MicroRNA-224 promotes tumor progression in nonsmall cell lung cancer

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Lung cancer is the leading cause of cancer-related deaths worldwide. Despite advancements and improvements in surgical and medical treatments, the survival rate of lung cancer patients remains frustratingly poor. Local control for early-stage nonsmall cell lung cancer (NSCLC) has dramatically improved over the last decades for both operable and inoperable patients. However, the molecular mechanisms of NSCLC invasion leading to regional and distant disease spread remain poorly understood. Here, we identify *microRNA-224* (*miR-224*) to be significantly up-regulated in NSCLC tissues, particularly in resected NSCLC metastasis. Increased *miR-224* expression promotes cell migration, invasion, and proliferation by directly targeting the tumor suppressors TNFα-induced protein 1 (TNFAIP1) and SMAD4. In concordance with in vitro studies, mouse xenograft studies validated that *miR-224* functions as a potent oncogenic miRNA in NSCLC in vivo. Moreover, we found promoter hypomethylation and activated ERK signaling to be involved in the regulation of *miR-224* expression in NSCLC. Up-regulated *miR-224*, thus, facilitates tumor progression by shifting the equilibrium of the partially antagonist functions of SMAD4 and TNFAIP1 toward enhanced invasion and growth in NSCLC. Our findings indicate that targeting *miR-224* could be effective in the treatment of certain lung cancer patients.

microRNA | NSCLC | metastasis | TNFAIP1 | SMAD4

Lung cancer is the second most common cancer and the leading cause of cancer-related death worldwide. In 2013, there were an estimated 228,190 new cases of lung cancer and 159,480 deaths in the United States. Despite advancements and improvements in surgical and medical treatments, the 5-y survival rate of lung cancer patients remains frustratingly poor (1). Although local control for early-stage nonsmall cell lung cancer (NSCLC) has dramatically improved over the last decades for both operable and inoperable patients (2, 3), ∼20% of early-stage patients, however, are developing distant metastasis (4, 5), and 10–15% of patients undergoing stereotactic ablative body radiation fail regionally (6). The molecular mechanisms of NSCLC invasion leading to regional and distant disease spread remain poorly understood. Understanding the molecular mechanisms that regulate invasion and disease spread would help to identify promising therapeutic targets and could be exploited to refine patient selection for already existing therapies.

MicroRNAs (miRNAs) are small endogenous noncoding RNAs that negatively regulate mRNA stability and/or repress mRNA translation (7). miRNAs have been proven to play essential roles in the initiation and progression of certain cancer types, such as chronic lymphocytic leukemia (8), breast cancer (9), and lung cancer (10, 11). Several miRNA expression profiling studies have shown that miRNAs could be used as diagnostic and prognostic biomarkers. For example, high expression levels of miR-155 and low levels of let-7a expression correlate with poor prognosis of lung cancer (10). In colorectal cancer (CRC), up-regulated miR-135b correlates with tumor stage and poor clinical outcome (12). Recently, we conducted genome-wide miRNA sequencing in primary lung cancer tissue from patients with lung adenocarcinoma (ADC), and we identified that miR-31 promotes lymph node metastasis and negatively correlates with survival in patients with lung ADC (13), emphasizing the impact of miRNAs in NSCLC biology.

TNFα-induced protein 1 (TNFAIP1) was originally identified as a TNFα- and LPS-inducible gene (14). It has been reported that TNFAIP1 interacts with the proliferating cell nuclear antigen and the small subunit of DNA polymerase-δ (P50) (15), suggesting that TNFAIP1 might be involved in DNA synthesis and apoptosis. Indeed, TNFAIP1 elicited proapoptotic activity, and coexpression of TNFAIP1 and RhoB markedly increased apoptosis in HeLa cells (16). SMAD4 plays a central role in the TGF-β family signaling pathways and is the only member of the SMAD family that is

Significance

Aberrant microRNA (miRNA) expression is involved in tumorigenesis, and *miR-224* was observed to be up-regulated in certain tumor types. However, the role of *miR-224* in the pathogenesis of lung cancer remains poorly understood. Here, we comprehensively analyzed and revealed mechanisms of *miR-224* up-regulation and its oncogenic role in nonsmall cell lung cancer (NSCLC). We showed that *miR-224* promotes cellular migratory, invasive, and proliferative capacity and tumor growth both in vitro and in vivo. Furthermore, we identified TNFα-induced protein 1 and SMAD4 as targets of *miR-224*. In addition, up-regulated *miR-224* expression in NSCLC is partially controlled by its promoter region's hypomethylation and activated ERK signaling. Our finding suggests that targeting *miR-224* might be a promising therapeutic strategy in the treatment of NSCLC.

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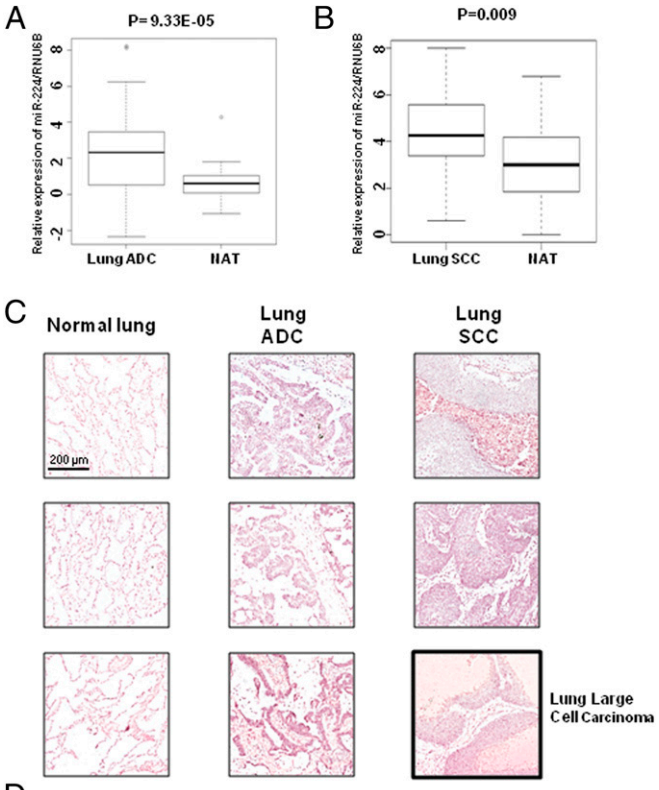
involved in TGF-β, activing, and bone morphogenetic protein signaling pathways (17, 18). SMAD4 functions as a tumor suppressor; loss of SMAD4 was frequently seen in pancreatic cancers and CRCs. Approximately 55% of pancreatic cancers have deletions or mutations in the *SMAD4* locus (19), and about 30% of biallelic loss of *SMAD4* was found in metastatic CRCs (20). To date, several studies have reported that TNFAIP1 and SMAD4 are targets of miRNAs in certain cancer types. For instance, oncogenic miRNAs, such as the miR-130a/301a/454 family, target SMAD4 in CRC, and miR-182 targets SMAD4 in bladder cancer (21, 22). In gastric cancer, miR-372/373 targets TNFAIP1, promoting carcinogenesis (23, 24).

Here, we show that increased *miR-224* in NSCLC promotes cell migration, invasion, and proliferation by direct targeting of TNFAIP1 and SMAD4. We further show that aberrant *miR-224* expression is partially controlled by hypomethylation of its promoter region and activated ERK signaling in NSCLC. Through both in vitro and in vivo analyses, we revealed the mechanisms of *miR-224* up-regulation and its oncogenic role in NSCLC pathogenesis.

