The feedback loop between miR-124 and TGF-β pathway plays a significant role in non-small cell lung cancer metastasis

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Abstract

Increasing evidence shows that micro RNAs (miRNAs) play a critical role in tumor development. However, the role of miRNAs in non-small cell lung cancer (NSCLC) metastasis remains largely unknown. Here, we found that miR-124 expression was significantly impaired in NSCLC tissues and associated with its metastasis. In vitro and in vivo experiments indicate that restoring miR-124 expression in NSCLC cells had a marked effect on reducing cell migration, invasion and metastasis. Mechanistic analyses show that Smad4, a co-binding protein in transforming growth factor-β (TGF-β) pathway, was identified as a new target gene of miR-124. Restoring Smad4 expression in miR-124-infected cells could partially rescue miR-124-induced abolition of cell migration and invasion. Notably, upon TGF-β stimulation, phosphorylation of Smad2/3 was modulated by alteration of miR-124 or Smad4 expression, followed by inducing some special transcription of downstream genes including Snail, Slug and ZEB2, all of which may trigger epithelial–mesenchymal transition and be associated with NSCLC metastasis. Moreover, activation of TGF-β pathway may enhance expression of DNMT3a, leading to hypermethylation on miR-124 promoter. Therefore, heavily loss of miR-124 expression further enhances Smad4 level by this feedback loop. Taken together, our data show for the first time that the feedback loop between miR-124 and TGF-β pathway may play a significant role in NSCLC metastasis. Targeting the loop may prove beneficial to prevent metastasis and provide a more effective therapeutic strategy for NSCLC.

Introduction

Lung cancer (LC) is the leading cause of cancer death throughout the world and is a major disease burden worldwide (1). Non-small cell LC (NSCLC), which accounts for >80% LC, and small cell LC (SCLC) are two major types of LC (2). Some favorite metastatic sites including the regional lymph node, liver, contralateral lung, brain and bone marrow are frequently observed in NSCLC, which is the most commonly diagnosed subtype and the major killer in Asian and Western populations (3,4). Due to LC was often diagnosed at late stage and lack of effective treatment, the overall 5-year survival remains ~17% (1). Therefore, inhibition of metastasis by targeting the candidate molecules will be critical for reducing LC mortality (5,6). Unluckily, the molecular mechanisms underlying its metastasis are not well understood.

Micro RNAs (miRNAs), typically ~21nt long, are one class of small non-coding RNAs that negatively regulate gene expression in eukaryotes (7). Increasing evidence shows that miRNAs have played important role in modulating many tumor development process such as proliferation, apoptosis, migration and invasion.
miR-124 is also documented to have made a marked contribution to NSCLC metastasis, including miR-143, miR-135b, miR-31 and miR-193a (9–11). However, identification of novel predictive and therapeutic target in NSCLC remains a large unmet need. MiR-124 is abundantly expressed in the brain and plays a role in gastrulation and neural development (12,13). Notably, miR-124 is also documented to have made a marked contribution to many solid tumors. For example, it has been shown that miR-124 can suppress proliferation by targeting STAT3 (14), PTB1 (15) and IASPP (16) in colorectal cancer, targeting SOS1 (17) and Clock (18) in glioma, targeting CDK4 (19) and CD151 (20) in breast cancer, targeting PIK3CA (21) in hepatocellular carcinoma, targeting androgen receptor (22) in prostate cancer, targeting extracellular signal-regulated kinase (23) in cutaneous squamous cell carcinoma and targeting SPHK1 (24) in gastric cancer, all of which suggest that a potential tumor suppressor function of miR-124 may be exerted in tumorigenesis. Additionally, miR-124 has been reported to inhibit the multiple cancer metastasis including breast cancer, gastric cancer, cervical cancer, prostate cancer, clear cell renal cell carcinoma and LC (25–30), which suggest that miR-124 can inhibit tumorigenesis at different cellular processes in vivo and in vitro. Recently, Zhang et al. (31) reported that miR-124 expression was downregulated in NSCLC cell lines and specimens. Similar finding was also observed by another group (32). However, Sun et al. (28) also indicated that miR-124 level was decreased in invasive subcell lines and node-positive specimens and depicted a nuclear factor-kappaB-centered inflammatory loop that was responsible for miR-124 deregulation. Therefore, it is meaningful to further elucidate the role and mechanism on which miR-124 exerts its function in NSCLC progression. Here, we disclose a novel feedback loop between miR-124 and transforming growth factor-β (TGF-β) pathway driving NSCLC metastasis, which might provide a new insight into treatment of NSCLC progression.

