Down-regulation of Micro-RNA-1 (miR-1) in Lung Cancer

SUPPRESSION OF TUMORIGENIC PROPERTY OF LUNG CANCER CELLS AND THEIR SENSITIZATION TO DOXORUBICIN-INDUCED APOPTOSIS BY miR-1

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Micro-RNAs are ~21–25-nucleotide-long noncoding RNAs that regulate gene expression primarily at the post-transcriptional level in animals. Here, we report that micro-RNA-1 (miR-1), abundant in the cardiac and smooth muscles, is expressed in the lung and is down-regulated in human primary lung cancer tissues and cell lines. In situ hybridization demonstrated localization of miR-1 in bronchial epithelial cells. The tumor suppressor C/EBPα, frequently suppressed in lung cancer, reactivated miR-1 expression in the lung cancer cells. Repressed miR-1 was also activated in lung cancer cells upon treatment with a histone deacetylase inhibitor. These observations led us to examine the role of miR-1 in lung cancer cells. miR-1 knockout cells reversed the tumorigenic properties of lung cancer cells. Enhanced activation of caspases 3 and 7, cleavage of their substrate PARP-1, and depletion of anti-apoptotic Mcl-1 contributed to the sensitivity of miR-1-expressing cells to doxorubicin. Thus, miR-1 has potential therapeutic application against lung cancers.

Lung cancer is the leading cause of cancer-related deaths both in men and women in the United States, with an incidence of ~213,000 new cases/year (1). Approximately 80% of lung cancers are classified histopathologically as non-small cell lung cancers. At early stages of non-small cell lung cancer, the only treatment is surgery, with a 5-year overall survival rate of 40% (2), whereas chemotherapy is mostly employed for small cell lung cancer. The majority of patients have developed an aggressive form of the disease by the time of diagnosis, limiting the scope for therapeutic intervention. At this stage, several genetic and epigenetic changes take place, resulting in epithelial cell transformation (3). They are treated by the administration of drugs that target the tumor cell lineages, which are a target of multiple miRNAs.

Withdrawing
This article has been withdrawn by the authors. The Journal questioned Figs. 8A and <zsi>S1<zsix>. The authors stand by the reproducibility of the experimental data and the conclusions of the paper. The paper, with confirmatory data supporting the results, can be obtained by contacting the authors.

5 The abbreviations used are: miR, micro-RNA; miR-1, micro-RNA-1; miRNAs, micro-RNAs; T5A, trichostatin A; DOXR, doxorubicin; 5-AzaC, 5-aza-cytidine; HDAC, histone deacetylase; SRF, serum response factor; UTR, untranslated region; RT, reverse transcription; MTT, 3-(4,5-dimethylthiazol-2-y)-2,5-diphenyltetrazolium bromide; LNA, locked nucleic acid.

8 This work was supported, in whole or in part, by National Institutes of Health Grants CA122695 and PO1CA101956. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

9 The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S3 and Figs. S1–S3.

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33394 JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 283 • NUMBER 48 • NOVEMBER 28, 2008
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miR-1 is a muscle-enriched micro-RNA that inhibits proliferation of progenitor cells and promotes myogenesis (25–27). Several key observations prompted us to examine the tumorigenic function of miR-1 in other tissues, such as the lung, which expresses miR-1, albeit at a lower level than the muscle (28). First, comparison of gene expression patterns in mouse tumor susceptibility loci with those of micro-RNAs deregulated in human malignancies revealed that the flanking micro-RNA genes of tumor suppressor loci in the mouse strain most susceptible to lung cancer were barely expressed, whereas those of the resistant strain were strongly expressed (29). Second, loss of function of mouse tumor susceptibility loci with those of mouse miRNA expression profiles yielded distinct miR signatures (15), inflammation (16), and cancer (17). Tumor-specific micro-RNAs, such as let-7 and miR-29a/b/c, are down-regulated in a variety of cancers, including lung cancer (18–20). Detection of miRNA genes at fragile sites that are frequently amplified, deleted, or rearranged in cancer provides further evidence of a causal role played by micro-RNAs in cancer pathogenesis (17, 21). Furthermore, depending upon the cell type, several miRs deregulated in human malignancies exhibit oncogenic or tumor suppressor properties (17). Some micro-RNAs, such as let-7 and miR-29a/b/c, are down-regulated in lung cancer (20, 22, 23). In fact, let-7 exhibited growth-inhibitory properties in lung cancer cells (24). Thus, it is conceivable that many more miRs will play a critical role in lung tumorigenesis and can potentially serve as biomarkers and targets for anticancer therapy.

