Smad3-related miRNAs regulated oncogenic TRIB2 promoter activity to effectively suppress lung adenocarcinoma growth

Yan-Xia Zhang1,2, Yun-Fei Yan1,3, Yue-Mei Liu1,3, You-Jie Li1, Han-Han Zhang1, Min Pang1, Jin-Xia Hu1, Wei Zhao1, Ning Xie3, Ling Zhou1, Ping-Yu Wang*1,1 and Shu-Yang Xie*1

MicroRNAs (miRNAs) and Smad3, as key transcription factors in transforming growth factor-β (TGF-β) signaling, help regulate various physiological and pathological processes. We investigated the roles of Smad3-regulated miRNAs with respect to lung adenocarcinoma cell apoptosis, proliferation, and metastasis. We observed that Smad3 and phospho-Smad3 (p-Smad3) were decreased in miR-206- (or miR-140)-treated cells and there might be a feedback loop between miR-206 (or miR-140) and TGF-β1 expression. Smad3-related miRNAs affected tribles homolog 2 (TRIB2) expression by regulating trib2 promoter activity through the CAGACA box. MiR-206 and miR-140 inhibited lung adenocarcinoma cell proliferation in vitro and in vivo by suppressing p-Smad3/Smad3 and TRIB2. Moreover, lung adenocarcinoma data supported a suppressive role for miR-206/miR-140 and an oncogenic role for TRIB2—patients with higher TRIB2 levels had poorer survival. In summary, miR-206 and miR-140, as tumor suppressors, induced lung adenocarcinoma cell death and inhibited cell proliferation by modifying oncogenic TRIB2 promoter activity through p-Smad3. MiR-206 and miR-140 also suppressed lung adenocarcinoma cell metastasis in vitro and in vivo by regulating EMT-related factors.

Cell Death and Disease (2016) 7, e2528; doi:10.1038/cddis.2016.432; published online 22 December 2016

Lung cancer causes more than 1 600 000 new lung cancers each year1 and contributes to greater than 1 370 000 cancer-related deaths2 worldwide, making it the most fatal of all cancers. Of all lung cancers,3 85% are non-small-cell lung cancers4,5 and adenocarcinomas are the most prevalent. Although non-small-cell lung cancers can be diagnosed early, they are often diagnosed late, when prognosis is poor.4 Over the past 30 years, overall 5-year lung cancer survival is ~16%.3 Therefore, new molecularly targeted therapy is urgently needed.

Transcription factor Smad3 is a central downstream modulator of transforming growth factor-β (TGF-β1)/Smad signaling, participating in the regulation of various physiological and pathological processes, including carcinogenesis.6 Cancer cell metastasis is a chief cause of lung cancer mortality. Smad3 is a central signaling molecule of TGF-β1, inducing epithelial-to-mesenchymal transition (EMT), during which early stage tumors are converted into invasive malignancies.7 Overexpression of Smad3 promoted metastasis in mice injected with human metastatic breast cancer cells (MCF10CA1a), but a COOH-terminally truncated dominant negative mutant of Smad3 suppressed cell metastasis.8

MiRNAs are noncoding RNAs of 20–22 nucleotides that bind to the 3'-untranslated regions (3'-UTRs) of cognate mRNAs, negatively regulating target mRNAs.9,10 miRNAs, as oncogenes or tumor suppressive genes, have been reported to modulate cell growth, metastasis, and cell death.11 MiR-20612 and miR-14013 act as tumor suppressive genes in the tumorigenesis.14 MiR-16 has also been verified to act as a tumor suppressor by downregulating BCL-2, whereas miR-150, by negatively regulating p53 expression, was confirmed to be an oncogene.14 MiR-27a can function as an oncogene by targeting MAP2K4, and inhibition of miR-27a increases MAP2K4 expression, which subsequently inhibits MG63 cell proliferation and migration.16

Considering potential roles of miRNAs and Smad3 in tumor cell growth and metastasis, we studied functions of Smad3-related miRNAs in lung cancer cell apoptosis, proliferation, and metastasis, and confirmed that miR-206 and miR-140 can suppress tumors as well as regulate phospho-Smad3 (p-Smad3)/Smad3, which can affect TRIB2 and suppress lung adenocarcinoma cell proliferation or metastasis.

Results

miR-206 and miR-140 inhibited lung adenocarcinoma cell proliferation. miRNAs act as tumor suppressive genes or oncogenes during tumor formation, and miR-20612 and miR-14013 have been reported to be tumor suppressive genes. To further investigate their roles in lung adenocarcinoma, we measured miR-206 and miR-140 expression in

1Key Laboratory of Tumor Molecular Biology in Binzhou Medical University, Department of Biochemistry and Molecular Biology, Binzhou Medical University, YanTai, ShanDong, P.R.China and 2Department of Chest Surgery, YanTaiShan Hospital, YanTai, ShanDong, P.R.China.
*Corresponding author: S-Y Xie or P-Y Wang, Key Laboratory of Tumor Molecular Biology in Binzhou Medical University, Department of Biochemistry and Molecular Biology, Binzhou Medical University, No.346 Guan Hai Road, Lai Shan District, YanTai, ShanDong 264003, P.R.China. Tel: +86 535 6913070; Fax: +86 535 6913163.
E-mail: shuyangxie@aliyun.com or wpingyugirl@163.com
These authors contributed equally to this work.
Received 27.9.16; revised 21.11.16; accepted 22.11.16; Edited by A Stephanou