Materials and methods

Cell culture

Human NSCLC cell lines A549 and H292 were obtained from American Type Culture Collection and cultured in RPMI-1640 (Hyclone) with 10% fetal bovine serum (FBS) (Gibco); 95-D, L-TEP-α-2 and SPCA-1 were obtained from the Institute of Health Sciences, SIBS, CAS/SJTUSM and cultured in RPMI-1640 (Hyclone) with 10% FBS (Gibco); CRL-2741 was obtained from American Type Culture Collection and cultivated in Keratinocyte-SFM (10749-019, Invitrogen) with Growth Supplement. The TNBC cell line MDA-MB-231 was maintained in Dulbecco’s modified Eagle’s medium (Hyclone) with 10% FBS (Gibco). A549 luciferase cells were constructed from A549 infected with luciferase reporter labeled virus as published previously (33). For TGF-β (HZ-1011, humanzyme) stimulation, the cells were treated at the final concentration of 10ng/ml for epithelial–mesenchymal transition (EMT) induction. The time for ‘transfection after induction’ or ‘induction after transfection’ was 48 and 24h, respectively. The cell lines were tested and authenticated by DNA typing in Shanghai Jiao Tong University Analysis Core and were cultured at 37°C water saturated 5% CO₂ atmosphere.

NSCLC tissues profile

Carcinoma and corresponding para-carcinoma tissue samples of 19 NSCLC patients were obtained from Department of Thoracic Surgery, Renji Hospital, Shanghai Jiao Tong University School of Medicine. All specimens were pathologically and clinically diagnosed as NSCLC, the lung carcinoma and para-carcinoma tissues (>5 cm away from the cancer lesions with appearance of normal non-cancerous infiltration) through surgical resection consisting of paired specimens. Patients’ consent and approval from the Shanghai Jiao Tong University School of Medicine Ethics Committee were obtained before using these clinical materials for research purposes.

Plasmids construction

Human Samd4 3′-untranslated region (UTR), containing putative miR-124 binding sites, was amplified by PCR from human genomic DNA. Fragments were double digested with restriction enzyme NotI and Xbal, and then cloned into pRL-TK (Promega). MiR-124 binding sequences in the Smad4 luciferase reporter were mutated using KOD-Plus-Mutagenesis Kit (SMK-101, Toyo-bo). Smad4 cDNA was amplified by RT-PCR using the whole RNA extracted from A549 and cloned into 3× Flag-pCMV 12.0, the expression vector. Mature miR-124 sequences were cloned into the retroviral vector pSuper-puro. Retroviral miR-124 or control vector (ctrl) plasmids were transfected into the phoenix cells and the supernatant were harvested 48h post-transfection. For infection, A549 cells were incubated for 16h at 37°C with filtered viral supernatants supplemented with polybrene (8 μg/ml). Stable cell lines were established using puromycin selection. Cells with restored expression of miR-124 were designated as A549-mir-124, the respective control cells infected with siRNA control vectors were designated as A549RNA control.

Dual-luciferase reporter assay

HEK293T cells were seeded in 24-well plate and cultured 24h. MiR-124 mimics or siRNA control with pRL-TK-Smad4-3′-UTR or mutant were cotransfected into 293T cells, respectively. After culturing 48h, cells were rinsed in cold phosphate-buffered saline (PBS) and lysed with luciferase assay buffer. Then, the luciferase activity was analyzed using the dual-luciferase reporter assay system (E1960, Promega) under manufacturer’s instructions. Measurements from triplicate transfections were determined as the ratio of Renilla luciferase activity to firefly’s and then analyzed. pGL-3 plasmid (firefly) was cotransfected as an endogenous control for normalization.