Micro-RNA-1 is a muscle-enriched micro-RNA that inhibits proliferation of progenitor cells and promotes myogenesis (25–27). Several key observations prompted us to examine the tumorigenic function of miR-1 in other tissues, such as the lung, which expresses miR-1, albeit at a lower level than the muscle (28). First, comparison of gene expression patterns in mouse tumor susceptibility loci with those of micro-RNAs deregulated in human malignancies revealed that the flanking micro-RNA genes of tumor suppressor loci in the mouse strain most susceptible to lung cancer were barely expressed, whereas those of the resistant strain were strongly expressed (29). Second, loss of function of mouse tumor susceptibility loci with those of mouse miRNA expression profiles yielded distinct miR signatures (15), inflammation (16), and cancer (17). Tumor-specific micro-RNAs, such as let-7 and miR-29a/b/c, are down-regulated in a variety of cancers, including lung cancer (18–20). Detection of miRNA genes at fragile sites that are frequently amplified, deleted, or rearranged in cancer provides further evidence of a causal role played by micro-RNAs in cancer pathogenesis (17, 21). Furthermore, depending upon the cell type, several miRs deregulated in human malignancies exhibit oncogenic or tumor suppressor properties (17). Some micro-RNAs, such as let-7 and miR-29a/b/c, are down-regulated in lung cancer (20, 22, 23). In fact, let-7 exhibited growth-inhibitory properties in lung cancer cells (24). Thus, it is conceivable that many more miRs will play a critical role in lung tumorigenesis and can potentially serve as biomarkers and targets for anticancer therapy.

EXPERIMENTAL PROCEDURES

Cell Culture and Tissue Procurement—Human lung cancer cell lines were obtained from ATCC. Human bronchial epithelial (BEAS-2B) cells and the lung cancer cell lines were cultured in RPMI 1640 medium containing 10% fetal bovine serum, cells were harvested for RNA isolation, and whole cell extracts were subjected to Western blot analysis. Primary human lung cancer and adjacent normal tissue samples were obtained from the Cooperative Human Tissue Network at the Ohio State University James Cancer Hospital. Tissue specimens were procured in accordance with the Ohio State University Cancer Internal Review Board guidelines.

RT in Situ PCR of miR-1—The protocol used has been previously described (36). Primers for the miRNA precursors were the same as used for solution RT-PCR (35).

Cell Motility Assays—Wounds were generated using a pipette in A549 cells plated at equal density in 6-well plates,
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grown to confluency, and rinsed with phosphate-buffered saline, and fresh culture medium was added. Wound areas were marked and photographed at different time points using a phase-contrast microscope (39).

For cell migration, A549 cells stably expressing pBabe or miR-1 were placed in a serum-free medium for 24 h. Chemotactic assays were done in 24-well trans-well inserts of 8-μm pore size (Corning Costar Corp.). Cells (1 × 10^4) were layered onto the top well in serum-free medium. The bottom chambers contained serum-supplemented medium that acted as a chemoattractant. The migration of cells was allowed to proceed for 48 h at 37 °C. Cells that migrated to the bottom of the insert were fixed, stained, and counted, and the percentage of migration was determined. Filters were washed thoroughly in water and suspended in 0.5 ml of water or phosphate-buffered saline containing 5% acetic acid and 5% methanol and stained with Hema-3 (Fisher), and absorbance was measured at 595 nm. Absorbance of cells incubated in the serum-free medium in the bottom chamber was used as negative control. Each experiment was performed at least three times.

Tumor Growth in Nude Mice—Nude mice (4–6 weeks old) were used for xenograft studies. A549 cells (2 × 10^6 in phosphate-buffered saline containing 50% Matrigel) expressing miR-1 or control micro-RNA were injected subcutaneously to the flanks of nude mice. After 4 weeks, tumor volume was measured using a slide caliper and was calculated as

\[ V = \frac{1}{2} \times d_1 \times d_2 \times d_3 \times 0.5236 \]

where \( d \) represents diameters.

Western Blot Analysis—Tumor tissues were immediately removed following a published protocol (39) and the antibodies used is provided in the supplementalmaterial). The signal was developed with ECLTM (GE Healthcare) or chemiluminescent peroxidase substrate (Sigma) after incubation with the appropriate secondary antibodies.

Doxorubicin-induced Apoptosis Assay—A549 cells expressing miR-1 or vector were seeded into 96-well plates (4000 cells/well) in complete medium. Twelve h later, doxorubicin (Sigma) (1 μg/ml) was added in the wells, and apoptotic cells were stained with Hoechst 33342 (5 μg/ml), as described (38), to visualize cells with and without fragmented nuclei under a fluorescence microscope. Extracts made from the cells were subjected to Western blot analysis with antibodies specific for pro-and antiapoptotic proteins. To quantify cells with fragmented DNA upon drug treatment, the cell cycle profile was analyzed with cells fixed overnight in 70% ethanol in the FACSCalibur after PI staining in the presence of RNase A, and the data were analyzed with the program Mod Fit LT (Verity, Topsham, ME).