Citation: Cell Death and Disease (2016) 7, e2528; doi:10.1038/cddis.2016.432
Official journal of the Cell Death Differentiation Association
www.nature.com/cddis
lung adenocarcinoma samples, and noted that miR-206 and miR-140 expression decreased in adenocarcinoma samples (n = 10) compared with para-carcinomas (n = 10). In *in situ* hybridization detection of miR-206 or miR-140. The brown color in cell indicates the expression of miRNAs. OD was less in miR-206- (P < 0.01) or miR-140- treated A549 cells (P < 0.01) compared with negative or control mutant-oligo-treated cultures. miRNAs measured with real-time PCR. miR-206 (or miR-140) were higher in miR-206- (or miR-140)-treated cells compared with scrambled-oligo controls. **P < 0.01. miR-206 (or miR-140) treatment versus control treatment.** Cell cycle distribution of A549 cells transfected with miR-206 and miR-140. All experiments were carried out in triplicate. A significant increase in A549 cells in the G1 phase occurred in miR-206- or miR-140-treated cells compared with control cultures. FACS analysis of miR-206-induced apoptosis. Apoptotic cells were shown in the upper left and right, and lower right quadrants of each panel. Apoptotic cells were increased in miR-206- or miR-140-treated cells compared with scrambled- or mutant mimics (Mu-206 or Mu-140) -treated cells after Annexin V-FITC/PI staining. Negative, vehicle-treated cells without oligos. Scrambled (scram), scrambled oligo control RNA. MiR-206 or miR-140, cells treated with miR-206 or miR-140 oligos. Mu-206 or Mu-140, cells treated with mutation sequence of miR-206 or miR-140. ASO-206 or ASO-140, cells treated with antisense RNA specific to miR-206 or miR-140.

**Figure 1** miR-206 and miR-140 regulate A549 cell growth. (a) Real-time PCR. Relative fold changes of miR-206 (or miR-140) were decreased in lung adenocarcinoma samples (n = 10) compared with para-carcinomas (n = 10). (b) *in situ* hybridization detection of miR-206 or miR-140. The brown color in cell indicates the expression of miRNAs. OD was less in miR-206- (P < 0.01) or miR-140- treated A549 cells (P < 0.01) compared with negative or control mutant-oligo-treated cultures. (c) miRNAs measured with real-time PCR. miR-206 (or miR-140) were higher in miR-206- (or miR-140)-treated cells compared with scrambled-oligo controls. **P < 0.01. miR-206 (or miR-140) treatment versus control treatment.** (d) Cell cycle distribution of A549 cells transfected with miR-206 and miR-140. All experiments were carried out in triplicate. A significant increase in A549 cells in the G1 phase occurred in miR-206- or miR-140-treated cells compared with control cultures. (e) FACS analysis of miR-206-induced apoptosis. (f) Cell cycle distribution of A549 cells transfected with miR-206 and miR-140. All experiments were carried out in triplicate. A significant increase in A549 cells in the G1 phase occurred in miR-206- or miR-140-treated cells compared with control cultures. (g) FACS analysis of miR-140-induced apoptosis. Apoptotic cells were shown in the upper left and right, and lower right quadrants of each panel. Apoptotic cells were increased in miR-206- or miR-140-treated cells compared with scrambled- or mutant mimics (Mu-206 or Mu-140) -treated cells after Annexin V-FITC/PI staining. Negative, vehicle-treated cells without oligos. Scrambled (scram), scrambled oligo control RNA. MiR-206 or miR-140, cells treated with miR-206 or miR-140 oligos. Mu-206 or Mu-140, cells treated with mutation sequence of miR-206 or miR-140. ASO-206 or ASO-140, cells treated with antisense RNA specific to miR-206 or miR-140.

Smad3 is a direct target of miR-206 and miR-140. We confirmed that the 3′-UTR of Smad3 contains the predicted target site (wild type) of miR-206 and miR-140 according to online miRNA analysis software (http://www.microrna.org/microrna/getMirnaForm.do, or http://www.targetscan.org/index.html. Figure 2a). Then, a pcDNA-GFP-smad-UTR vector was cloned with human Smad3 3′-UTR. pcDNA-GFP-smad-UTR was transfected with miR-206 or miR-140 into A549 cells. GFP expression was significantly decreased in miR-206- or miR-140-treated cells compared with controls (Figure 2b). FACS results revealed fewer GFP-positive cells in miR-206- and miR-140- treated cultures compared with NC-treated cultures (Figure 2c).
Western blot confirmed that not only Smad3 expression decreased, but also p-Smad3 levels were downregulated in miR-206- (or miR-140)-transfected cells compared with control treatment (Figure 2d and e). Similar data were obtained in miR-206- and miR-140-transfected LTEP-a-2 cells compared with controls. Smad3/actin (or p-Smad3/actin) is shown in the upper panel. **P < 0.01, miR-206 (or miR-140) treatment versus scrambled or mutant Mu-206 (or Mu-140) control. P < 0.01, siRNA treatment versus control. Negative, mock transfections. Scrambled, cells treated with scrambled oligo control RNA. MiR-206 or miR-140, cells treated with miR-206 or miR-140 oligos. Mu-206 or Mu-140, cells treated with mutation sequence of miR-206 or miR-140. SiRNA-smad3, small interfering RNA specific for knocking down Smad3 expression.