Results

Up-Regulated *miR-224* Is Associated with NSCLC Metastasis. We conducted genome-wide miRNA sequencing (miR-seq) on four primary lung ADCs with lymph node metastasis and six primary lung ADCs without lymph node metastasis as previously described (13). We selected 16 deregulated miRNAs with *P* values less than 0.01 and fold changes larger than five (*SI Appendix*, Table S1). Among 12 up-regulated miRNAs, we have shown that miR-31 was positively associated with lymph node metastasis and negatively correlated with survival in patients with lung ADC (13). Subsequent analyses using 87 NSCLC cases and 48 normal adjacent tissues (NATs) from the Ohio State University Comprehensive Cancer Center (OSUCCC) Tissue Procurement Shared Resource indicate that *miR-224* was significantly up-regulated in both lung ADC and squamous cell carcinoma (SCC) tissues compared with the NATs (Fig. 1 *A* and *B*). Reanalysis of 666 lung ADC and SCC cases and 86 NATs for which *miR-224* expression was available in The Cancer Genome Atlas (TCGA) miR-seq dataset confirmed that *miR-224* was up-regulated in both lung ADC and SCC compared with NATs (*SI Appendix*, Fig. S1 *A* and *B*). In addition, of the evaluable 424 lung ADC patients with available staging and miRNA expression data, 281 patients had N0 disease, and 143 patients had positive lymph nodes (N1+) metastasis. We found that *miR-224* was markedly up-regulated in patients with lymph node metastasis (N1+) compared with those patients without lymph node metastasis (N0) (*SI Appendix*, Fig. S1*C*). In situ hybridization analyses on human lung tissue microarrays were conducted to further examine *miR-224* expression in NSCLC. Analyzing 51 evaluable paired NSCLC tissues and NAT revealed that *miR-224* was overexpressed in 61% of cases, in which *miR-224* was measured in cancer tissues compared with corresponding NATs (Fig. 1*C* and *SI Appendix*, Fig. S1*D*). We further evaluated *miR-224* expression in metastasized lung cancer tissues and primary lung cancer tissues and found stronger *miR-224* expression in metastasized lung cancers (90%) compared with that in primary lung cancers (58%) (Fig. 1*D*). These results suggest that *miR-224* might also play an important role in lung cancer metastasis. Kaplan–Meier survival analysis using available data from 642 TCGA NSCLC patients indicates that there is no significant association between *miR-224* expression and survival (*SI Appendix*, Fig. S1*E*). Interestingly, *miR-224* was significantly associated with poor prognosis of patients with lung ADC having Kirsten rat sarcoma viral oncogene homolog (*kras*)/*p53* mutations (n = 59), important genes involved in lung ADC pathogenesis (*SI Appendix*, Fig. S1*F*). Significantly up-regulated *miR-224* expression in the N1+ group compared with the N0 group was also observed in lung ADC patients with *kras*/*p53* mutations (*SI Appendix*, Fig. S1*G*). Subsequent analysis indicates that there is no robust association between *miR-224* expression and T stage in the unselected NSCLC TCGA cohort. We, however, found



D

	miR224 expression levels		
	0	1+	2+
Normal (9)	3 (33%)	4 (44%)	2 (22%)
Primary (38)	2 (5%)	14 (37%)	22 (58%)
Metastases (10)	0 (0%)	1 (10%)	9 (90%)

Fig. 1. *miR-224* expression in primary NSCLC and metastasized lung cancer. (*A* and *B*) Box plots showing *miR-224* expression in patients with (*A*) lung ADC and (*B*) lung SCC; the RNA samples were purified from 56 lung ADCs and 21 corresponding NATs and 31 lung SCCs and 27 corresponding NATs, respectively. The RNAs were subject to quantitative RT-PCR with an *miR-224* probe, and the expression was normalized by RNU6B. (*C*) Representative pictures for in situ hybridization analysis of *miR-224*. (*D*) Summary of tissue microarray data for metastasized lung cancer.

significantly increased *miR-224* expression in patients with T3 stage compared with patients with T1 stage (*SI Appendix*, Fig. S1*H*).

***miR-224* Promotes Migration, Invasion, and Proliferation of Lung Cancer Cells.** Given the high expression of *miR-224* in lung cancer and its association with metastasis, we hypothesized that *miR-224* might play a role in the carcinogenesis and progression of lung ADC and SCC. To investigate the function of *miR-224* in lung cancer cells, we transduced a Lenti-miR vector containing *miR-224* precursor in three NSCLC cell lines (H1299, H1573, and H460) to overexpress *miR-224*. A549 cells were transduced with a vector containing an miRZip-224 anti–*miR-224* miR construct to knockdown *miR-224*. The expression levels of *miR-224* after overexpression and/or knockdown were confirmed by quantitative RT-PCR (*SI Appendix*, Fig. S2 *A*–*D*). In response to overexpression of *miR-224*, the H1299 and H1573 cells' migratory, invasive, and proliferative abilities were significantly increased (Fig. 2 *A*–*C*). Migrated cell counts were shown in *SI Appendix*, Fig. S2 *E* and *F*. Similar results were also observed in *miR-224*–overexpressing H460 cells (*SI Appendix*, Fig. S2 *G* and *I*). Conversely, knockdown of *miR-224* in A549 cells markedly

reduced cell migration and proliferation (Fig. 2 *D* and *E* and *SI Appendix*, Fig. S2*H*).

***miR-224* Directly Targets the 3′-UTR of TNFAIP1 and SMAD4.** To identify putative mRNA targets of *miR-224*, we performed bioinformatics analyses and found several candidate targets. Among them, the 3′-UTRs of TNFAIP1 and SMAD4 mRNAs contained sequences complementary to the *miR-224* seed sequence (*SI Appendix*, Fig. S2*K*). We focused on these two genes based on their tumor-suppressive role in carcinogenesis. To verify whether TNFAIP1 and SMAD4 are direct targets of *miR-224*, we cotransfected each of the 3′-UTRs and *miR-224* mimics in 293T cells. Consistent reduction in luciferase activity for both of 3′-UTRs by *miR-224* was observed. To validate target specificity, we generated mutated forms of the 3′-UTRs, where the binding sites of *miR-224* were destroyed using the QuikChange Mutagenesis Kit. In detail, there were two *miR-224* binding sites for 3′-UTRs of TNFAIP1 and SMAD4. Cotransfection of *miR-224* with mutated forms of the 3′-UTRs (TNFAIP1 3′-Mut2 UTR, SMAD4 3′-Mut1 UTR, and SMAD4 3′-Mut2 UTR) significantly attenuated the reduction of luciferase activities on WT 3′-UTRs (Fig. 3 *A* and *B*), suggesting the specificity of miRNA and target 3′-UTRs. Next, we examined the effect of *miR-224* on target proteins and mRNAs using overexpression and knockdown systems. Overexpression of *miR-224* markedly reduced mRNA and protein expressions of TNFAIP1 and SMAD4, respectively, in H1299, H1573, and H460 cells (Fig. 3 *C* and *D* and *SI Appendix*, Fig. S2*J*). Conversely, knockdown of *miR-224* increased expression of TNFAIP1 and SMAD4 mRNA and protein levels, respectively, in A549 cells (Fig. 3 *E* and *F*).