Protein was estimated using the Bio-Rad protein assay kit with bovine serum albumin as a standard. Kodak Imaging software was used to quantify ethidium bromide-stained gels and scanned x-ray films (Western blot data).

Statistical Analysis—Statistical significance of differences between groups was analyzed by unpaired Student’s t test, and \( p \leq 0.05 \) was considered to be statistically significant.

RESULTS

miR-1 Is Down-regulated in Primary Human Lung Cancer—To determine whether miR-1 is deregulated in lung cancer, we measured the mature miR-1 level in human primary lung tumors and pair-matched lung tissues by real time RT-PCR. We used both 18S rRNA and miR-191 that is not deregulated in tumors and pair-matched controls. Ten μg of RNA was separated in urea-acrylamide gel, transferred to Zetaprobe, and sequentially hybridized to 32P-labeled antisense miR-1 and 5 S rRNA deoxyoligonucleotides. The signal was captured in a PhosphorImager and quantified using ImageQuant software. D, representative in situ hybridization data showing miR-1 expression in epithelial cells lining the bronchi. Expression of miR-1 in human lung tissues was detected by in situ hybridization with LNA-modified antisense miR-1 probe and RT in situ PCR, respectively. Tissue sections were hybridized to biotin-labeled oligonucleotide (antisense miR-1 or scrambled), which was captured with alkaline phosphatase-conjugated streptavidin, and the signal (blue) was developed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate. The cell body was stained with Nuclear fast red.
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FIGURE 2. A, C/EBPα is down-regulated in primary lung cancer. Real-time RT-PCR analysis of C/EBPα mRNA was performed with 12 pairs of tumor and matching lung tissues. B and C, ectopic C/EBPα expression in lung cancer cells. Top, miR-1 level in A549 and H1299 cells transfected with expression vector or empty vector. Bottom, Western blot analysis. D, depletion of endogenous C/EBPα level in lung cancer (N417) cells. Cells were transfected with pretroSuper harboring C/EBPα or the vector followed by analysis of miR-1 (top) and C/EBPα (bottom) levels. D, three independent experiments ± S.D. E, F, TSA induces miR-1 expression treated with 1 μM of 5-AzaC or 300 μM of TSA for 24 h, and DNase I-mediated RT-PCR. The results are means of three independent experiments ± S.D.

miR-1 has been studied in great detail (26, 27), regulation of human miR-1 gene expression has not been explored, especially in nonmuscle cells. In muscle cells, ubiquitously expressed serum response factor (SRF) in concert with muscle-specific transcription factors activates these genes by binding to the upstream enhancer elements. To identify the molecular mechanism of down-regulation of miR-1 in lung cancer, we measured the SRF mRNA level in primary lung cancers and matching lung tissues. Like many other growth factors, SRF mRNA was elevated in the majority of primary lung cancers (Fig. S1). However, lesser expression of SRF in cell lines that express relatively high levels of miR-1 (Table S2) compared with A549, H1155, and H792 (which do not express miR-1). Thus, SRF may not play a critical role in miR-1 expression in nontumorigenic bronchial epithelial (BEAS-2B) cells.

To identify the transcription factor that may play a role in miR-1 expression in the lung, we analyzed the promoter of the intronic miR-1-1 and miR-1-2 using the TESS data base (41) (available on the World Wide Web), which revealed potential cognate sites for C/EBPα, a member of the basic leucine zipper family of transcription factors. C/EBPα is a tumor suppressor that is frequently inactivated in different cancers due to mutation, transcriptional repression, or promoter methylation (42–45). Real-time RT-PCR analysis showed a significant decrease in its expression in primary lung cancer compared with matching lung tissues (Fig. 2A). Furthermore, a higher level of miR-1 (Fig. 2D, top) in N417 cells that express C/EBPα (bottom) as opposed to A549 or H1299 cells, in which both C/EBPα and miR-1 are almost undetectable (Fig. 2, B and C) (45), suggested to us its potential role in trans-activation of miR-1 genes. To test this possibility, we measured the miR-1 level in A549 and H1299 cells expressing ectopic C/EBPα (Fig. 2, B and C, bottom). The result showed an increase in endogenous miR-1 level of ~6- and ~5-fold (top) in A549 and H1299 cells, respectively. We also performed the reverse experiment by depleting C/EBPα from N417 cells and measured the miR-1 level. Indeed, transfection of N417 cells with pretroSuper harboring short hairpin RNA specific for C/EBPα reduced its level by 50% (Fig. 2D, bottom), with a concomitant decrease in miR-1 level by ~40% (top). These results suggest that the loss of C/EBPα expression in lung cancer cells is one of the factors causing miR-1 repression.