Figure 2 Smad3 expression is regulated by miR-206 and miR-140 in A549 cells. (a) The site of Smad3 3′-UTR is targeted by miR-206 and miR-140. (b) Fluorescent analysis (bar = 100 μM). (c) FACS analysis. GFP-positive cells and GFP fluorescent intensity in miR-206- and miR-140-treated cultures were decreased significantly compared with control culture. (d and e) Smad3 and p-Smad3 expression. Data showed that p-Smad3 and Smad3 expression decreased in miR-206 and miR-140-treated cells compared with controls. Smad3/actin (or p-Smad3/actin) is shown in the upper panel. **P < 0.01, miR-206 (or miR-140) treatment versus scrambled or mutant Mu-206 (or Mu-140) control. P < 0.01, siRNA treatment versus control. Negative, mock transfections. Scrambled, cells treated with scrambled oligo control RNA. MiR-206 or miR-140, cells treated with miR-206 or miR-140 oligos. Mu-206 or Mu-140, cells treated with mutation sequence of miR-206 or miR-140. SiRNA-smad3, small interfering RNA specific for knocking down Smad3 expression.

Western blot confirmed that not only Smad3 expression decreased, but also p-Smad3 levels were downregulated in miR-206- (or miR-140)-transfected cells compared with control treatment (Figure 2d and e). Similar data were obtained in miR-206- and miR-140-transfected LTEP-a-2 cells compared with control oligo treatment (Supplementary Figure 2a–c), suggesting that Smad3 is a direct target of miR-206 (or miR-140).

Negative roles of miR-206 and miR-140 in the TGF-β1 pathway. Because TGF-β1 can induce p-Smad3 expression,17 we treated lung adenocarcinoma cells with different concentrations of TGF-β1 (0–20 ng/ml). We noted that 10 ng/ml TGF-β1 enhanced the ratio of p-Smad3 to Smad3 obviously in A549 cells (Figure 3a), but this was inhibited gradually after cells were treated with 0.5–5 μmol/ml the TGF-β1 inhibitor (SB431542). The greatest effects were observed with 5 μmol/ml SB431542 (Figure 3b). Thus, the concentrations of 10 ng/ml TGF-β1 and 5 μmol/ml SB431542 were used in this study.

Next, real-time PCR data indicated that miR-206 and miR-140 were decreased in 10 ng/ml TGF-β1-treated cultures compared with untreated A549 cells (Figure 3c) and decreased expression of miR-206 (or miR-140) by TGF-β1 in A549 cells was restored after 5 μmol/ml SB431542 treatment. Expression of miR-206 (or miR-140) in LTEP-a-2 cells was inhibited by TGF-β1 (Supplementary Figure 3), indicating that the expression of miR-206 and miR-140 was regulated by TGF-β1.

Then we asked whether there is any relationship between miR-206 (or miR-140) and the TGF-β1 pathway or whether TGF-β1 levels are affected by miR-206 and miR-140 in turn? The results analyzed by online miRNA analysis software did not show that 3′-UTR of TGF-β1 was targeted by miR-206 (or
miR-140), but the TGF-β1 expression was downregulated in miR-206- (or miR-140)-treated A549 cells compared with controls (Figure 3d), suggesting there might be a feedback loop between miR-206 (or miR-140) and TGF-β1 in which miR-206 (or miR-140) can decrease TGF-β1 levels indirectly.

miR-206 and miR-140 regulate TRIB2 promoter activity through Smad3-binding ‘CAGACA’. Previously, a Tribble family member TRIB3 was reported to interact with Smad3. TRIB2, another Tribble family member, acts as an oncogene in acute myeloid and T-cell acute lymphoblastic leukemias and some lung cancers, but whether TRIB2 and Smad3 interact is unclear. Thus, we reduced p-Smad3 expression with miR-206 or miR-140, and noted that TRIB2 was reduced in miR-206- (or miR-140-)transfected A549 cells, and the TRIB2 levels inhibited by miR-206 (or miR-140) was abolished in the mutation mimics (Mu-206 or Mu-140)-treated cultures (Figure 4a, Supplementary Figure 4), indicating that TRIB2 was also regulated by miR-206 and miR-140.

siRNA can be used to investigate gene function and signal pathways, so we used this method to study whether miR-206 and miR-140 regulated TRIB2 expression through Smad3. Using siRNA specifically designed for Smad3, we observed that this siRNA could inhibit Smad3 mRNA compared with siRNA control-treated cells (Figure 4b). Smad3 and p-Smad3 were lower in siRNA-treated A549 cells compared with control cells, indicating that siRNA decreased Smad3 expression (Figure 2d and e, Supplementary Figure 2). To understand whether these siRNAs (siRNA-Smad3, specific to Smad3) affected the expression of TRIB2, we observed that TRIB2 expression decreased in siRNA-Smad3-treated cells compared with control treatment (Figure 4a, Supplementary Figure 4), an outcome similar to the effects of miR-206 and miR-140 on TRIB2 expression. siRNA experiments in LTEP-a-2 cells were also similar as well regarding TRIB2 expression. Therefore, Smad3-related miR-206, miR-140, and siRNA could regulate TRIB2 expression, suggesting that Smad3 may affect TRIB2 expression.