TNFAIP1 and SMAD4 Play Essential Roles in the *miR-224*–Induced Phenotypes. First, we examined *TNFAIP1* and *SMAD4* expression in lung ADC and SCC using the TCGA dataset. We assessed a total of 334 lung ADC patients with 57 matched NATs, and 349 lung SCC patients with 51 matched NATs with available gene expression data. Notably, significant decreases of both genes were observed in both lung ADC and lung SCC compared with the corresponding NATs. In lung ADC, the TNFAIP1 (P = 4.9E-15) and SMAD4 (P = 2.2E-16) expressions were dramatically reduced in primary tumor tissues (*SI Appendix*, Fig. S3 *A* and *B*). Similar reductions of TNFAIP1 (P = 1.8E-7) and SMAD4 (P = 6.2E-9) expression levels were also observed in lung SCC (*SI Appendix*, Fig. S3 *C* and *D*). We further analyzed *TNFAIP1* and *SMAD4* expression in an OSU cohort containing 63 NSCLC cases and 48 matched NATs. Consistent reductions of *TNFAIP1* (5.23E-14) and *SMAD4* (2.67E-06) in NSCLC were also observed in the OSU cohort (*SI Appendix*, Fig. S3 *G* and *H*). Second, we examined the correlation between *miR-224* and its target genes (*TNFAIP1* and *SMAD4*) using the OSU cohort and the TCGA dataset to understand if the *miR-224*–mediated *TNFAIP1* and *SMAD4* suppression might occur in human lung cancers. The samples (n = 111) from NSCLC patients from the OSU cohort or the TCGA lung ADC (n = 306) samples having both *miR-224* and *TNFAIP1* or *SMAD4* expression data available were selected for Pearson correlation analysis. *miR-224* showed a significant anticorrelation with both *TNFAIP1* (r = –0.34, P = 0.000288 in the OSU cohort; r = –0.12, P = 0.038 in the TCGA dataset) and *SMAD4* (r = –0.37, P = 6.286E-05 in the OSU cohort; r = –0.33, P = 3.2E-9 in the TCGA dataset), suggesting that reduced *TNFAIP1* and *SMAD4* expressions might be related to the up-regulation of *miR-224* expression in lung cancer (Fig. 3 *G* and *H* and *SI Appendix*, Fig. S3 *E* and *F*).

To determine whether the oncogenic functions of *miR-224* were attributable to direct targeting of tumor suppressors TNFAIP1 and SMAD4, we conducted several overexpression and knockdown experiments for TNFAIP1 and SMAD4, respectively. Knockdown of TNFAIP1 significantly increased migratory and proliferative ability of H460 (Fig. 4 *A* and *B* and *SI Appendix*, Figs. S4*A* and S7*B*) and H1299 cells (*SI Appendix*, Fig. S4 *B*–*D*). In addition, TNFα

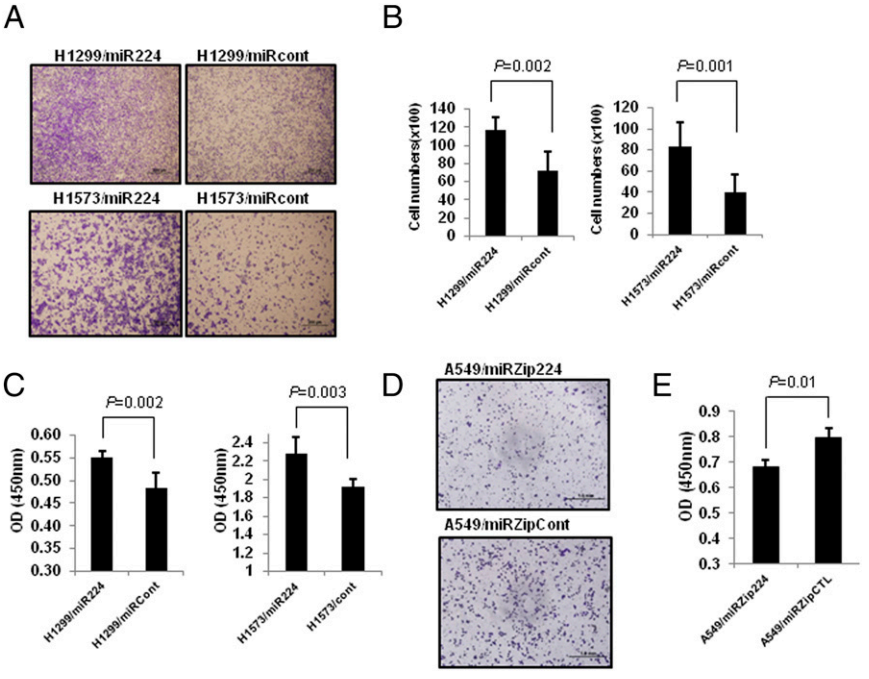


Fig. 2. Ectopic expression of *miR-224* promotes cell proliferation, migration, and invasion. (*A*) Cell migration assay for *miR-224*–overexpressing H1299 and H1573 cells using transwell membranes. Representative pictures of migration chambers are shown (40× magnification). (*B*) Cell invasion assay for *miR-224*–overexpressing H1299 and H1573 cells. The average counts were derived from 10 random microscopic fields. (*C*) Cell proliferation assay for *miR-224*–overexpressing lung cancer cells. The cell growth rates were measured by cell counting kit 8. (*D*) Representative pictures of migration assay for *miR-224* knockdown A549 cells (100× magnification). (*E*) Cell proliferation assay for *miR-224* knockdown A549 cells. The values present means ± SDs as determined by quintuplex assays.

