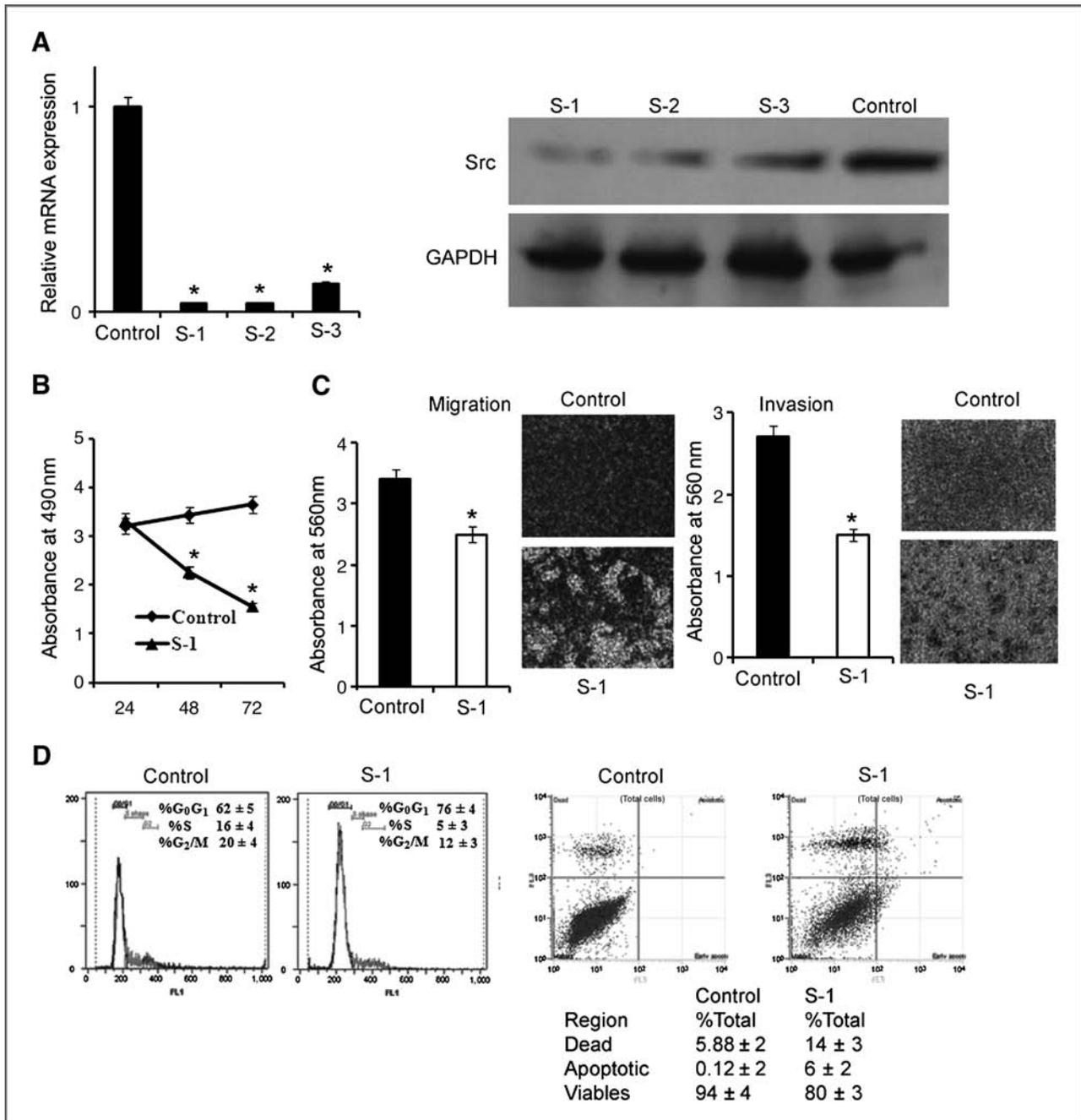


Figure 5. Knockdown of Src by siRNA. **A**, relative Src mRNA levels assessed by qRT-PCR in A498 cells transfected with 50 nmol/L siRNA duplexes (S-1, S-2, and S-3) and a nonsilencing siRNA duplex (control). Src protein levels were assessed by Western blot in A498 cells transfected with 50 nmol/L siRNA duplexes and a nonsilencing siRNA duplex. **B**, proliferation of A498 cells after S-1 transfection was significantly reduced compared with control. **C**, a significant decrease was observed in the migratory capability of A498 cells after siRNA (S-1) transfection compared with control. Invasion assay shows a significant decrease in the number of invading A498 cells transfected with S-1. **D**, cell-cycle analysis showing an increase in the G₀/G₁ phase of A498 cells transfected with S-1. Apoptosis assay showing induction of apoptosis after Src knockdown by S-1. *, $P < 0.05$. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

with nonspecific control (Fig. 5D). These results provide evidence that inhibition of Src by miR-205 reconstitution is responsible for the observed phenotype in renal cancer cells. We also knocked down the expression of miR-205 in HK-2 cells that expressed high levels of miR-205 (Fig. 6A) and determined

its effect on cell growth, migration, and invasion. Our results showed that cells transfected with anti-miR-205 showed increased proliferation (Fig. 6B), migration (Fig. 6C), and invasion (Fig. 6D) compared with control anti-miRNA. These results show that miR-205 is an important tumor suppressor