Next we addressed whether an epigenetic mechanism, such as DNA methylation or histone deacetylation, plays any role in the repression of miR-1 in lung cancer cells. For this purpose, we treated A549 and H1299 cells with 5-AzaC (an inhibitor of...
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![Image](https://example.com/image.png)

**FIGURE 3. Ectopic Expression of miR-1 inhibits proliferation of A549 cells.** Stable cell lines expressing miR-1 (miR-1 #1 and #2) or the retroviral vector (pBabe) were selected with puromycin and used for the following experiments. A, total RNA (5 μg) from these cells was subjected to Northern blot analysis, as described in the legend to Fig. 1B. B, cells (3000/well) were seeded in a 96-well plate, and cell growth was monitored every 24 h for 4 days using an MTT assay. Each cell type was analyzed in quadruplicate. The results are means of three independent experiments ± S.D. C, cells (10,000/well) were serum-starved overnight, followed by the addition of serum and [3H]thymidine incorporated into DNA was measured in a scintillation counter. Each experiment was performed in triplicate and was repeated twice.

**FIGURE 4. Ectopic expression of miR-1 reduces growth and motility of lung cancer cells.** A549 cells were seeded in serum-free medium on the top chamber of a two-chamber trans-well cell culture plate, and the cells migrated to the lower chamber containing complete medium after 48 h were photographed (Fig. 4A) and 48 h. Representative experiment was performed twice, generating similar results.

DNA methyltransferase) or trichostatin A (TSA; an HDAC inhibitor) for 24 h, followed by quantification of miR-1. The results showed that treatment with TSA increased miR-1 level 25- and 250-fold in A549 and H1299 cells, respectively, whereas 5-AzaC had minimal effect (Fig. 2, E and F). Prolonged treatment with 5-AzaC could not activate miR-1 expression in A549, H1155, and H792 cells (data not shown). These results suggest that DNA methylation plays a causal role in the suppression of miR-1 in these lung cancer cells. Since the promoter of the intronic miR-1-1 gene is embedded in Cpg islands, we analyzed its methylation status by COBRA (combined bisulfite restriction analysis) in primary lung tumor and matching normal tissues. The absence of tumor-specific methylation (data not shown) suggests that DNA methylation does not play a major role in suppressing miR-1-1 in lung tumors.

Ectopic Expression of miR-1 Reduces Growth and Replication Potential of Lung Cancer Cells—

Next we examined the antitumorigenic function of miR-1 in lung cancer cells. For this purpose, we generated stable A549 cell lines expressing miR-1. We used two clones (clones 1 and 2) expressing comparable levels of miR-1 (~20-fold) relative to the control (vector-transfected) cells (Fig. 3A).

We first studied the effect of ectopic miR-1 on cell proliferation. The results showed that cell growth was significantly reduced in both A549 cells compared with the control (vector-transfected) cells (Fig. 3B). After 48 h, growth of miR-1-expressing clones 1 and 2 was reduced by 18 and 30%, respectively, relative to control cells. Replication of cells was significantly reduced, as assayed by [3H]thymidine incorporation into DNA (Fig. 3C) by ~56 and 38%, respectively, relative to control cells (Fig. 3C). These results indicate that miR-1 possesses growth-inhibitory property of miR-1.

Next we examined the antitumorigenic function of miR-1 in the ability of A549 cells to migrate. In the first technique, cells were seeded in serum-free medium on the top chamber of a two-chamber trans-well cell culture plate, and the cells migrated to the lower chamber containing complete medium after 48 h were photographed (Fig. 4A) and counted. As expected, very few cells (1%) migrated to the lower chamber containing serum-free medium. Migration of miR-1-expressing clones 1 and 2 was inhibited by 27 and 35%, respectively, compared with control cells (Fig. 3C).