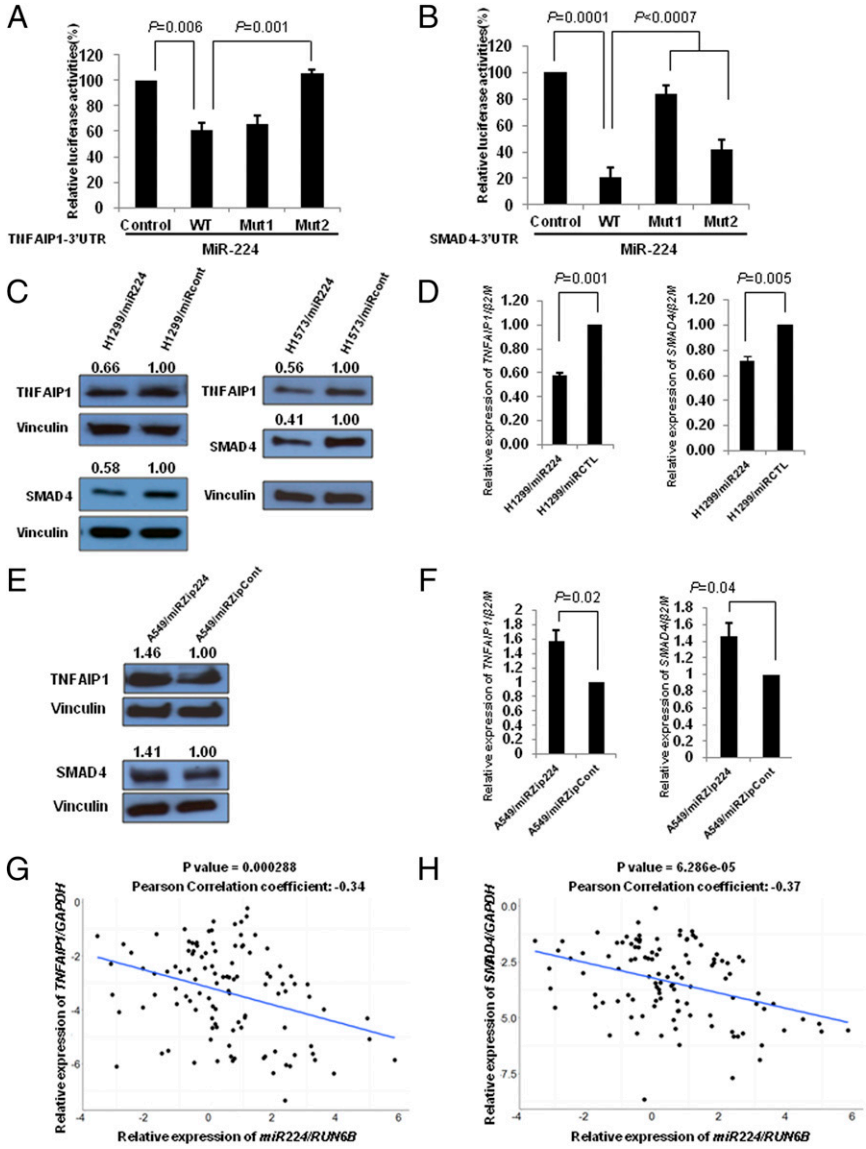


Fig. 3. TNFAIP1 and SMAD4 are direct targets of *miR-224*. (*A* and *B*) Luciferase reporter constructs containing WT or mutated TNFAIP1 and SMAD4 3′-UTRs were cotransfected with *miR-224* mimic into 293T cells. (*C* and *D*) Western blot and quantitative RT-PCR to measure TNFAIP1 and SMAD4 protein and mRNA levels in lung cancer cells infected with lentivirus expressing pre*miR-224* or control vector. (*E* and *F*) Western blot and quantitative RT-PCR to measure TNFAIP1 and SMAD4 protein and mRNA levels in lung cancer cells infected with lentivirus expressing miRZip-224 anti*miR-224* or control vector. The bands were quantified using Image J software, and relative values were obtained by normalizing to the value of each corresponding vinculin. Data are presented as means ± SDs, and each assay was conducted three times. (*G* and *H*) Correlation between *miR-224* and (*G*) *TNFAIP1* or (*H*) *SMAD4* in samples from NSCLC patients from the OSU cohort (n = 111).

markedly induced TNFAIP1 expression in H1299/miRCont cells but not in H1299/*miR-224* cells, indicating that *miR-224* might functionally prevent TNFα signaling-mediated induction of TNFAIP1. We also observed reduced cleaved poly (ADP-ribose) polymerase 1 (PARP1) expression level in H1299/*miR-224* cells compared with H1299/miRCont cells after TNFα treatment (Fig. 4*C*). Additional analyses showed that casp3/7 activity was significantly reduced in H1299/*miR-224* cells compared with H1299/miRCont cells after TNFα treatment (*SI Appendix*, Fig. S4*F*). Accordingly, *miR-224* significantly attenuated TNFα-induced cell growth inhibition in H1299 cells (Fig. 4*D*). These results suggest that *miR-224* is involved in TNFα-induced apoptosis by targeting TNFAIP1 in lung cancer cells.

Next, to evaluate the function of SMAD4 on *miR-224*–mediated cell growth and migration in lung cancer cells, we transduced SMAD4 in stable *miR-224*–overexpressing lung cancer cells. Overexpression of SMAD4 dramatically abrogated *miR-224*–mediated promotion of cell proliferation and migration (Fig. 4 *E* and *F* and *SI Appendix*, Figs. S4*E* and S7*C*) in both H1299 and H1573 lung cancer cells. SMAD4 plays an essential role in the TGF-β signaling pathway, because it binds to SMAD2/3 and translocates to the nucleus upon TGF-β stimulation (17). To evaluate whether *miR-224* affects TGF-β signaling by targeting SMAD4, we treated *miR-224*–overexpressing and control cells with TGF-β. *miR-224* markedly impaired TGF-β–induced nuclear transportation of SMAD4 (Fig. 4*G*), and accordingly, *miR-224* significantly attenuated the TGF-β–induced inhibitory effect of cell growth and migration in H460 cells (Fig. 4 *H* and *I* and *SI Appendix*, Fig. S7*D*). These results suggest that *miR-224* plays a crucial role in the TGF-β signaling pathway by targeting SMAD4 in lung cancer.