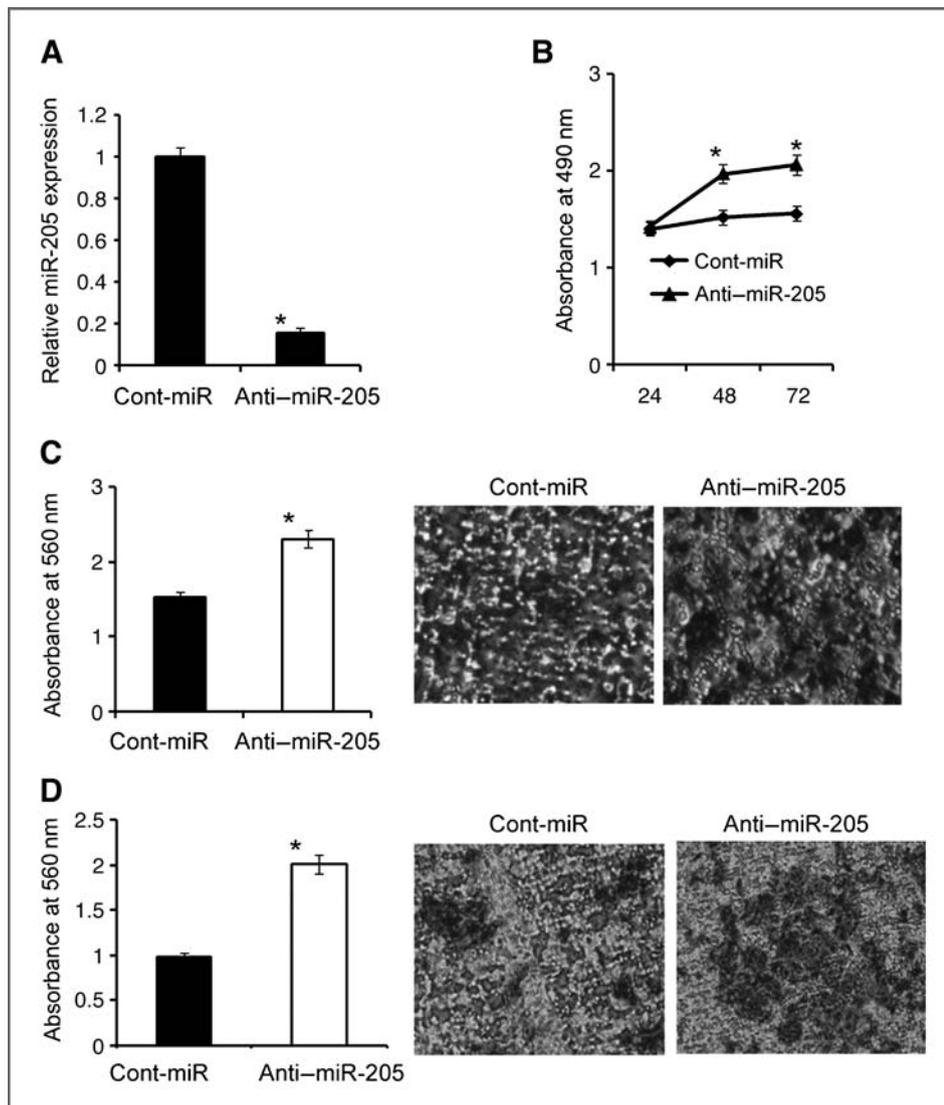


Figure 6. Attenuation of miR-205 expression by anti-miR-205 in HK-2 cells. A, relative miR-205 expression. B, HK-2 cells had increased proliferation after anti-miR-205 transfection compared with the anti-miR control (Cont-miR). C and D, migration and invasion assays. *, $P < 0.05$.

miRNA in renal cancer and attenuation of this miRNA in overexpressing nonmalignant renal cells increases their proliferative, migratory, and invasive capability.

miR-205 inhibits tumor growth *in vivo*

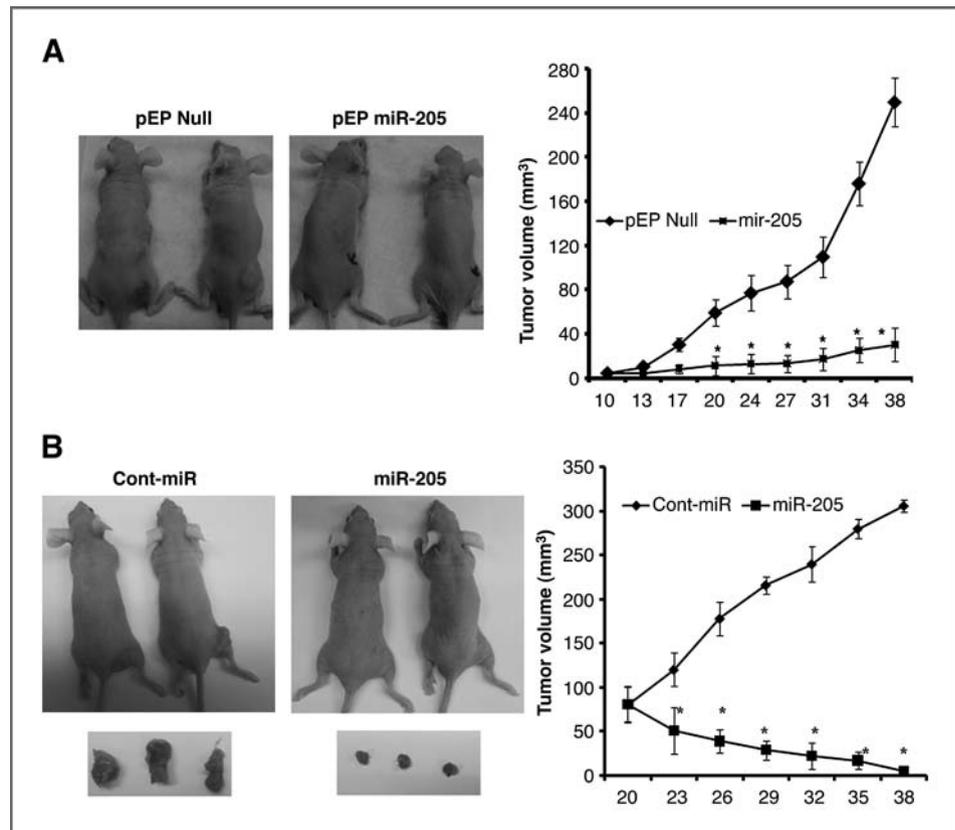
The antitumor effect of miR-205 stably transfected in A498 cells was determined by carrying out phenocopy experiments *in vitro* (Supplementary Fig. S3) and confirmed by *in vivo* experiments. Stable overexpression of miR-205 dramatically suppressed tumor growth *in vivo* on subcutaneous injection into nude mice when compared with cells expressing control vector (Fig. 7A). We further checked the expression of miR-205 or Src, Lyn, and Yes in 8 harvested tumors, 4 from pEP Null control group, and 4 from pEP miR-205 group. Our results showed that miR-205 expression was significantly high, with a corresponding significant decrease in the target gene expression in tumors that had pEP miR-205 compared with the pEP Null control (Supplementary Fig. S4A and B). Because over-