Analysis of miRNA expression by real time quantitative PCR

Total RNA was extracted from NSCLC patient’s tissues after homogenization or cell samples using Trizol reagent (15596-026, Invitrogen) according to manufacturer’s instructions. RNA samples were reverse transcribed (DD0037A, Takara) into cDNA with specific RT primers and miRNA relative expression level then had been analyzed by real time quantitative PCR (qRT-PCR) using One Step SYBR PrimeScript™ RT-PCR Kit II (RR042A, Takara) in an ABI 7500 fast fluorescence temperature cycler. U6 was chosen as the endogenous control for miRNAs normalization in the assay, whereas glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was also chosen as the control for genes normalization. The expression of each gene was defined from the threshold cycle (Ct), and the relative expression levels were calculated using the 2^ΔΔCt method after normalization. All experiments were repeated three times. Primers are provided in the Supplementary Methods, available at Carcinogenesis Online.

Western blot assay and antibodies

The whole cell lysate was prepared in RIPA buffer (88900, Thermo Scientific) with phenylmethylsulfonyl fluoride and protease inhibitors. Supernatants were isolated by centrifugation and prepared with loading buffer. Samples were first separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride membrane (Millipore, Billerica, MA). The membranes were
incubated with different primary antibodies: anti-E-cadherin (3195S, 1:1000, CST), anti-N-cadherin (4061S, 1:1000, CST), anti-Vimentin (5741S, 1:1000, CST), anti-Smad4 (sc-7966, 1:300, Santa Cruz), anti-Smad2/3 (56785, 1:1000, CST), anti-phosphorylated-Smad2/3 (88285, 1:1000, CST), anti-DNMT3a (ab2850, 1:1000, Abcam), anti-DNMT3b (ab2851, 1:1000, Abcam), anti-GAPDH (1:5000, KanChen, China) in 5% milk/TBST buffer overnight at 4°C and then probed for 1h with secondary horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Jackson, 1:5000). After extensive wash with TBST, signals of target proteins were obtained with enhanced chemiluminescent plus substrate (WBKLS0500, Millipore).

Methylation-specific PCR and bisulfate-sequencing PCR

Methylation-specific PCR (MSP) analysis was performed to evaluate the methylation status of miR-124 promoter associated with three genomic loci [miR-124-1 (8p23.1), miR-124-2 (8q12.3) and miR-124-3 (20q13.33)]. In brief, genomic DNA was obtained from NSCLC cells with genome isolation kit (DP323, Tiangen, China) and then treated with EZ DNA Methylation kit from Zymo Research (D5001, CA) according to manufacturer’s protocol. Methylation-specific PCR Kit from Zymo Research (D5001, CA) was used to detect the differential level of DNA methylation in NSCLC cells. Methylation-specific PCR was then performed with specific primers and treated DNAs.

For bisulfate-sequencing PCR assay, genomic DNA isolation and bisulfate conversion was performed according to MSP protocol. Primers design, clones construction and selection, and sequencing were performed by Songon Biotech (Shanghai, China). All primers are provided in the Supplementary Methods, available at Carcinogenesis Online.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (CHIP) was performed by chromatin immunoprecipitation kit (17-371, EZ-CHIP, Millipore) according to the instructions. Briefly, A549 cells with or without TGF-β stimulation were prepared and cross-linked by 1% (wt/vol) formaldehyde in PBS then stopped by adding glycine to a final concentration of 0.125 M. The samples were pelleted, resuspended in nuclear lysis buffer and sonicated to obtain chromatin fractions with an average size of 500bp using a BioRuptor (Diagenode, Liege, Belgium). The supernatants were diluted and incubated with protein G agarose for 1h at 4°C. Then, Protein G agarose was pelleted and supernatants were reincubated with 1–3 μg primary antibody: anti-DNMT3a (ab2850, Abcam), anti-DNMT3b (ab2851, Abcam) at 4°C with rotation overnight. After washing the beads, the protein/DNA samples were eluted twice. Cross-link was reversed by adding 5M NaCl overnight at 65°C. The immunoprecipitated DNAs were purified by treatment with RNase A and proteinase K as well as cell extract DNAs (input). Purified DNA was evaluated and analyzed by PCR with specific primers.