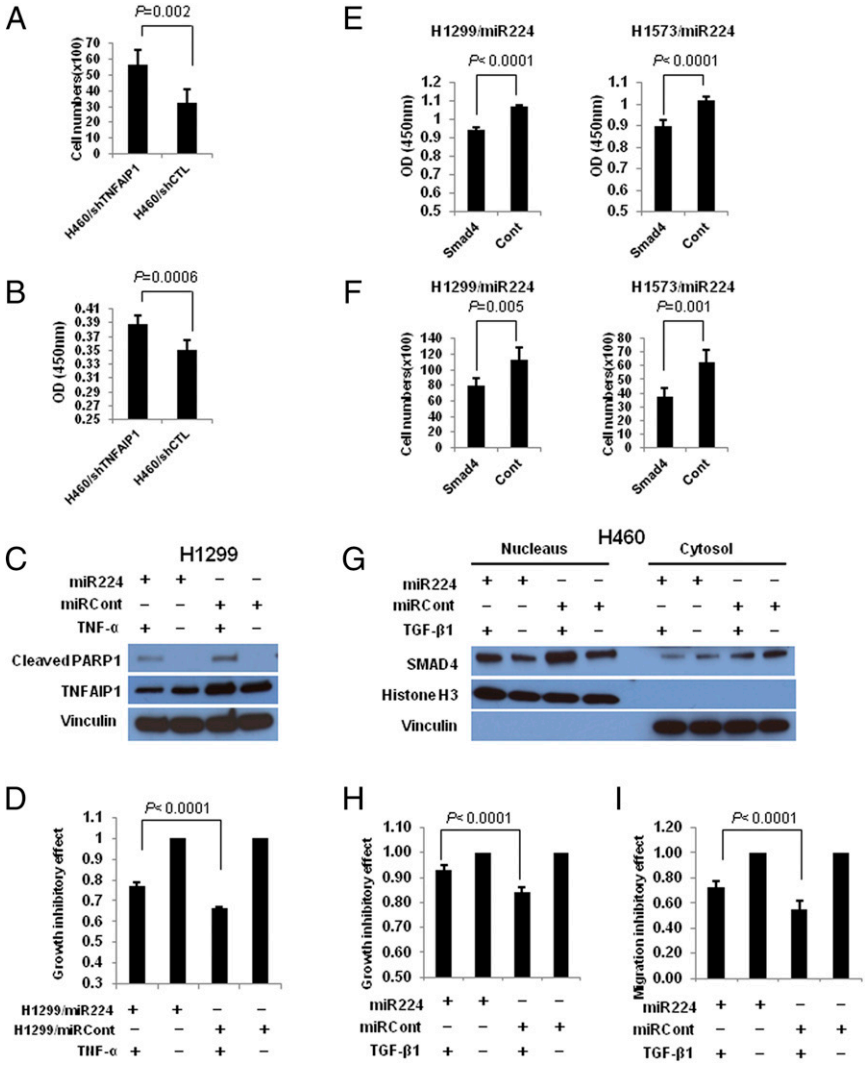


Fig. 4. TNFAIP1 and SMAD4 play a crucial role in *miR-224*–induced cell growth and migration. (*A*) Cell migration assay for TNFAIP1 knockdown H460 cells using transwell membranes. The average counts were derived from six random microscopic fields. (*B*) Cell proliferation assay for TNFAIP1 knockdown H460 lung cancer cells. The cell growth rates were measured by cell counting kit 8. (*C*) Western blot analysis of H1299/*miR-224* and H1299/miRCont cells treated with or without TNFα for 4 h. (*D*) Cell proliferation assay for H1299/*miR-224* and H1299/miRCont cells treated with or without TNFα for 24 h. (*E*) Cell proliferation assay and (*F*) migration assay of *miR-224*–overexpressing cells transfected with SMAD4 or empty vector. (*G*) The H460/*miR-224* and H460/miRCont cells were treated with or without TGF-β for 8 h. Both nuclear and cytosol proteins were extracted and subject to Western blot analysis. (*H*) Cell proliferation assay and (*I*) migration assay for H460/*miR-224* and H460/miRCont cells treated with or without TGF-β. The values present means ± SDs as determined by quintuplex assays.

CpG Island in the Promoter Region of *miR-224* Is Hypomethylated in Lung Cancer. miRNAs are an important molecule class regulating gene expression. miRNA expression is controlled by epigenetic modifications and/or transcriptional activations (25). *miR-224* and *miR-452* are located in intron 6 of the host gene *γ-Aminobutyric Acid A Receptor* (*GABRE*), and the *GABRE* promoter region has a CpG island (*SI Appendix*, Fig. S5*A*). We investigated the correlations between expressions of *miR-224*, *miR-452*, and *GABRE* using the TCGA dataset and found significant positive correlations between all three (*SI Appendix*, Fig. S5*B*), suggesting that these three genes might be transcriptionally coregulated under the same promoter. Next, we examined whether up-regulated *miR-224* expression in NSCLC was associated with hypomethylation of its promoter region's CpG island. We selected nine methylation probes, which were located in the CpG island of the promoter region, from the TCGA Illumina Infinium Human DNA Methylation 450,000 Bead Chip Data to evaluate *miR-224* promoter methylation status. TCGA lung ADC ($n = 221$) samples and lung SCC ($n = 125$) samples containing both methylation and *miR-224* expression were extracted for Pearson correlation analyses. Among nine methylation probes that were analyzed in our study, eight probes displayed significant anticorrelations with *miR-224* expression in NSCLC (*SI Appendix*, Table S3). Anticorrelations between methylation probe cg27049053 and *miR-224* expression in lung ADC ($r = -0.16$, $P = 0.02$) and lung SCC ($r = -0.42$, $P = 1.25\text{E-}6$) are shown in Fig. 5 *A* and *B*. These results suggest that promoter methylation could be involved in the repression of *miR-224* transcription. To validate the results that we found in the TCGA methylation data, we conducted methylation specific-high resolution melting (MS-HRM) analysis using two cell lines (469NAT and 489NAT) from normal lung tissues and two lung cancer cell lines (A549 and H647). Comparing melting curves from each cell line (green lines in Fig. 5*C*) with standard melting curves (orange lines in Fig. 5*C*), 10–30% methylation was estimated in both 469NAT and 485NAT cells, and 0% methylation was estimated in the two lung cancer cell lines (Fig. 5*C*). The bisulfate sequencing results confirmed methylation levels at each CpG dyad (*SI Appendix*, Fig. S5*C*) in the cells from normal lung tissue. Expression of *miR-224* in both 469NAT and 485NAT cells was

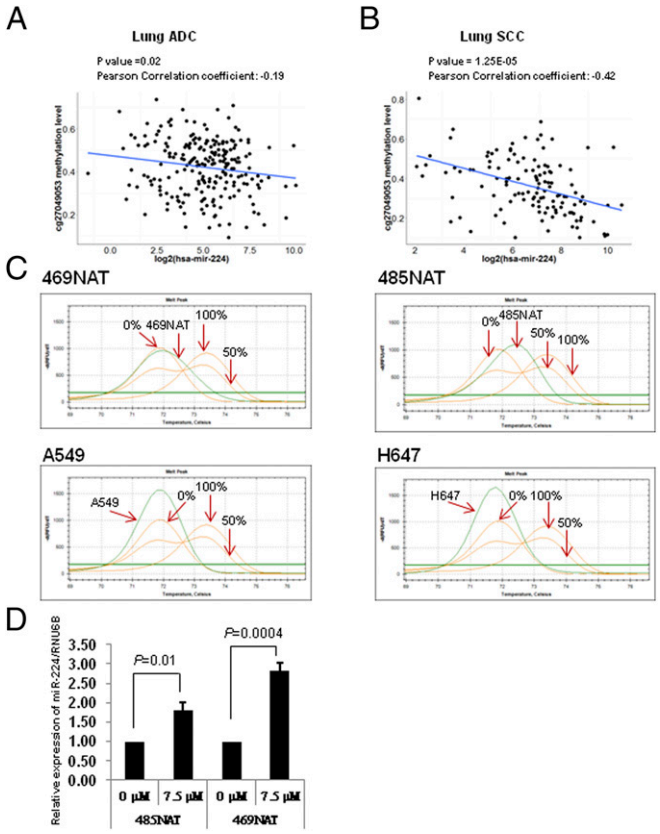


Fig. 5. Correlation between *miR-224* expression and its promoter region's methylation status. (*A* and *B*) *miR-224* expression from the TCGA dataset and methylation probe cg27049053 (chromosome X: 151142517) from the TCGA Illumina Infinium Human DNA Methylation 450,000 Bead Chip in (*A*) lung ADC and (*B*) SCC. (*C*) Methylation specific-high resolution melting (MS-HRM) analyses of the cells from normal lung tissues and lung cancer cells. (*D*) Quantitative RT-PCR to measure *miR-224* expression in cells from normal lung tissues after treatment with 7.5 μM 5-aza-CdR. The values present means ± SDs as determined triplicated assays.

increased after treatment with the demethylation reagent, 5-aza-CdR, for 3 d (Fig. 5*D*). We then validated reduced methylation levels in the promoter region of *miR-224* after 5-aza-CdR treatment (*SI Appendix*, Fig. S5*D*). Taken together, these results suggest that hypomethylation at the *miR-224* promoter-associated CpG island in NSCLC at least partially contributes to the higher *miR-224* expression in lung cancer.