expression of miR-205 inhibited cell growth *in vitro*, we conducted an additional experiment to check the antitumor effect of miR-205 after local administration in established A498 tumors. Indeed, the tumor volume regressed from 81 to 5 mm³ with miR-205 compared with Cont-miR, in which tumor volume increased from 80 to 306 mm³ (Fig. 7B). These results show that miR-205 suppressed cancer growth both *in vitro* and *in vivo*.

Discussion

In this study, we provide evidence that miR-205 interdicts SFK pathways by inhibiting their expression at both the mRNA and protein levels. Our results show that Src inhibition by miR-205 leads to growth suppression and cell-cycle arrest in renal cancer and are accompanied by inactivation of ERK1/2 and downregulation of *c-Myc* and cyclin D1. FAK and STAT3 phosphorylation were also decreased by diminished Src

Figure 7. miR-205 inhibits tumor growth *in vivo*. A, tumor volume following subcutaneous injection of stable A498 cells expressing miR-205 was significantly reduced. B, tumor volume following intratumoral injection of Cont-miR or miR-205 precursor into established tumors. *, $P < 0.05$.



activity, leading to significantly reduced cell migration and invasion.

Expression of miR-205 in cancer is controversial because it has been found to be either upregulated (42) or downregulated (43) in tumors. In this study, we examined the expression pattern and functional significance of miR-205 in renal cancer. We found miR-205 to be significantly downregulated in tumor samples when compared with adjacent normal samples. The downregulation of miR-205 expression was also observed in RCC cell lines when compared with a nonmalignant cell line. This is consistent with a previous microarray analysis of 27 samples of kidney cancer tissues that showed downregulation of miR-205 (42). The significant suppression of miR-205 expression in tumors and cancer cell lines suggests a tumor suppressor role in renal carcinoma. However, neither the functional role nor the targets of miR-205 in renal cancer have been previously defined.

An obstacle to understanding miRNA function has been the relative lack of experimentally validated targets. To determine potential targets of miR-205 action, several *in silico* algorithms were utilized to identify SFKs as putative targets of miR-205. The SFKs play an important role in the regulation of cellular proliferation and cell-cycle progression (5). Our results indicate an inverse correlation between expression of miR-205 and that of phospho-Src, Lyn, and Yes in cell lines and tissues samples. We showed that miR-205 directly targets the 3'-UTR of phospho-Src, Lyn, and Yes, as its overexpression was

associated with suppression of luciferase activity. In addition, a significant downregulation of phospho-Src, Lyn, and Yes protein and mRNA levels was observed after miR-205 overexpression, indicating that phospho-Src, Lyn, and Yes mRNAs are targets of miR-205.