In the TGF-β1 pathway, p-Smad3 was reported to bind other Smad proteins together to form a protein complex, which then moves to the cell nucleus and promotes Smad3-responsive promoter activity to drive gene expression. Therefore, we studied whether Smad3 could drive trib2 gene expression by affecting trib2 gene promoter. Then, different lengths of TRIB2 promoter luciferase plasmids were cloned (Figure 4c) and Hela cells were treated with these plasmids. The 2.9 kb of the
TRIB2 promoter (WT1) had the most activity for driving luciferase expression with TGF-β1 treatment for 24 h compared with the 2.4 kb (WT2) or 1.2 kb (WT3) length promoter (Figure 4d). Activity of the 2.9 kb section of the TRIB2 promoter (WT1) induced by TGF-β1 was blocked in cells treated with 5 μmol/ml SB431542 or siRNA-Smad3 oligos (Figure 4e). We further found that miR-206 and miR-140 also effectively blocked the activity of TRIB2 promoter (WT1) induced by TGF-β1 (Figure 4f), which proved that TGF-β1/Smad3 promoted TRIB2 promoter activity. Moreover, experiments with cells treated mouse trib2 promoter-luciferase reporter also demonstrated that TGF-β1 treatment enhanced mouse TRIB2 promoter activity, whereas SB431542 treatment inhibited mouse TRIB2 promoter activity induced by TGF-β1.
for 24 h (Supplementary Figure 5), which further proved that TGF-β1/Smad3 could promote TRIB2 promoter activity obviously. The activity of promoter induced by TGF-β1 might be related to the Smad3-binding consensus sequence CAGACA. We found that there was a sequence box ‘CAGACA’ at position −2698 and −2692 of the human TRIB2 promoter and mutated this sequence to further prove whether TGF-β1/Smad3 promoted TRIB2 promoter activity through CAGACA. When the CAGACA box at position −2698 was mutated in the Mut-promoter plasmid using site-directed Gene Mutagenesis Kit (Supplementary Figure 6), the luciferase expression decreased in Mut-promoter plasmid-treated cultures compared with wild type (WT) cultures. Moreover, TGF-β1 treatment cannot increase the luciferase levels in Mut-promoter plasmid-treated cultures (Figure 4g), which proved that Smad3 promotes TRIB2 responsive promoter activity to drive TRIB2 expression by the ‘CAGACA’ box. To further investigate the role of Smad3 on TRIB2 responsive promoter activity, the transcriptional levels of TRIB2 mRNA were analyzed after TGF-β1 treatment. Our results showed that 10 ng/ml TGF-β significantly increased TRIB2 mRNA levels (Figure 4h).

The CCAAT/enhancer-binding proteins α and β (C/EBPα and β) are reported to be downstream factors of TRIB2. To investigate the effects of Smad3-related miRNAs on C/EBPα and β expression, lung adenocarcinoma cells were treated with miR-140 and miR-206. Western blot revealed that expression of C/EBPα and β increased in miR-206- and miR-140-treated A549 cells compared with control treatment (Figure 4i and j). Similar results occurred in siRNA oligo-treated cultures, indicating a regulatory role for miR-206 and miR-140 with respect to TRIB2 and its downstream factors.

miR-206 and miR-140 inhibited cell metastasis through Smad3. MiRNAs have been reported to inhibit tumor metastasis in hepatocellular or ovarian cancer cells. In our studies to learn how miRNAs function in lung adenocarcinoma metastasis, we found that fewer cells migrated to the lower chamber in miR-206- (or miR-140)-treated cultures compared with scrambled-oligo-treated cells (Figure 5a and b), suggesting that miR-206 or miR-140 can inhibit lung adenocarcinoma metastasis. Smad3, a target of miR-206 or miR-140, has a role in tumor cell metastasis and E-cadherin and α-SMA expression, so we measured these proteins in miR-206- (or miR-140)-treated A549 cells. We found that E-cadherin was elevated, and α-SMA was decreased in miR-206- (or miR-140)-treated A549 cells, similar to data observed after SB431542 treatment (Figure 5c). Western blot confirmed that miR-206 and miR-140 increased E-cadherin and downregulated α-SMA expression in A549 cells (Figure 5d, Supplementary Figure 7). However, using TGF-β1 to induce p-Smad3 overexpression enhanced α-SMA and downregulated E-cadherin in TGF-β1-treated cells, which could be ameliorated in miR-206- (or miR-140)-treated cultures compared with control treatment (Figure 5c and d, Supplementary Figure 7). Because miR-206 and miR-140 also affects Smad3-related TRIB2 expression, we next investigated how TRIB2 contributes to cell migration.