Activation of ERK Signaling Pathway Induces *miR-224* Expression. The RAS-proto-oncogene, serine/threonine kinase (RAF)-mitogen-activated protein kinase kinase (MEK)-ERK signaling pathway is aberrantly activated in many cancers (26), and considerable evidence suggests that the activated ERK signaling pathway might regulate miRNA expression (27). In our preliminary studies, we observed a trend toward RAS mutated lung cancer cell lines having higher *miR-224* expression than RAS WT lung cancer cell lines, with some exceptions (*SI Appendix*, Fig. S6*A*). Analyzing 188 lung ADC patients for which both *miR-224* and total ERK2 protein expressions were available in the TCGA miR-seq dataset and reverse phase protein array dataset also showed significant positive correlation between total ERK2 protein and *miR-224* expression (*SI Appendix*, Fig. S6*B*). Thus, we sought to determine whether ERK signaling is involved in *miR-224* induction. To investigate the role of ERK signaling on *miR-224* expression, we activated ERK1/2 by phorbol 12-myristate 13-acetate (PMA) in two lung cancer cell lines, H460 and A549. The expression of *miR-224* was significantly up-regulated in both lung cancer cell lines with PMA treatment compared with those without PMA treatment (Fig. 6*C* and *SI Appendix*, Fig. S6*D*). Enhanced pERK and c-Jun protein levels were observed in the cells with PMA treatment (Fig. 6*B* and *SI Appendix*, Fig. S6*C*). In addition, a luciferase construct containing an *miR-224* promoter region markedly increased luciferase activity compared with the control vector, and it was more evident after treatment with PMA (Fig. 6*A*), indicating that *miR-224* might be regulated by the ERK signaling pathway. Activated ERK1/2 phosphorylates and activates the c-Jun and c-Fos protooncoproteins, which participate to form the AP1 transcription factor. To investigate the direct involvement of c-Jun and c-Fos in the transcription of *miR-224*, we analyzed the promoter region of *miR-224* using the PROMO 3.0 transcription factor binding site prediction server and found two predicted c-Jun binding sites but none for c-Fos in the promoter region (*SI Appendix*, Fig. S6*E*). Interestingly, knockdown of c-Jun significantly reduced luciferase activity induced by the *miR-224* promoter vector (Fig. 6*E*). Moreover, knockdown of c-Jun resulted in reduced *miR-224* expression in lung cancer cells (Fig. 6*D*). Taken together, these results suggest that *miR-224* expression might be regulated by ERK signaling through c-Jun binding to the *miR-224* promoter region in lung cancer.

Effects of *miR-224* on in Vivo Tumorigenicity. *miR-224* has been reported to be deregulated in several solid tumors, including lung cancer (28, 29). To investigate the role of *miR-224* in tumor growth in vivo, H460/*miR-224* and control cells or A549/miRZip224 and control cells were s.c.-injected into the flanks of nude mice. Overexpression of *miR-224* in H460 cells significantly increased tumor growth in vivo compared with control cells (Fig. 6 *F* and *G*). Inversely, knockdown of *miR-224* in A549 cells caused a substantial reduction in tumor volume in vivo (*SI Appendix*, Fig. S7*E*). These results clearly indicate that *miR-224* functions as an oncogenic miRNA in lung cancer. In addition, we observed a microscopically visible lung metastasis in two of five mice with H460/*miR-224* xenografts but none in the mice with H460/Cont xenografts (*SI Appendix*, Fig. S7*F*). Additional studies need to be done to validate this observation.

Discussion

Accumulated evidence has indicated that aberrant expression of miRNAs contributes to the pathogenesis of most human malignancies (30). A number of miRNAs functions as oncogenes or tumor suppressors in the majority of cancers. *miR-224* has dual functions: either as tumor suppressor or oncogene dependent on the specific cancer tissue type. It has been reported that *miR-224* was up-regulated in a number of solid tumors, including hepatocellular carcinoma (31, 32), CRC (33), and breast cancer (34), and plays an oncogenic role by targeting API5, SMAD4, PHLPP1, PHLPP2, and RKIP. Inversely, *miR-224* plays a tumor-suppressive role in prostate cancer by targeting TPD52 and/or TRIB1 (35, 36).

Recent reports identify distinct biological functions of *miR-224* in NSCLC and suggest a prognostic value of *miR-224* in NSCLC (28, 29). Although one report suggested that high expression of *miR-224* is associated with resistance to cisplatin therapy and poor prognosis, another report suggested that high expression of *miR-224* is associated with favorable prognosis, clearly indicating that the role of *miR-224* in the pathogenesis of lung cancer remains to be determined. In fact, our Kaplan–Meier survival analysis using the TCGA dataset showed no significant association between *miR-224* expressions and NSCLC patients' survival. Possible explanations and contributing factors for the apparently opposite results include differences in stage and treatment received and differences in the ethnic origin of the analyzed populations. Subgroup analysis indicates that the expression of *miR-224* is significantly associated with poor prognosis in lung ADC patients having *kras*/*p53* mutations, suggesting that *miR-224* might be of prognostic significance in some lung cancers.