In the second approach, we used a “scratch wound healing” assay (Fig. 4B). Motility of cells at different time points after generation of the wound was monitored. Closure of the wound was complete within 48 h in control A549 cells (Fig. 4D). In contrast, miR-1-expressing cells migrated toward the wound at a much slower rate. These results demonstrate that miR-1 expression markedly reduces the migration/motility of lung cancer cells.
Oncogenic MET Is a Target of miR-1 in Lung Cancer Cells

Next, we explored the underlying molecular mechanism of the antitumorigenic property of miR-1 in lung cancer cells. Since micro-RNAs primarily mediate their biological functions in动物 cells by impeding the expression of target genes, we searched different data bases (TargetScan and PicTar) for its potential targets that exhibited oncogenic properties. MET (hepatocyte growth factor receptor), which harbors two conserved miR-1 cognate sites (Fig. 5A), is a predicted target of miR-1. MET is a receptor-type tyrosine kinase, overexpressed in many human cancers (46, 47). It consists of two subunits of 50 and 145 kDa processed from a 170-kDa precursor polypeptide. To determine whether MET expression is indeed regulated by miR-1, we generated pIS0-MET-3′-UTR, transfected the construct along with pSV40-β-gal (as internal control). After 48 h, firefly luciferase and β-galactosidase activities were measured. MET-3′-UTR deleted of both or individual miR-1 complementary sites were transfected following the same protocol. The results are means of three independent experiments ± S.D. The levels of miR-1 targets are reduced in A549 cells expressing miR-1. Cell extracts were subjected to Western blot analysis with specific antibodies. C, quantification of Western blot data in B. A reproducible result was obtained in two independent experiments.

**FIGURE 5.** MET proto-oncogene is a target of miR-1. A, the 3′-UTR of MET harbors two miR-1 cognate sites. Luciferase activity regulated by 3′-UTR of MET is inhibited by ectopic expression of miR-1. A549 cells were co-transfected with firefly luciferase-3′-UTR (MET) and hsa-miR-1 or control RNA (60 nM) along with pSV40-β-gal (as internal control). After 48 h, firefly luciferase and β-galactosidase activities were measured. MET-3′-UTR deleted of both or individual miR-1 complementary sites were transfected following the same protocol. The results are means of three independent experiments ± S.D. A reproducible result was obtained in two independent experiments. D, Pim-1 expression in miR-1 expressing A549 clones was measured by real time RT-PCR.
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**FIGURE 6.** Ectopic expression of miR-1 inhibited growth, clonogenic survival, and anchorage-independent growth of its target proteins in H1299 cells. Cells were transfected with pre-miR-1 (100 nM) or control RNA. After 24 h, cells were trypsinized and used for different assays. A, Northern blot analysis of total RNA (10 μg). The signal was detected by autoradiography. B, growth of cells as measured by an MTT assay. Cells (4000/well) were seeded in a 96-well plate, and cell growth was monitored every 24 h for 5 days. C and D, assay of clonogenic survival. 1 × 10³ cells/60-mm dish were seeded in a 100-mm dish, and colonies were stained with 0.05% crystal violet after 2 weeks. E and F, anchorage-independent growth of H1299 cells in soft agar. 1.5 × 10⁶ cells/60-mm dish were used for the soft agar assay. A representative set of colonies formed after 4 weeks is shown. Each sample in B–D was analyzed in triplicate, and the data are means of two independent experiments ± S.D. G, Western blot analysis of miR-1 targets in H1299 cells transfected with hsa-miR-1. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

**FIGURE 7.** miR-1 targets are up-regulated with concomitant increase in growth rate of N417 cells depleted of endogenous miR-1. A, miR-1 level in N417 cells transfected with 50 nM anti-miR-1 or control RNA. Total RNA isolated from cells after 48 h was analyzed by real time RT-PCR. B, Western blot analysis of miR-1 targets in transfected N417 cells. Reproducible results were obtained in two independent experiments. C, growth of N417 cells transfected with anti-miR-1 (50 nM) or control RNA was measured by an MTT assay beginning 48 h post-transfection (0 h). The optical density at 570 nm at 0 h was assigned a value of 1. The results are means of three independent experiments ± S.D.

RNA (Fig. 5A). Moreover, miR-1-dependent repression of luciferase activity was abrogated upon the deletion of both miR-1 cognate sites, whereas deletion of each site individually inhibited luciferase activity by 50%. These results showed that the MET-3'-UTR was instrumental in miR-1-mediated negative regulation of the reporter gene activity. Western blot analysis showed significant reduction in MET (~50 and 65%) in clones 1 and 2, respectively) in both miR-1-expressing clones (Fig. 5B and C). The decrease in MET mRNA level in these cells (Fig. 5D) indicates that miR-1 also induces destabilization of its message.