Clinical data set analysis

Data on miR-124 expression and clinical information of patients were obtained from TCGA dataset. miRNAs-seq data of a total of 364 patients were downloaded from TCGA website (cancergenome.nih.gov), bcgsc.ca. LUAD.illuminaHiSeq_miRNASeq.Level_3.1.9.0.tar.gz; miRNAs expression abundance was represented by RPKM value in above file. The clinical information was also from TCGA website: nationwidechildrens.org.clinic_patient_luad.txt. For overall survival (OS) analysis, we then stratified the patients into two groups, high/low expression, according to miR-124 expression using the median of miRNAs abundance as the threshold and then conducted standard log-rank test and draw the K-M plot. For clinical information analysis, we adopted two-sample Kolmogorov-Smirnov test (K-S test) to evaluate the significance correlation. (h, P) = ktest2(x1, x2) returns a test decision for the null hypothesis that the data in vectors x1 and x2 are from the same continuous distribution, using the two-sample K-S test. The alternative hypothesis is that x1 and x2 are from different continuous distributions. The result h is 1 if the test rejects the null hypothesis at the 5% significance level, and 0 otherwise. The function also returns the asymptotic P value.

In vivo metastasis assay

All experimental animal procedures were performed in compliance of the institutional ethical requirements and were approved by the Shanghai Jiao Tong University School of Medicine Committee for the Use and Care of Animals. A549 cells with stable-expressed miR-124 or with negative control were cultured and harvested. 2×10⁵ cells were injected into tail vein per nude balb/c male mice (Slaccas Laboratory Animal, Shanghai, China). Eight weeks after injection, mice were anesthetized by inhalation and then intraperitoneally injected with 200 μl of mouse of 15mg/ml β-luciferin (Caliper, Hopkinton, MA). Tumor growth and metastasis were examined using the Xenogen IVIS Imaging System. All mouse experiments were performed in an animal center after obtaining approval from the Shanghai Medical Experimental Animal Care Committee. Mice were killed and eviscerated, and lungs with metastasis were then analyzed by immunohistochemistry.

Immunohistochemistry assay

Tumor samples generated from nude mice were taken for paraffin embedding, and serial 5 μm sections were used for immunohistochemistry staining. Deparaffinized by xylene and rehydrated in a graded alcohol series (100, 95, 80 and 70%, 5 min each). Heat-induced antigen retrieval was performed for 20min at 95°C with 10 mM citrate sodium buffer (pH 6.0). After the sections were cooled at room temperature for 40min, they were blocked in 3% hydrogen peroxide for 10min and then blocked in normal goat serum (5%) for 40min at room temperature. After this, the sections were allowed to react overnight at 4°C with primary antibody for E-cadherin (1:200, CST) and Vimentin (1:200, CST). After washing three times with PBS, the sections were reacted with biotinylated goat anti-rabbit antibody (1:200, Vector Laboratories, Burlingame, CA) for 30min at room temperature. Then, tissue sections were incubated with the avidin-biotin-complex–PO (Vector Laboratories) and developed in DAB Coloring Agent (Sigma, St Louis, MO) and counterstained with hematoxylin.

Statistical analysis

Statistical analysis was performed by Student’s t-test, and P values <0.05 were considered as statistically significant. Data were expressed as mean ± standard deviation of no smaller than three biological repeats. Survival curves were estimated using the Kaplan–Meier method and compared using the log-rank test. Statistical correlation was analyzed by K-S test.

Results

Expression of miR-124 is impaired in NSCLC cell lines and tissues

It has been demonstrated that miRNAs play an essential role in many cancer metastasis (5,34). Here, we set out to explore miR-124 function in NSCLC development. Figure 1A indicates that miR-124 expression was lower in NSCLC tissues than its para-carcinoma tissues (Figure 1A). Similar results were also observed in NSCLC cell lines as compared with the control cell line CRL-2741 (Figure 1C). Interestingly, miR-124 expression was also significantly reduced in metastatic tissues compared with the respective primary NSCLC tissues (Figure 1B). The findings
MiR-124 inhibits NSCLC metastasis in vivo

To determine whether miR-124 plays a role in NSCLC development in vivo, we transplanted A549miR-124-luc and respective A549GloRNA control-luc control cells tagged with luciferase into nude balb/c mice by tail vein injection. The bioluminescence imaging show that A549miR-124-luc cells significantly reduced lung metastasis compared with A549GloRNA control-luc control cells regardless of whether the animal was imaged from ventral surface (Figure 2A). Examination of the number of micrometastasis also shows that examination significantly reduced lung metastasis compared with the control (Figure 2A). The results indicate that Smad4 expression was greatly increased in A549 cells transfected with miR-124 mimics (Figure 2B). Representative image was showed in Figure 2C, suggesting that miR-124 expression significantly inhibits the lung metastasis.