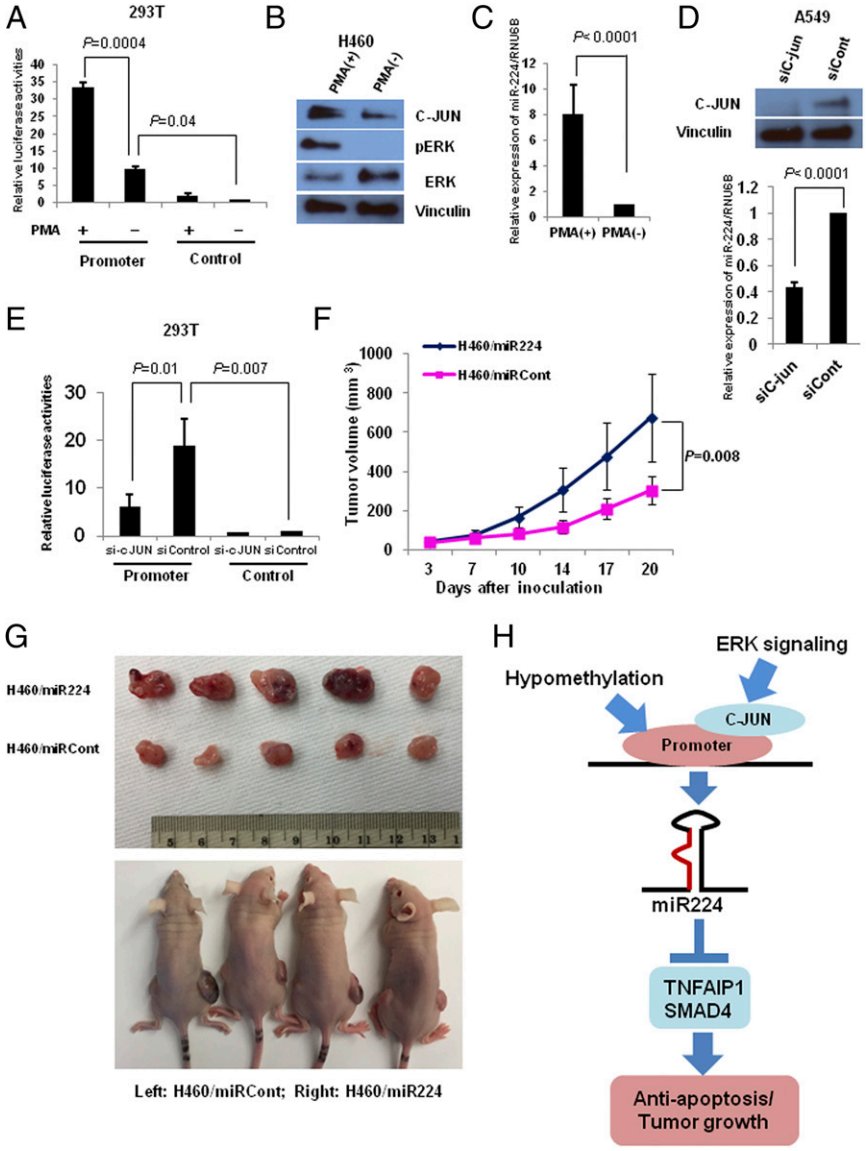


Fig. 6. *miR-224* expression was controlled by ERK signaling pathway. (*A*) Luciferase reporter construct-containing promoter region of *miR-224* and control vector were transfected into 293T cells and treated with PMA for 24 h. (*B*) Western blot analysis and (*C*) quantitative RT-PCR for H460 cells with and without PMA treatment to measure *miR-224* expression. (*D*) Western blot analysis and quantitative RT-PCR for A549 cells with and without c-Jun knockdown to measure *miR-224* expression. (*E*) Luciferase reporter constructs containing *miR-224* promoter and control vector were cotransfected with si-Cjun into the 293T cells. (*F* and *G*) Effects of *miR-224* on tumor growth in mouse model. (*F*) Tumor growth in nude mice s.c.-injected into flanks with H460/*miR-224* or H460/miRCont. Data are presented as means ± SDs ($n = 6$ per group). (*G*) Representative photographs of the tumors at day 20 after inoculation with either the H460/*miR-224* or H460/miRCont cells. (*H*) Proposed dysregulated *miR-224* expression involved in lung cancer pathogenesis in our research. The data are presented as means ± SDs, and each assay was performed three times.

In this study, we identified that *miR-224* is an oncogenic miRNA promoting lung cancer cell migration, invasion, and proliferation by direct targeting of TNFAIP1 and SMAD4, indicating an important role of *miR-224* in lung cancer progression and metastasis. These findings are consistent with the results from the work by Wang et al. (28) but inconsistent with the results from the work by Zhu et al. (29), which reported that overexpression of *miR-224* mimics inhibits lung cancer cell migration and proliferation. Because A549 cells have relatively high *miR-224* expression, we knocked down *miR-224* in A549 cells and found reduced cell migration and proliferation. Additional analysis showed that knockdown of *miR-224* was involved in cell cycle arrest (*SI Appendix*, Fig. S7*A*). Wang et al. (28), using the same cell line, conducted both knockdown and overexpression experiments and observed consistent results with our study. Wang et al. (28) generated stable *miR-224*–overexpressing A549 cells to investigate phenotypic change of A549 cells. However, Zhu et al. (29) transiently overexpressed *miR-224* mimics in A549 cells. These differences in experimental methods could be a reason for the observed discrepancy.

We evaluated *TNFAIP1* expression levels in NSCLC and found that *TNFAIP1* is significantly down-regulated in both lung ADC and SCC. We also observed a significant inverse correlation between *miR-224* and *TNFAIP1* in NSCLC. In addition, knockdown of TNFAIP1 induced the same phenotype with overexpression of *miR-224* in lung cancer cells. Moreover, we found that *miR-224* attenuated lung cancer cell growth inhibition induced by TNFα through targeting of TNFAIP1 and cleaved PARP1 expressions. These results suggest that TNFAIP1 has essential functions in *miR-224*–mediated invasion and progression in NSCLC.

SMAD4, a candidate tumor suppressor, plays an important role in mediating TGF-β superfamily (TGF-βs, activins, and bone morphogenetic proteins) signal transduction (37). Aberrant TGF-β signaling is common in human lung cancer, and loss of SMAD4 is thought to play an important role in the inactivation of TGF-β signaling (38, 39). However, the frequency of mutation and/or deletion on SMAD4 is relatively low in NSCLC (40, 41); therefore, the actual mechanism of aberrant expression of SMAD4 in NSCLC remained unclear. Given the significant up-regulation of *miR-224* in NSCLC and the fact that SMAD4 is a direct target of *miR-224*, it is possible that loss of SMAD4 might be attributable to up-regulated *miR-224* expression in NSCLC. As expected, we observed down-regulated *SMAD4* expression in NSCLC compared with normal lung tissue and a significant inverse correlation between *miR-224* and *SMAD4* in NSCLC. Overexpression of SMAD4 in lung cancer cells significantly attenuated *miR-224*–induced cell proliferation and migration. We also identified that *miR-224* is involved in the TGF-β signaling network by inhibiting nuclear transportation of SMAD4. Taken together, our findings suggest that *miR-224*–mediated reduction of TNFAIP1 and SMAD4 might contribute, at least in part, to the invasion and progression of NSCLC.

We know that *miR-224* is up-regulated in a number of cancers; however, the mechanisms of its up-regulation in lung cancer are uncertain. Considering that the *miR-224* promoter region has a CpG island, we speculate that promoter methylation status might be involved in *miR-224* expression. To test our hypothesis, we conducted MS-HRM analyses and bisulfate sequencing and found promoter methylation in cells from normal lung tissues; however, no methylation was found in two lung cancer cell lines. Methylation probe cg27049053 from the TCGA Methylation 450,000 dataset was significantly anticorrelated with *miR-224* expression in both lung ADC and SCC. In addition, treatment of the cells from normal lung tissues with 5-aza-CdR significantly restored *miR-224* expression. These results suggest that up-regulated *miR-224* expression in NSCLC can be, at least partially, attributed to the hypomethylation of the *miR-224* promoter.

KRAS mutation is frequently seen in lung ADC (42). Given our observation that there is a trend toward RAS-mutated lung cancer cell lines having higher *miR-224* expression, we sought to determine whether increased expression of *miR-224* was associated with ERK signaling pathway activation status. We performed luciferase reporter assay and found that activation of ERK signaling significantly increased luciferase activity induced by *miR-224* promoter vector. Interestingly, knockdown of c-Jun markedly reduced luciferase activity of the *miR-224* promoter vector. In the same way, we showed that activation of ERK signaling by PMA markedly increased *miR-224* expression. In addition, reduced *miR-224* expression was observed in c-Jun knockdown lung cancer cells. Overall, our results suggest that c-Jun might bind to the *miR-224* promoter region, subsequently regulating *miR-224* expression in lung cancer cells. Knoll et al. (43) recently showed that *miR-224* can also be induced by E2F1 driving epithelial mesenchymal transition through thioredoxin interacting protein down-regulation in a melanoma model. The fact that ERK signaling can activate E2F1 suggests that a possible interaction of E2F1 and c-Jun could lead to the activation of GABRE/*miR-224* transcription, mediating invasion and metastasis.

To explore the relationship between ERK signaling and hypomethylation of the promoter of *miR-224*, we treated the cells from normal lung tissues with PMA and evaluated methylation levels of the promoter of *miR-224*. No changes were found with or without PMA treatment (*SI Appendix*, Fig. S5*E*), indicating that ERK signaling and hypomethylation of the *miR-224* promoter might be independent. Additional studies are necessary to clarify this issue. Lastly, we investigated the effects of *miR-224* on in vivo tumorigenicity using a nude mouse model. Mice harboring *miR-224*–overexpressing or knockdown cell lines showed significant increase or reduction in tumor size compared with control mice, respectively. Our in vivo studies strongly support the results from in vitro analyses indicating that *miR-224* plays an oncogenic role in NSCLC.

NSCLC metastasis represents a very frequently encountered challenging clinical problem. Currently available tools to detect metastasis have limited sensitivity, and patients with distant metastases are generally considered incurable. Biomarker development to predict who will develop metastasis after definitive therapy and development of metastases prevention strategies for NSCLC patients are in their infancy. A better understanding of the biology of NSCLC metastasis will hopefully allow us to overcome some of these limitations in the near future. In this study, we show promoter hypomethylation and activated ERK signaling to be involved in the regulation of *miR-224* expression in lung cancer. We further show the important functions of *miR-224*, modulating proliferation, migration, and invasion by direct targeting of TNFAIP1 and SMAD4 (Fig. 6*H*). Our study suggests previously unidentified therapeutic strategies for lung cancer by targeting *miR-224*.