**Ectopic Expression of miR-1 Inhibits Growth, Clonogenic Survival, and Anchorage-independent Growth of H1299 Cells**—To demonstrate that the antitumorigenic property of miR-1 is not restricted to A549 cells, we transfected nonexpressing H1299 cells with pre-miR-1 and measured cell growth, clonogenic survival, and anchorage-independent growth. Northern blot analysis showed a 45-fold increase in miR-1 level in these cells compared with those transfected with control RNA (Fig. 6A). Proliferation of miR-1-expressing cells was inhibited by 44 and 49% on day 3 and 4, respectively, compared with that of control cells (Fig. 6B). Similarly, clonogenic survival of H1299 cells (a pair of representative plates is shown in Fig. 6C) was inhibited 62% upon ectopic expression of miR-1 (Fig. 6D). Furthermore, anchorage-independent growth, a hallmark of cancer cells, dramatically (80%) inhibited in miR-1-expressing H1299 cells compared with controls (Fig. 6E and F). It is noteworthy that the sizes of the colonies formed by miR-1-expressing cells are significantly smaller than the controls. These properties of miR-1-expressing cells correlated with reduced expression of its targets MET and FoxP1 in these cells (Fig. 6G).

We also performed the reverse experiment in which growth rate and MET level were measured in N417 cells following depletion of miR-1. Transfection of antisense miR-1 resulted in a ~75% decrease in its expression compared with cells transfected with control RNA (Fig. 7A). Western blot analysis revealed a ~2-fold increase in MET in miR-1-depleted cells (Fig. 7B). Depletion of miR-1 with concomitant elevation of its oncogenic target MET was associated with an ~20% increase in cell growth (Fig. 7C).

Next, we measured MET mRNA level in primary lung tumors by RT-PCR analysis, which showed its up-regulation in 12 of 16 tumors compared with matching control tissues (Fig. 8A). Immunohistochemical analysis of tumors and normal lung tissues demonstrated that MET is expressed at a low level in lung tissues, localizing to less than 1% of pneumocytes and cells lining bronchi in a given section (Fig. S3). In contrast, it was markedly up-regulated in the lung cancer cells. Representative data (Fig. S3) clearly demonstrate a dramatic increase in MET level in the lung cancer. Thus, down-regulation of miR-1 is probably one of the mechanisms by which its oncogenic target MET is up-regulated in lung cancer.
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Since HDACs have been identified as therapeutic targets in a variety of cancers, including lung cancer (49), and HDAC4 is a validated target of miR-1 (26), we measured its level in A549 cells expressing miR-1. Western blot analysis revealed a dramatic decrease in HDAC4 following miR-1 expression (Fig. 5, B and C). FoxP1 RNA level increased in 75% of lung tumors analyzed (Fig. 8A). Immunohistochemical analysis showed that FoxP1 and HDAC4 were also up-regulated in primary lung cancer tissues (a representative figure is shown, Fig. S2).

Oncogenic Ser/Thr Kinase Pim-1, a Target of miR-1, Is Significantly Regulated in Lung Cancer—Since miR-1 expression reversed the tumorigenic property of A549 cells in vitro, it was logical to examine whether ectopic miR-1 could regress tumor growth ex vivo. For this purpose, A549 cells expressing miR-1 and vector-transfected cells were injected subcutaneously into the left and right flanks of nude mice, respectively, and tumor volume and weight were measured. The results showed that the tumor growth was significantly reduced in miR-1-expressing cells compared with the control cells (Fig. 9A). The reduction in tumor volume and weight in miR-1-expressing A549 cells were 55.27 and 62%, respectively (Fig. 9, B and C). Real time RT-PCR analysis showed that the miR-1 level was ~9-fold higher in A549 clones expressing miR-1 relative to the control cell. Real time RT-PCR analysis demonstrated that ectopic miR-1 reduced the mRNA levels of FoxP1 (Fig. 5E).

Pim-1, a Ser/Thr kinase, induces tumorigenesis by promoting cell cycle progression and cell apoptosis (50, 51). Since its 3'-UTR harbors one miR-1 cognate site (Fig. 5F), we explored whether miR-1 can regulate Pim-1 expression in lung cancer. Firefly luciferase activity driven by Pim-1–3'-UTR-luciferase, we confirmed that miR-1 negatively regulates reporter activity through Pim-1 3'-UTR (31). Western blot analysis of the tumor tissue extract from nude mice showed that the targets of miR-1 MET and FoxP1 were indeed down-regulated (Fig. 5G). Real time RT-PCR analysis showed that miR-1 reduced lungs tumor cell growth by suppressing the expression of its targets.