EMT is considered as one of the main reasons for metastasis, characterized by downregulation of epithelial markers (e.g., E-cadherin) and upregulation of mesenchymal markers (e.g., Vimentin and N-cadherin) (35). In this regard, using immunohistochemical staining, we found that the epithelial marker, E-cadherin expression was markedly reduced and the mesenchymal markers, Vimentin expression was greatly increased in A549 cells transfected with control compared with the control (Figure 2D), suggesting that miR-124 inhibits NSCLC metastasis is potentially responsible for EMT process.

Smad4 is a downstream target of miR-124

Based on the above findings, to investigate the role and mechanism of how miR-124 modulates NSCLC development, TargetScan6.2 was used to predict its downstream target. Figure 3A indicates that the finding show that three conserved binding sites in 3′-UTR of Smad4 was excellently matched the binding sequences of miR-124. To verify the prediction that miR-124 may regulate Smad4 expression through targeting its 3′-UTR, we constructed the plasmids with luciferase reporter containing the normal or mutant 3′-UTR of Smad4. Figure 3B indicates that luciferase activity with normal 3′-UTR was dramatically repressed by miR-124 mimics, whereas luciferase activity with mutant 3′-UTR was almost not affected compared with the respective controls.

Moreover, the data show that compared with wild-type and siRNA control, Smad4 protein level was markedly reduced in A549 cells transfected with miR-124 mimics (Figure 3C). In contrast, Smad4 expression was greatly increased in the cells treated with miR-124 inhibitors (Figure 3D). The regulatory tendency was also similarly observed in other LC cells, but not in the control cell (Figure 3E). Together, these findings indicate that Smad4 is a downstream target of miR-124.

MiR-124 inhibits TGF-β-induced EMT

Previous report indicates that Smad4 is a cobiolding protein in TGF-β pathway and plays an important role in cancer
metastasis through inducing EMT (36). Under TGF-β stimulation, A549 cell would initiate EMT, followed by losing cell–cell adhesion and gaining cell mobility. To define the role of miR-124 in NSCLC metastasis, miR-124 transfection before or after TGF-β induction was performed to eliminate the effects of experiment method. Although E-cadherin expression was only enhanced when miR-124 was transfected after TGF-β induction compared with the control, Supplementary Figure 2B and D, available at Carcinogenesis Online, indicate that miR-124 expression had the ability to partly induce MET by decreasing expression of N-cadherin and Vimentin whenever it was transfected before or after TGF-β induction. Moreover, the morphologic phenomenon of MET in A549 cell was observed after miR-124 transfec-
tion (Supplementary Figure 2A and C, available at Carcinogenesis Online). Notably, without TGF-β stimulation, we also observed that expression of N-cadherin and Vimentin was reduced and E-cadherin expression was not markedly affected by miR-124 expression in A549 cells (Supplementary Figure 3A, available at Carcinogenesis Online). Similar results were also shown in breast cancer MDA-MB-231 cells (Supplementary Figure 3B and C, available at Carcinogenesis Online). These findings indicate that miR-124 expression enables EMT process to be partly reversed.

**MiR-124 suppresses migration and invasion of NSCLC and Smad4 expression**

To exclude the off-target effect of miR-124, the siRNA targeting Smad4 was transfected into A549 cells before TGF-β induction. Figure 4A indicates that silence of Smad4 expression reduced expressions of N-cadherin and Vimentin while E-cadherin expression was increased, almost consistent with the results when the cells were transfected by miR-124 in the same condition. Meanwhile, similar results were also observed for these EMT relevant markers at mRNA level using Q-PCR assays (Figure 4A). Next, we explored the effect of miR-124 overexpression on migration and invasion of NSCLC cells. Figure 4C and E show that miR-124 overexpression markedly inhibited migration and invasion compared with the respective controls when it was transfected before TGF-β induction as well as siRNA-mediated silence of Samd4 did.