Materials and Methods

Patients and Tissue Samples. Human lung tissue microarrays [cancer (IMH-305), normal (IMH-340), and metastasized lung cancer (IMH-358)] were purchased from Novus Biologicals. Metastasized lung cancers consisted of seven cases with lymph node, two cases with bone, and one case with soft tissue metastases; 76 frozen tissue specimens from patients with lung ADC and 59 frozen tissue specimens from patients with lung SCC were obtained through the OSUCCC Tissue Procurement Shared Resource based on The Ohio State University Institutional Review Board (IRB)-approved research protocol. We obtained written informed consent from patients before sample analyses. Tissue samples were flash-frozen using liquid nitrogen within 2 h of surgical resection and stored at −80 °C until analyses.

Genome-Wide miR Sequencing and Data Analysis. The detailed procedures are described in *SI Appendix*.

Quantitative Real-Time PCR and Western Blot Analysis. Detailed procedures are described in *SI Appendix*.

Treatment of Cells with TNFα and/or TGF-β1. The cells were treated with TNFα and cycloheximide at final concentrations of 20 ng/mL and 10 μg/mL, respectively. After 4 h of treatment, the proteins were extracted from cells and subject to Western blot. The cell proliferation assay was conducted after 24 h of treatment with TNFα. To evaluate caspase 3/7 activity, the H1299 cells were cultured in 96-well plates in quadruplicates and treated with TNFα at a final concentration of 15 ng/mL. After 2 h of incubation, the caspase 3/7 activities were analyzed using the Caspase-Glo 3/7 Assay Kit (Promega) according to the manufacturer's instructions. The TGF-β1 was used to treat cells with a final concentration of 5 ng/mL. After 8 h of incubation, both nuclear and cytosol proteins were extracted from the TGF-β1–treated cells. The cell proliferation assay was conducted after 4 d of treatment with TGF-β1.

TCGA Dataset. The TCGA miR-seq, RNA-seq data, and Infinium Human DNA Methylation 450,000 Bead Chip with clinical information were downloaded on July 31, 2013. Only $\log_2$-transformed level 3 data were used for analysis. For analysis of the TCGA dataset, the Welch *t* test was conducted to test if *miR-224* expression is different between patients with and without cancer to account for unequal variances. For the correlation analysis between *miR-224* and target gene expression/promoter methylation, Pearson correlation coefficients were calculated. We performed survival analysis using the Cox proportional hazards model to determine the prognostic value of *miR-224* in the TCGA dataset.

In Situ Hybridization of *miR-224*. In situ hybridization was performed using the *miR-224* probe tagged with 5′ digoxigenin and locked nucleic acid-modified (Exiqon). The detailed procedures are described in *SI Appendix*.

Luciferase Reporter Assay. To determine if *miR-224* directly targets the 3′-UTRs of *TNFAIP1* and *SMAD4*, 5×10^4 293T cells were seeded in 24-well plates overnight and then transfected with *miR-224* mimic (Thermo Scientific) plus empty 3′-UTR vector or 3′-UTR vectors containing WT or mut–3′-UTR. After 48 h, the cells were lysed and assayed using Dual Luciferase Assay (Promega) according to the manufacturer's instructions. To investigate the role of ERK signaling on *miR-224* expression, empty vector or promoter vector containing C-jun binding sites was transfected to 293T cells. After 12 h, PMA was added with a final

concentration of 100 nM and further incubated for 24 h. To study the direct involvement of C-jun on *miR-224* promoter, we cotransfected C-jun siRNA pluses empty vector or promoter vector containing C-jun binding sites to the 293T cells. After 24 h, the cells were lysed and assayed using Dual Luciferase Assay (Promega) according to the manufacturer's instructions.

Isolation of Cells from Normal Lung Tissues from Lung Cancer Patients. The detailed procedures are described in *SI Appendix*.

Bisulfite Modification. The cell line DNA was bisulfite-modified using the EZ DNA Methylation Kit (Zymo Research Corp). The dsDNA was denatured in M-Dilution Buffer for 15 min at 37 °C, and then, CT Conversion Reagent was mixed with an ssDNA sample and incubated at 50 °C overnight. After mixing with M-Binding Buffer, the DNA purification and desulphonation were performed on the Zymo-Spin IC Column. Finally, the bisulfite-modified DNA was eluted by M-Elution Buffer for methylation analysis.

High-Resolution Melting Analysis. Methylation-specific high-resolution melting is based on PCR amplification of bisulfite-modified genomic DNA with subsequent high-resolution melting analysis of PCR amplicons. The primers were designed to amplify both methylated and unmethylated DNA. The MethPrimer (www.urogene.org/methprimer/index1.html) was used specifically for primer design in this *miR-224* promoter methylation study. The primer sequences flanking methylation probe cg27049053 are cg27049053F: AAGTAAGTAATGTTAGAGTTGTTTGG and cg27049053R: ACAAACTTCAACTACTAAAAAAACC.

The T_{melt} for the unmethylated sequence is 71.8 °C, and the T_{melt} for the methylated sequence is 73.4 °C. The amplicon length is 184 bp. PCR amplification and high-resolution melting analyses were carried out sequentially on a CFX96 Real-Time PCR System (Biorad). High-resolution melting analyses were performed at a temperature ramping from 70 °C to 95 °C by 0.2 °C per second, and florescence acquisition was set per the manufacturer's recommendation.

Demethylation by 5-aza-CdR. The 469NAT and 485NAT cells were seeded on 10-cm tissue culture dishes and cultured with or without 7.5 μM 5-aza-CdR for 3 d. The medium-containing agent was replaced every 24 h. RNAs were isolated, and quantitative real-time PCR was carried out to evaluate the restoration of *miR-224* expression after 5-aza-CdR treatment.

Target Analysis. Bioinformatics analysis was performed by using these specific programs: Targetscan (www.targetscan.org), Pictar (pictar.mdc-berlin.de/), and RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/).

Animal Study. Animal studies were performed according to OSU Institutional Animal Care and Use Committee guidelines. Stable *miR-224*–overexpressing H460 cells (H460/*miR-224*) or *miR-224* knockdown A549 cells (A549/miRZip-224) were harvested by trypsin, washed with PBS, and resuspended in Matrigel:RPMI medium (1:1); 2 million H460/*miR-224* and control cells or 10 million A549/miRZip-224 and corresponding control cells were s.c. injected into the flanks of nude mice. Injections were conducted in two flanks of each mouse. Tumor volumes were calculated from the length (*a*) and the width (*b*) by using the following formula: volume (millimeters^3) = $ab^2/2$. To see the role of *miR-224* in metastasis in vivo, 4 million miR-224–overexpressing H460 cells or control cells were s.c. injected into the flanks of nude mice. Lung metastases were evaluated by microscope after H&E stains.

Statistical Analysis. Statistical analyses were performed with the R Program (version 3.0.2). Data are represented as means with SDs, and statistical significance was determined with unpaired Student's *t* tests unless indicated otherwise. *P* values less than 0.05 were considered statistically significant. Pearson correlation analysis was conducted to determine the correlation between expression of *miR-224* and its target genes.

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