Relatively fewer cells migrated to the lower chamber in siRNA (specific to TRIB2)-treated cultures compared with siRNA control-treated cells (Figure 5e and f), supporting that the inhibition to cell migration by miR-206 and miR-140 may also attribute to Smad3-related TRIB2 expression. To study the effects of miR-206 and miR-140 on the metastasis of A549 cells in vivo, 2 × 10⁶ GFP-positive A549 cells transfected with miRNAs were injected into male nude mice by tail vein. Seven weeks after injection, small animal in vivo imaging results showed that fewer GFP-positive A549 cells migrated to the lungs in nude mice treated with miR-206 or miR-140 compared with scrambled control treatment (Figure 6a). In addition, HE staining of lung sections also supported that miR-206 or miR-140 decreased migratory tumors in miRNAs-treated tumors (n = 3) compared with scrambled control treatment (n = 3, Figure 6b). As a cell adhesion molecule, human CD44 increases the migratory capacity of various cancers. We further detected human CD44 expression to analyze the migratory capacity and number of A549 cells in oligo-treated metastatic nodules by using anti-human specific CD44 primary antibody. Interestingly, human CD44 expression decreased in miR-206- (or miR-140)-treated metastatic A549 cell nodules compared with that in scrambled control-treated metastatic nodules (n = 3, Figure 6c). Therefore, Smad3-related miR-206 and miR-140 could effectively inhibit lung cancer cell metastasis in vitro and in vivo.

miR-206 and miR-140 suppressed cell proliferation in vivo through TRIB2. To evaluate the roles of miR-206 and miR-140 in the regulation of cell proliferation in vivo, A549 lung cancer xenografts were established in BALB/C-nu mice. Tumor volumes and weights were smaller in miR-206- (or miR-140)-treated xenografts compared with scrambled-oligo controls (Figure 7a and b). qRT-PCR data show that miR-206 (or miR-140) increased in miR-206- (or miR-140)-treated xenografts compared with control tumors (Figure 7c). P-Smad3 (or Smad3), the target of miR-206 (or miR-140), was lower in tumors treated with miR-206 (or miR-140) compared with control tumors (Figure 7d). TRIB2 expression also decreased in miR-206- (or miR-140)-treated xenografts compared with control treatment (Figure 7d). The suppressive action of miRNA to tumorigenicity may be attributed to downregulation of TRIB2, which was supported by our previous study, demonstrating that lower levels of TRIB2 lead to inhibiting lung adenocarcinoma cell growth in vivo.

Smad3 and TRIB2 expression in lung adenocarcinoma samples and clinical outcomes. We measured Smad3 and TRIB2 expression in lung adenocarcinoma samples. Different to lower levels of miR-206 and miR-140 in adenocarcinoma samples, we noted that p-Smad3, Smad3, and TRIB2 were higher in adenocarcinoma samples compared with para-carcinomas (n = 10, Figure 8a and b, P < 0.01), and this was negatively correlated with miR-206 and miR-140 expression, which supporting that the suppressive roles of miR-206 (or miR-140) in regulating the expression of p-Smad3/Smad3 and TRIB2.
Kaplan–Meier survival analysis indicated that patients with greater TRIB2 had a poor survival (Figure 8d). TRIB2 was significantly positively correlated with Smad3 \( (n=111, r_s = 0.227, P=0.016, \text{ Figure 8d}) \), suggesting a positive regulation of Smad3 to TRIB2 promoter activity. These data with the luciferase experiments \emph{in vitro} indicates that Smad3 can increase TRIB2 expression.
Discussion

MiRNAs are involved in cell proliferation, metastasis, apoptosis, and stress responses\(^\text{29}\) and miR-206 and miR-140 are important suppressors of lung adenocarcinoma cell proliferation and metastasis. We observed there might be a negative feedback loop between miR-206 (or miR-140) and TGF-\(\beta_1\) whereby these two miRNAs, downregulated by TGF-\(\beta_1\), participated in Smad3-dependent TGF-\(\beta_1\) signaling and negatively regulated TGF-\(\beta_1\)/Smad3 signals in lung adenocarcinoma (Figure 8e). After decreasing p-Smad3 expression, we observed that oncogenic TRIB2 was also regulated by miR-206/miR-140. As tumor suppressors, miR-206 and miR-140 can inhibit lung adenocarcinoma proliferation and metastasis by decreasing E-cadherin and decreasing \(\alpha\)-SMA expression, and suppress lung adenocarcinoma cell growth \textit{in vivo} by decreasing oncogenic TRIB2 promoter activity through Smad3. MiR-140 was first identified (in chondrocytes) to play a role in cartilage development and homeostasis.\(^\text{30}\) Compared with normal control tissues, miR-140 was downregulated in human ovarian cancer and basal cell carcinoma\(^\text{31,32}\) and gastric cancer. MiR-140 overexpression inhibited HGC-27 cell viability and colony formation, and resulted in G0/G1 arrest by suppressing SOX4 expression.\(^\text{33}\) In this study, we found that miR-140, as a novel miRNA directly regulating Smad3, acted as a tumor suppressor to inhibit lung adenocarcinoma proliferation and was downregulated in lung adenocarcinoma samples compared with para-carcinomas, a finding supported by Tan et al.’s study.\(^\text{34}\) MiR-206 has also been reported to be a tumor suppressor that can block cell proliferation, migration, invasion, and tumorigenesis and induce apoptosis by regulating VEGF expression.\(^\text{35}\) Recently, miR-206 was found to effectively inhibit stemness and metastasis of breast cancer by targeting MKL1/IL11 pathway.\(^\text{36}\) Here we further explored the roles of miR-206 in lung adenocarcinoma and its new target. We found that miR-206 was down-expressed in lung adenocarcinoma samples and that miR-206 and miR-140 can inhibit lung adenocarcinoma cell proliferation \textit{in vitro} and \textit{in vivo} by downregulating new target-Smad3. Moreover, miR-206 and miR-140 can suppress A549 cell metastasis via regulating the expression of E-cadherin and \(\alpha\)-SMA, a finding supported by Wang et al.’s group who reported that