Doxorubicin-induced Apoptosis Is Augmented in A549 Cells Expressing Ectopic miR-1—It has been shown recently that certain micro-RNAs can alter sensitivity of cancer cells to therapeutic agents (52–54). We hypothesized that ectopic miR-1

FoxP1 and HDAC4, Targets of miR-1, Are Also Differentially Regulated in Human Lung Cancer—FoxP1, a member of the Fox family of transcription factors, is also a predicted target of miR-1 harboring three cognate sites. It is differentially regulated in human cancers and can act as an oncprotein or a tumor suppressor, depending upon the cellular context (48). By transfecting pS0-FoxP1–3'-UTR-luciferase, we confirmed that miR-1 negatively regulates reporter activity through FoxP1–3'-UTR (31). Western blot analysis of A549 cell extracts showed a significant decrease in the endogenous FoxP1 level in miR-1-expressing cells (Fig. 5, B and C). The steady state level of FoxP1 was reduced by ~80% in A549 clones expressing miR-1 relative to the control cell. Real time RT-PCR analysis demonstrated that ectopic miR-1 reduced the mRNA levels of FoxP1 (Fig. 5E).

**FIGURE 8.** A, RT-PCR analysis of MET and FoxP1 in human primary lung cancers and matching lung tissues. One hundred ng (for MET), 200 ng (for FoxP1), and 10 ng (for 18 S rRNA) cDNA was amplified with gene-specific primers. The PCR products were separated in an agarose gel, stained with ethidium bromide, and photographed. The asterisks denote samples in which MET and FoxP1 were up-regulated. B, real time RT-PCR analysis of Pim-1 in human primary lung cancers and matching lung tissues. The data are shown using a box-whisker plot. A horizontal line in each box represents the median value of Pim-1 mRNA normalized to 18 S rRNA in each group. The box denotes the 25th and 75th percentile range of scores, whereas whiskers represent the highest and lowest values.

**FIGURE 9.** Ectopic Expression of miR-1 in A549 cells inhibits tumor growth in athymic nude mice. miR-1-expressing cells were trypsinized and counted and then mixed with 50% Matrigel, and 2 × 10⁶ cells were injected subcutaneously into the left and right flanks, respectively, of nude mice. After 4 weeks, the animals were sacrificed, and the tumor volume and weight were measured. The results showed that the tumor growth was significantly reduced in miR-1-expressing A549 cells expressing miR-1 and vector-transfected cells (Fig. 9A). The reduction in tumor volume and weight in miR-1-expressing A549 cells were 55.27 and 62%, respectively (Fig. 9, B and C). Real time RT-PCR analysis showed that the miR-1 level was ~9-fold higher in A549 clones expressing miR-1 relative to the control cell. Real time RT-PCR analysis demonstrated that ectopic miR-1 reduced the mRNA levels of FoxP1 (Fig. 5E).
may sensitize cells to anticancer drugs, because it targets MET that protects cells against DNA damaging agents (35). We therefore investigated whether miR-1 can alter sensitivity of lung cancer cells to anticancer drugs. Doxorubicin (DOXR) is one of the major drugs used in the treatment of a variety of cancers, including lung cancer (39). Induction of apoptotic cell death is a key mechanism by which DOXR inhibits cancer cell growth (55). We treated A549 cells with DOXR (1 μg/ml) and counted cells undergoing nuclear fragmentation by Hoechst staining (Fig. 10A). The results showed that 17 and 23% of the cells were apoptotic after 24 h of DOXR treatment in miR-1-expressing clones 1 and 2, respectively, whereas only 2% of the vector-transfected cells were apoptotic (Fig. 10B). After 36 h, 28 and 38% of cells of clones 1 and 2 were apoptotic, as opposed to only 4% of the control cells undergoing cell death (Fig. 10C).

We also measured the number of cells undergoing apoptosis by fluorescence-activated cell sorting analysis of cells with fragmented DNA after propidium iodide staining. The population of cells with sub-G₀ DNA content was increased from 0% in the vector-transfected cells to 23% in miR-1-expressing cells after DOXR exposure for 24 h (Fig. 10D). The population of cells with fragmented DNA was negligible in cells that were not exposed to DOXR (data not shown). It is notable that the population of cells at different stages of the cell cycle is distinct in miR-1-expressing cells from that in control cells (Fig. 10D).

To elucidate the underlying mechanism of miR-1-mediated sensitization of A549 cells to DOXR, we measured the levels of a few critical factors that mediate apoptosis by Western blot analysis. As expected, DNA damage due to DOXR exposure resulted in significant induction of p53 (Fig. 11A) and its target gene PUMA (data not shown) at a comparable level in the control and miR-1-expressing A549 cells at all time points tested. In contrast, the basal level of cleaved caspase 9, an activator caspase, was relatively high both in the control and in miR-1-expressing cells (Fig. 11A, lanes 1, 5, and 9). However, in the vector-transfected cells, its level gradually decreased with time of DOXR exposure (Fig. 11, A lanes 5–8 and B), whereas it increased to some extent in miR-1-expressing cells (Fig. 11A, lanes 1, 5, and 9).
miR-1 Suppresses Lung Tumorigenesis