Almost similar inhibitory effects of migration and invasion were also observed in A549 cells when miR-124 or knockdown of Smad4 expression was transfected after TGF-β induction as shown in Figure 4B, D and F.
Restoring expression of Smad4 rescues miR-124-suppressed EMT
To further demonstrate that Smad4 is a critical target gene involved in miR-124-induced phenotypes of NSCLC cells, we firstly examined the effect of Smad4 on EMT. Supplementary Figure 4A, available at Carcinogenesis Online, shows that Smad4 overexpression could enhance Vimentin expression and reduce E-cadherin expression. Then, we restored the Smad4 expression in A549 cells before TGF-β stimulation. As expected, A549 cells transfected by Smad4 markedly rescued the reduction of migration and invasion caused by miR-124 re-expression (Supplementary Figure 4D and G, available at Carcinogenesis Online), followed by reversing the miR-124-suppressed EMT through upregulation of Vimentin expression and reduction of E-cadherin expression compared with the respective controls (Supplementary Figure 4B, available at Carcinogenesis Online), whereas A549 cells transfected by Smad4 after TGF-β induction, almost similar results were also observed (Supplementary Figure 4C, E and H, available at Carcinogenesis Online).

MiR-124 reverses EMT through repressing TGF-β signaling
To further explore the mechanism of how miR-124 reverses EMT, we assayed its downstream signaling and targets. Our findings show that both Smad2/3 and phosphorylated Smad2/3 (p-Smad2/3) were downregulated when miR-124 or knockdown of Smad4 expression was transfected after TGF-β induction (Figure 5A). Furthermore, expressions of Snail, Slug and ZEB2 were also markedly inhibited on both mRNA and protein level compared with the respective controls (Figure 5C and E). Meanwhile, almost similar results were also observed when miR-124 or knockdown of Smad4 expression was transfected before TGF-β induction (Figure 5B, D and F). These results suggest that EMT inhibited by miR-124 is associated with suppressing some key transcriptional factors of TGF-β pathway.

To further prove that miR-124 may inhibit TGF-β pathway by targeting Smad4, we also examined the level of p-Smad2/3 at different time points. While we firstly treated the cells with TGF-β stimulation after miR-124 transfection, the p-Smad2/3 level could be rapidly and sharply increased at 30 min, although that is already downregulated by miR-124 without TGF-β stimulation. On the other hand, although the cells were transfected by miR-124 after TGF-β induction, the results showed that the p-Smad2/3 level was not changed until 24 h, whereas A549 cells transfected by miR-124 marked rescued the reduction of migration and invasion caused by miR-124 re-expression (Supplementary Figure 5A, available at Carcinogenesis Online). Taken together, the findings suggest that miR-124 inhibits the p-Smad2/3 level within long time through potentially targeting Smad4 in TGF-β pathway, but not other targets.

TGF-β stimulation reversely inhibits miR-124 expression
It has been reported that TGF-β pathway was responsible for regulation of a herd of miRNAs (37,38). Here, we set out to explore whether TGF-β has an effect on regulating miR-124 expression. Supplementary Figure 6A, B and C, available at Carcinogenesis Online, indicate that miR-124 expression was greatly reduced under TGF-β stimulation and dependent on its time and concentration. In contrast, whenever it was transfected before or after TGF-β induction, miR-124 expression was markedly enhanced by knockdown of Smad3 or Smad4 expression (Supplementary Figure 6D, E and F, available at Carcinogenesis Online), two critical targets in TGF-β pathway (Supplementary Figure 6G and H, available at Carcinogenesis Online). Meanwhile, miR-124 expression was only slightly increased without TGF-β treatment when Smad4 was silenced (Supplementary Figure 6I, available at Carcinogenesis Online).
MiR-124 is hypermethylated under TGF-β stimulation

Previous findings suggest that miR-124 expression is potentially repressed by its promoter methylation in pancreatic and cervical cancer (39,40), which offer the clue that suppression of miR-124 expression induced by TGF-β may be associated with methylation modification on its promoter. In this regard, MSP analyses initially indicate that miR-124 promoter hypermethylation indeed occurred in NSCLC cells (Supplementary Figure 7A, available at Carcinogenesis Online). Furthermore, the level of miR-124 expression in these cells was able to be significantly increased under 5-azacytidine (5-Aza) treatment (Supplementary Figure 7B, available at Carcinogenesis Online). Notably, we observed that hypermethylation on miR-124-1/2 promoter could be deeply enhanced after TGF-β stimulation, whereas no change was occurred on miR-124-3 promoter (Figure 6A). Bisulfate-sequencing PCR further confirmed that miR-124-2 promoter was hypermethylated by TGF-β induction (Figure 6B), which had no effect on miR-124-1 and miR-124-3 promoters (Supplementary Figure 8A and B, available at Carcinogenesis Online).