---

**Figure 6** MiRNAs suppressed cell metastasis \textit{in vivo}. (a) An experimental metastasis mouse model was injected with control miR-206, miR-140, or scrambled control oligo-treated A549/34 R cells. (b) Visualization of the HE-stained lung section. Arrow, the migratory A549 cells. (c) Immunohistochemistry was conducted to detect CD44 expression. Brown color indicates the migratory A549 cells. Bar = 100\(\mu\)m.
increasing miR-206 leads to cell proliferation arrest and weaker lung cancer cell invasiveness.\(^{37}\)

The TGF-\(\beta\)\(_1\) signaling pathway is critical to cell differentiation, development, proliferation, and migration.\(^{38}\) Constitutive activation of TGF-\(\beta\) signaling seems to promote tumor progression through tumor-host cell interactions.\(^{39,40}\) Smad3 is a central downstream modulator, which plays important roles in TGF-\(\beta\)/Smad pathway. In this study, we further investigated the roles of miRNAs in lung adenocarcinoma through TGF-\(\beta\)/Smad pathway, and found that miR-206 and miR-140, as signal factors in TGF-\(\beta\)/Smad pathway, could inhibit lung adenocarcinoma cell proliferation and metastasis by downregulating p-Smad3/Smad3 and that this may be related to p-Smad3's promotion of cell growth or invasion\(^{41}\) in TGF-\(\beta\) pathway. It was reported that p-Smad3 binds other Smad proteins into complexes to promote gene expression via regulating Smad3 responsive promoter activity.\(^{22}\) Indeed, we constructed a luciferase vector driven by \(\text{trib2}\) promoter to study whether p-Smad3 binds the promoter to activate \(\text{trib2}\) transcription, and found that the 2.9 kb component of the \(\text{trib2}\) promoter had the greatest activity for driving luciferase expression. Moreover, our results demonstrated that TGF-\(\beta\) promoted \(\text{trib2}\) promoter activity, which was abolished by miR-206 and miR-140 treatment. By mutating the Smad-binding consensus sequence CAGACA,\(^{23}\) we confirmed that p-Smad3 could bind CAGACA to regulate \(\text{trib2}\) promoter activity.

Tribbles, which are inhibitors of mitosis, regulate cell proliferation, migration, and morphogenesis during development. In mammals, three Tribble homologs exist: TRIB1, TRIB2, and TRIB3, and all are associated with human malignancies.\(^{42-44}\) Several studies indicated that TRIB2 can act as an oncogene involved in a mouse model of AML by inhibiting transcription factor C/EBP\(\alpha\).\(^{24,45}\) In previous studies, we reported an oncogenic role of TRIB2 in lung adenocarcinoma, and proved that miR-511 and miR-1297 could suppress A549 cell proliferation\(^{20}\) and increasing TRIB2 expression decreased as p-Smad3/Smad3 was downregulated by miRNA or siRNA. Specifically, miR-206 and miR-140 suppressed lung adenocarcinoma cell proliferation and metastasis by decreasing TRIB2 through Smad3 in TGF-\(\beta\) pathway. Collectively, our results show that miR-206 or miR-140 can suppress lung cancer cell proliferation by reducing oncogenic TRIB2 through Smad3 regulating \(\text{trib2}\) promoter.
During tumor progression, EMT is critical for conversion of early stage tumors into invasive ones, because it promotes tumor cell infiltration into adjacent tissue and the formation of subsequent metastasis. TGF-β1/Smad3 signaling regulates EMT through Smad3-dependent or -independent mechanisms. During EMT, epithelial markers E-cadherin and zona occludin-1 are downregulated, whereas mesenchymal markers α-SMA and fibronectin are upregulated. In this study, we investigated a miRNA-mediated mechanism of lung cancer cell migration and found that miR-206 and miR-140 could suppress A549 cell metastasis by regulating p-Smad3 and oncogenic TRIB2. Moreover, E-cadherin expression was upregulated, whereas α-SMA was downregulated in miR-206- or miR-140-treated cells, suggesting that the mechanism of suppressing EMT by miR-206 and miR-140 might be related to regulating expression of E-cadherin and α-SMA.

Thus, we report that miR-206 and miR-140, as tumor suppressors, induce lung adenocarcinoma cell apoptosis and promote lung adenocarcinoma cell apoptosis.
inhibit cell growth by reducing oncogenic trib2 promoter activity through Smad3 binding CAGACA box and that they suppress lung cancer metastasis by regulating EMT-related factors. Our work offers essential information about novel targets for the development of new therapeutics for treating lung cancers.