Because of the reported importance of phospho-Src in renal cancer (11), we further characterized its role in response to miR-205. It has been reported that Src is involved in multiple signaling pathways including Ras/Raf/ERK1/2, PI3K/AKT, β -catenin/*c-Myc*/cyclin D1, and FAK/p130CAS/MMP-9 that induce growth, survival, and migration in various types of cancer cells (35). We observed that inhibition of phospho-Src by miR-205 overexpression reduced signaling via the ERK1/2 pathway. A previous study by Chang and colleagues (35) and others (44) have shown that Src inhibition by small molecule inhibitors induced apoptosis and cell-cycle arrest at the G₀/G₁ phase of the cell cycle in prostate cancer cell lines. Our results revealed that inhibition of phospho-Src by miR-205 overexpression induced apoptosis and G₀/G₁ arrest in renal cancer cells. This effect on the cell cycle prompted us to study the effect on *c-Myc*, a Src target gene (39), cyclin D1, the rate limiting factor for cellular proliferation (41), and phospho-STAT3, a Src target and key transcriptional factor for *c-Myc* and *cyclin D1* (37). We found that all these genes were downregulated at the protein level. Our results indicate that miR-205 inhibited renal cell migration and invasion and also downregulated phospho-FAK, a Src substrate in renal cancer

cells. Inhibition of Src has been found to decrease the invasion and migration of prostate cancer cells (9) through selective inhibition of phosphorylation of Src substrates, such as FAK. To determine whether Src inhibition is responsible for the phenotype observed after miR-205 reconstitution, we conducted phenocopy experiments inhibiting Src expression by siRNA. Our results showed that inhibition of Src was responsible for decreased cell viability, migratory, and invasive capability of A498 cancer cells. We also observed a G₀/G₁ cell-cycle arrest (14%) whereas there was a decrease of 11% in S-phase cell population. Almost 5% apoptotic cells were observed in Src siRNA-transfected cells compared with non-specific control. These results prove that tumor-suppressive effect of miR-205 is mediated by Src inhibition in renal cancer. We further attenuated miR-205 expression in nonmalignant HK-2 cells that expressed higher levels of miR-205 and determined its effect on cell growth, migration, and invasion. Our results showed that cells transfected with anti-miR-205 showed more proliferation, migration, and invasion than those transfected with control anti-miRNA. These results indicate that miR-205 is important tumor suppressor miR in renal cancer and attenuation of this miRNA in overexpressing nonmalignant renal cells increases their proliferative, migratory, and invasive capability.

The antiproliferative effects of miR-205 observed in this study, mediated by suppression of phospho-Src and downstream target genes, were confirmed following stable overexpression of miR-205 in A498 cells. *In vivo* studies showed a striking reduction in subcutaneous tumor cell growth in mice injected with stable A498 cancer cells overexpressing miR-205. Furthermore, results from local administration of miR-205 in established tumors revealed a dramatic regression of tumor growth compared with the Cont-miR. In conclusion, our study shows an important tumor suppressor role for miR-205 in renal cancer.

The SFKs are essential for many important tumorigenic phenotypes including proliferation, invasion, migration, epithelial-to-mesenchymal transition (5, 45), apoptosis, survi-

val, angiogenesis, etc. Thus, the activity of SFKs increase in progressive stages of tumors, with the highest activity observed in metastatic lesions (46). Increasing evidence from molecular and pharmacologic studies suggests that inhibition of Src, the prototype SFK member, inhibits tumor function associated with metastasis, including migration, invasion, and expression of the proangiogenic molecules, such as interleukin-8 and VEGF (47). In addition, recent studies indicate that Src plays critical roles in host cells in the tumor microenvironment and the tumor cells that contribute to metastasis (4). Several studies have shown that Src-mediated phosphorylation of VE-cadherin, a cell adhesion molecule that is essential to vascular cell-to-cell junctional integrity, directly leads to increased vascular permeability, thus facilitating intravasation and extravasation of migratory tumor cells (48). Thus, Src plays pleiotropic roles in cancer, making it a promising therapeutic target for intervention. Our study is the first report showing that miR-205 inhibits the proto-oncogenic SFKs, indicating the therapeutic potential of miR-205 in the treatment of renal cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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