Although micro-RNA signatures for different types of cancer have been established, elucidation of the role of altered expression of specific micro-RNAs and its consequence on oncogenic transformation remains in the early stage of development. The data presented herein shed light on the regulatory role of miR-1 in lung cancer. miR-1 is predominantly expressed in cardiac tissues and smooth and skeletal muscles, where it inhibits cell cycle progression of myoblasts and promotes their differentiation (57). In cardiac tissues, miR-1 inhibits expression of some transcription repressors, such as Hand2 (27) and HDAC4 (26). In the present study, we showed that miR-1 expression is reduced in lung cancers and that it inhibits the tumorigenic potential of lung cancer cells by down-regulating oncogenic targets, such as MET and FoxP1. To our knowledge, this is the first report that correlates loss of miR-1 with tumorigenic potential of lung epithelial cells. This contention is based upon the following observations. First, miR-1 expression was significantly reduced in primary lung cancer. Second, almost all lung cancer cell lines tested showed either very low or undetectable levels of miR-1. Third, ectopic expression of miR-1 in the lung cancer cell line (A549) significantly reduced cell growth, replication potential, and tumor growth in nude mice. miR-1 facilitated lung cancer cell transformation of A549 cells to the metastatic phenotype. Ectopic miR-1 underexpression is common against lung cancer. The transcriptional regulation of miR-1 is likely to be one of the transcription factors involved in the activation of the miR-1 gene. Indeed, frequent loss of C/EBPα and miR-1 in bronchial epithelial cells suggests its potential role in miR-1 gene activation in these cells. Further, frequent loss of C/EBPα by genetic (11) and epigenetic mechanism (52) could explain miR-1 suppression in lung cancer cells. C/EBPα can up-regulate gene expression by directly binding to cognate sites or indirectly through protein-protein interactions (36). miR-1 is likely to be one of the targets of C/EBPα, through which it inhibits cell proliferation and induces apoptosis (59). It would be of interest to determine whether binding of C/EBPα to miR-1-1 and miR-1-2 gene promoters is essential for their transactivation. The miR-1 suppression in lung cancer cells is also mediated by histone hypoacetylation, since treatment of A549 cells with trichostatin A, an HDAC inhibitor, significantly induced miR-1 expression. The lack of activation of miR-1 by a DNA hypomethylating agent suggests that DNA methylation may not play a key role in silencing micro-RNA genes in lung cancer cells. In contrast, methylation-mediated silencing of miR-1 occurs in human primary hepatocellular carcinomas and cell lines (31). It therefore appears that different epigenetic mechanisms may contribute to regulation of miR-1 expression, depending upon the cell type. Suppression of miR-1 in lung cancer cells expressing a high level of SRF suggests that it may not play a key role in miR-1 expression. Activation of miR-1 by TSA suggests repressed chromatin structure may play a causal role in its silencing in lung cancer cells. The lack of activation of miR-1 or any other micro-RNA (23) by a DNA-hypomethylating agent supports the notion that DNA methylation may not play a key...
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Like protein-coding genes, some micro-RNAs also function as oncogenes or tumor suppressors (58, 59). The global reduction of micro-RNA is associated with increased tumorigenesis (60). In fact, mRNA and miRNA interactions are tightly regulated, and even a small change between these interactions may cause severe consequences to cell physiology (61). Accordingly, alterations in the expression of target genes could lead to disease states. We further showed that this decrease in miR-1 expression is associated with an increase in oncogenic MET, Pim-1, FoxP1, and HDAC4. Pim-1 is a Ser/Thr kinase overexpressed in various human cancers and plays a causal role in lymphoma and leukemogenesis (50, 51). Our study shows that it is also up-regulated in the majority of human primary lung tumors and cancer cell lines. FoxP1 is an essential transcription factor required for mammalian development (62). It is also noteworthy that it maps to chromosome 3p14.1, a region that is amplified or translocated, resulting in its up-regulation in patients with diffuse large B cell lymphoma. Thus, the nuclear FoxP1 expression is associated with poor prognosis of cells. Our data support the notion that the loss of miR-1 at the transcriptional level should be tightly regulated for normal function in patients with diffuse large B cell lymphoma. Thus, the miR-1 level should be tightly regulated for normal function in patients with diffuse large B cell lymphoma. The FoxP1 FoxP1 level could be tightly regulated for normal function in patients with diffuse large B cell lymphoma. Thus, the nuclear FoxP1 expression is associated with poor prognosis of cells. Our data support the notion that the loss of miR-1 at the transcriptional level should be tightly regulated for normal function in patients with diffuse large B cell lymphoma. 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