Materials and Methods

Lung adenocarcinoma samples. Fresh lung adenocarcinoma and para-
carcinoma tissues from patients who underwent surgery at YanTaiShan Hospital were obtained after surgery and immediately prepared for pathological diagnosis, western blot or RNA analysis. All experiments were performed in accordance with relevant guidelines of the Medical Ethics Committee of Binzhou Medical University. Before study inclusion, patients provided written informed consent after study procedures were fully explained.

RT-PCR and real-time PCR. MiRNAs of lung adenocarcinoma cells, tissues, or mouse xenografts were isolated by mirVana miRNA Kit (Ambion, Austin, TX, USA) and poly (A) was added using poly(A) polymerase (Ambion). cDNA was synthesized by RT primer 5'-AACATGTAGACCTCTGAGTTGATGGIdT30N (A,G,C or T)-3'. Forward primer was used to amplify miR-140 was: 5'-CCAGTGTGTTTACCTCATTGATG-3', reverse: 5'-AACATGTACAGCCCATCGATG-3'. Forward primer of miR-206: 5'-TGCGAATTGAAAGTGTCGTG-3', reverse: 5'-AACATGTACAGCCCATCGATG-3'. 

Construction of pcDNA-GFP-Smad-UTR vector. Smad3-3'UTR was amplified by PCR from human genomic DNA. Forward primer: 5'-TGGAGCTTACCTAAGGAAAAACT-3', reverse: 5'-TATCGATCCGGAACGCTGATGTCT-3'. The Quantitect SYBR-Green kit (Qiagen, Valencia, CA, USA) was used to measure miR-140 and miR-206 with an RG3000 system (Corbett Research, Mortlake, Australia) as follows: denaturing at 95 °C for 3 min; 40 cycles of 95 °C for 30 s, 60 °C annealing for 20 s and extension at 72 °C for 20 s. Then, fluorescence was measured (585 nm).

GFP assays. GFP-positive cells were observed 24 h after transfection. A549 and LETP-a-2 cells were trypsinized and gently washed with serum-containing medium. Cells were then collected and centrifuged at 400 x g for 5 min. Then, GFP-positive cells were counted by FACS (Beckman).

Cell culture and miRNAs transfection. Lung adenocarcinoma cells (A549/LETBP-a-2) and human cervical cancer (HeLa) cells were obtained from Shanghai Institute of Cell Biology, China. Cells were maintained in 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% calf serum (HyClone, Logan, UT, USA), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C with 5% CO2.

Supernatant was removed and 100 μl DMSO (Sigma) was added and OD was measured (570 nm) using an ELISA reader (Multiskan FC, Thermo Fisher Scientific, Boston, MA, USA).

Apoptosis was measured by flow cytometry (FACS). Briefly, cells (8 x 104) in each well of 12-well flat-bottom microtiter plates were treated with miR-140 and miR-206 for 48 h. Then, cells were dyed with Annexin V-FITC/PI according to the manufacturer's instructions (KeyGEN Biotech. Co. Ltd., Nanjing, China). Finally, Annexin V-FITC/PI positive cells were counted (Beckman Coulter, Inc., Kraemer Boulevard Brea, CA, USA).

Construction of pcDNA-GFP-Smad-UTR vector. Smad3-3'UTR was amplified by PCR from human genomic DNA. Forward primer: 5'-TGGAGCTTACCTAAGGAAAAACT-3', reverse: 5'-TATCGATCCGGAACGCTGATGTCT-3'. The Quantitect SYBR-Green kit (Qiagen, Valencia, CA, USA) was used to measure miR-140 and miR-206 with an RG3000 system (Corbett Research, Mortlake, Australia) as follows: denaturing at 95 °C for 3 min; 40 cycles of 95 °C for 30 s, 60 °C annealing for 20 s and extension at 72 °C for 20 s. Then, fluorescence was measured (585 nm).

Western blot. Lung adenocarcinoma or mouse xenograft cells were lysed with lysis buffer (Western of Beyotime, Shanghai, China) according to the manufacturer's instructions. Then, 30 μg of protein was loaded into individual lanes and separated via SDS-PAGE. Protein was then transferred to PVDF membranes, which were blocked with 5% non-fat milk in TBST (50 mmol Tris-HCl (pH 7.6), 150 mmol NaCl, 0.1% Tween-20) for 2 h at room temperature. Membranes were incubated with rabbit anti-human p-Smad3/Smad3/E-cadherin/TRIB2 /C-EBP-β antibodies (1:1000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or α-SMA antibody (1:1000, BioWorld Technology, Inc., Minneapolis, MN, USA) in TBST at 4 °C overnight. Membranes were washed with TBST three times. HRP-labeled goat anti-

rabbit IgG (1:6000, Beijing Zhong Shan-Golden Bridge Technology Co., Ltd., Beijing, China) was added and samples were incubated for 1 h at room temperature. Finally, membranes assessed with ECL (Boster Immunoleader, Wuhan, China). Actin or GAPDH for each sample was used as a control.

Promoter and luciferase. Different lengths of TRIB2 (NM_021643) promoter elements were amplified by PCR (Primers appear in Supplementary Table 1). Promoters were cloned into the T vector (Takara) to construct T-promoter vectors. Then, promoters were cut from T-promoter vectors by KpnI/Stall, which were inserted before the luciferase sequence of pGL-basic (Promega, Madison,WI, USA) using KpnI/Xhol, constructing promoter-luciferase expression vectors. The mutated promoters/ luciferase vector was constructed using a site-directed Gene Mutagenesis Kit (Beyotime). All constructs were confirmed by DNA sequencing.