DNMT3a induced by TGF-β enhances the status of methylation on the miR-124 promoter

Although our findings suggest that TGF-β stimulation has been linked with hypermethylation of miR-124 promoter and makes a contribution on metastasis of NSCLC, the mechanism of how TGF-β regulates miR-124 still remains unclear. As shown in Figure 6C, we interestingly find that expression of DNMT3a and DNMT3b was greatly increased upon TGF-β stimulation while DNMT1 was not changed. To determine whether DNMT3a and DNMT3b interacted directly with miR-124 promoter, CHIP assays were performed. Figure 6D shows that only DNMT3a, not DNMT3b, could bind with more miR-124 promoter under TGF-β stimulation compared with the control. When the activity of DNMTs was blocked by 5-Aza, miR-124 expression suppressed by TGF-β was markedly increased (Figure 6E) as well as siRNA-mediated silence of DNMT3a did (Figure 6F, Supplementary Figure 9, available at Carcinogenesis Online). Notably, silencing of DNMT3b also upregulated miR-124 expression, but it seems to play an indirect effect on it.

Figure 4. MiR-124 inhibits migration and invasion ability of NSCLC cells by targeting Smad4. (A) EMT markers were determined by western blot and Q-PCR (right) when the cells were induced by TGF-β after transfection with miR-124 or siSmad4. *P < 0.05. (B) EMT markers were determined by western blot and Q-PCR (right) when the cells were transfected with miR-124 or siSmad4 after induction. *P < 0.05. (C) miR-124 or siSmad4 inhibited the ability of cell migration by wound healing assay when the cells were induced by TGF-β after transfection. (D) miR-124 or siSmad4 inhibited the ability of cell migration by wound healing assay when the cells were transfected after TGF-β induction. (E) miR-124 or siSmad4 inhibited the ability of cell migration by transwell assay when the cells were transduced after TGF-β induction. *P < 0.05. (F) miR-124 or siSmad4 inhibited the ability of cell invasion by transwell assay when the cells were transfected after TGF-β induction. *P < 0.05. Three independent experiments were performed in above assays.
Finally, we also detected core components of miRNAs production machinery by Q-PCR assay. Supplementary Figure 10, available at Carcinogenesis Online, shows that most genes were upregulated under TGF-β stimulation except TRBP2, suggesting that DNMT3a and DNMT3b induced by TGF-β may play an essential role in reducing expression of miR-124 rather than through miRNA itself production machinery.

Schematic diagram showing a novel mechanism for miR-124 and TGF-β pathway-modulated NSCLC progression

The feedback loop between miR-124 and TGF-β pathway may play a significant role in NSCLC metastasis (Supplementary Figure 11, available at Carcinogenesis Online).

Discussion

The TGF-β signaling pathway is a key regulator in metazoan biology, and its misregulation can lead to tumor development. TGF-βs are multifunctional cytokines that regulate several cellular process including proliferation, differentiation, metastasis, survival and microenvironment [41]. Basically, TGF-β exerts its function by binding to type I and type II (TGFβRI/II). After binding TGF-β, type II receptors phosphorylate and activate type I, which then propagate the signal by phosphorylating Smad2 and Smad3 (R-Smad). Once activated, R-Smad forms a complex with Smad4 and accumulates in the nucleus where they, in conjunction with other cofactors, regulate specific genes [42]. Notably, the effects of TGF-β pathway can be both tumor suppressive and tumor promoting that has been investigated over the past years. The current dogma is that the suppressive role exists in normal cells or precancerous cells whereas tumor-promoting role exists in advanced stage of tumor [42]. TGF-β signaling is known to induce the EMT and be associated with multiple tumor progression. However, the role and mechanism underlying TGF-β in LC progression remain largely unclear. Here, using the database [43], we initially found that NSCLC patients with high TGF-β expression had greatly poorer OS time than low TGF-β.

Figure 5. MiR-124 inhibits the activity of TGF-β signaling pathway. (A) The level of p-Smad2/3 was decreased when miR-124 or siSmad4 was transfected after TGF-β induction. Quantification of p-Smad2/3 was measured by Image J software, and ratio of each p-Smad2/3 band was calculated upon normalizing to Smad2/3 control. (B) The effect on the status of p-Smad2/3 by miR-124 was detected by western blot when the cells were induced by TGF-β after transfection. Quantification of p-Smad2/3 was measured by Image J software, and ratio of each p-Smad2/3 band was calculated upon normalizing to Smad2/3 control. (C) Some transcriptional factors related to EMT were downregulated by miR-124 when the cells were transfected after TGF-β induction. (D) MiR-124 inhibited some transcriptional factors related to EMT, such as Snail, Slug and ZEB2, when the cells were induced by TGF-β after transfection. (E) Some transcriptional factors related to EMT were determined by Q-PCR when the cells were transfected after TGF-β induction. *p < 0.05. (F) Some transcriptional factors related to EMT were determined by Q-PCR when the cells were induced by TGF-β after transfection. *p < 0.05. Three independent experiments were performed in above assays.
expression (Supplementary Figure 12, available at Carcinogenesis Online), suggesting that TGF-β pathway acts as the promoting role in patients with LC.

Smad4 is a pivotal transducer of TGF-β pathway and plays complex and contradictory roles during tumorigenesis (42). Accumulating evidence suggests that abnormal Smad4 expression is closely associated with a variety of human cancers and Smad4 mutations were found in colorectal (43) and pancreatic (44) cancer. Smad4 must be strictly controlled to ensure the normal cell growth. Here, we identified Smad4 as a new target gene of miR-124, involved in NSCLC progression by regulating TGF-β-induced EMT. Interestingly, as a suppressive regulator of Smad4, miR-124 could block the activity of TGF-β pathway by decreasing the level of p-Smad2/3.

MiR-124 has been documented to inhibit the cancer development in many solid tumors (26,28,29). Here, we observed that miR-124 expression was heavily impaired in NSCLC cells and tissues and associated with its metastasis. In vivo and in vitro experiments show that miR-124 could suppress the migration and invasion of NSCLC through inhibiting TGF-β-induced EMT. It has been reported that aberrant epigenetic regulations such as methylation may be associated with aberrant expression of miRNAs (46). We also found that miR-124 promoter was hypermethylated in NSCLC cells, consistent with the previous reports (39). In light of the finding that miRNA expression was also regulated by TGF-β pathway (38), we set out to assay the relationship between miR-124 and TGF-β pathway. Here, we found that expression of miR-124 was inhibited by TGF-β stimulation. Moreover, MSP and bisulfate-sequencing PCR experiments further confirmed that methylation on the promoter of miR-124 was enhanced by TGF-β stimulation. DNMTs are mainly responsible for hypermethylation of the promoter regions of genes. Mechanistically, we interestingly found that expression of DNMT3a, not DNMT1, was upregulated under TGF-β treatment, potentially responsible for enhancing the hypermethylation of miR-124 promoter. In contrast, loss of DNMT3a function by 5-Aza or siRNA-mediated silence led to the restoration of miR-124 expression.

In this investigation, on the one hand, TGF-β pathway was modulated by miR-124, followed by altering some special transcription of downstream genes including Snail, Slug and ZEB2, all of which may trigger EMT and be associated with NSCLC.
metastasis. On the other hand, activation of TGF-β pathway may enhance expression of DNMT3a, leading to hypermethylation on miR-124 promoter. Therefore, heavily loss of miR-124 expression further enhances Smad4 level by this feedback loop.

Although we proposed a novel mechanism of how miR-124 and TGF-β pathway function in NSCLC metastasis, some questions require further investigation. For example (1), other miRNAs may also function in this process as we found that miR-124 only partially reversed TGF-β-induced EMT (2); Although CHIP assay does not show that DNMT3b could bind with miR-124 promoter, silencing DNMT3b expression also restored miR-124 expression, suggesting that it plays an indirect effect.

In conclusion, our data show for the first time that the feedback loop between miR-124 and TGF-β pathway may play a significant role in NSCLC metastasis. Targeting the loop may prove beneficial to prevent metastasis and provide a more effective therapeutic strategy for NSCLC.

Supplementary material
Supplementary Figures 1 and 12 and Supplementary Methods can be found at http://carcin.oxfordjournals.org/

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