The Hela cells were treated with both luciferase reporter plasmid and TGF-f1 or other factors. After 24 h, cells were collected and luciferase activity was measured with a Dual-Luciferase Reporter Assay according to the manufacturer's instructions (Promega).

Transwell cell migration assays. Transwell migration assays were performed using Corning Costar Transwell chambers with filter membranes of 8 μm pore size (Sigma). Cells treated with miRNAs or TGF-f1 were seeded into the upper chamber (104 cells per well in 100 μl 1640 medium, PBS-free). The lower chamber was filled with 600 μl 1640 medium supplemented with 10% calf serum. After 24 h, the liquid in the upper chamber were removed and the upper surface was carefully washed with PBS three times. In the upper chamber, —20 °C methanol was added for 10 min and samples were washed with PBS twice. Then, lower chamber cells were stained with 1% crystal violet (Sigma) in 2% ethanol for 20 min. Excess crystal violet was removed by quickly merging the insert in ddH2O for 3-4 s. Lower chamber cells were counted under a microscope (DM6000B, Leica). Each migration condition was tested three times.

Tail vein injection and migratory cell detection. GFP-positive A549 cells transfected with miRNAs or controls were collected from petri dish in 100 μl normal saline at 1.5 x 106 cells. Subsequently, these cells were injected into the tail veins of nude mice (5 weeks old). Seven weeks later, the migration of GFP-positive

Cell Death and Disease
electroplasmid in PEB, permeabilized with 0.5% Triton X-100 in PBS, and incubated with rabbit anti-human E-cadherin (1:50; Santa Cruz Biotechnology), α-SMA (1:100; BioWorld Technology) at 4 °C overnight. Then, cells were incubated with Alexa Fluor 488 donkey anti-rabbit IgG (H+L) and Alexa Fluor 594 donkey anti-mouse IgG (H+L) (Molecular probes, Eugene, OR, USA) at 37 °C for 1 h. Fluorescent images were captured under a microscope (DM6000B, Leica).

Immunofluorescent analysis. Cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.5% Triton X-100 in PBS, and incubated with rabbit anti-human E-cadherin (1:50; Santa Cruz Biotechnology), α-SMA (1:100; BioWorld Technology) at 4 °C overnight. Then, cells were incubated with Alexa Fluor 488 donkey anti-rabbit IgG (H+L) and Alexa Fluor 594 donkey anti-mouse IgG (H+L) (Molecular probes, Eugene, OR, USA) at 37 °C for 1 h. Fluorescent images were captured under a microscope (DM6000B, Leica).

A549 lung adenocarcinoma cell xenografts. Briefly, after treatment with miR-140 or miR-206 for 48 h, A549 cells were cultured, collected, washed, and resuspended in culture medium (1×10⁷/ml) and injected into the lower back of 6–8-week old female BALB/C-nu mice (nude mice, HFK Biotechnology, Beijing, China). Once mice developed palpable tumors, tumor volume was measured with calipers daily. All mice were killed after 4 weeks and tumors were collected. All animal experiments were approved by the Committee on the Ethics of Animal Experiments of Binzhou Medical University.

Statistics. SPSS Statistics Client 22 (IBM) software was used to analyze the significance of all results. Group means comparisons were calculated using an unpaired, two-sided, Student’s t-test. ANOVA was applied to compare different groups with respect to continuous variables. Array data of TRIB2 and Smad3 were downloaded from data link Data Link(s): http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE33141. Overall survival was determined using Kaplan–Meier survival analysis. Correlations were calculated with a Spearman rank test. P-values < 0.05 were considered statistically significant differences.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements. We thank Professor Karen Keesmah (Paul O’Gorman Lekuakesa Research Centre, Institute of Cancer Sciences, University of Glasgow, Scotland) for the kind help in donating mouse trib2 promoter-luciferase reporter vector. This work was supported by the National Natural Science Foundation of China (Nos. 31440061, 31371321), and the Shandong Science and Technology Committee (Nos. 2015GSF118073, ZR2016LC09, ZR2014HP04).

1. Chen J, Ye L, Xie F, Yang Y, Zhang L, Jiang WG. Expression of bone morphogenetic protein 7 in lung cancer and its biological impact on lung cancer cells. Anticancer Res 2010; 30: 1113–1202.
2. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Perkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 2010; 127: 2893–2917.
3. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. CA Cancer J Clin 2010; 60: 277–300.
4. Herbst RS, Heymach JV, Lippman SM. Lung cancer. N Engl J Med 2004; 350: 1243–1255.
5. Selvaggi G, Scaglioni GV. Histologic subtype in NSCLC: does it matter? J Thorac Oncol 2011; 6: 1173–1185.
6. Kim IY, Kim MM, Kim SJ. Transforming growth factor-beta: biology and clinical relevance. Int J Cancer 2006; 118: 2893–2903.
7. Oft M, Heider KH, Beug H. TGFbeta signaling is necessary for carcinoma cell invasiveness and for epithelial-mesenchymal transition in lung cancer cells. Int J Oncol 2014; 44: 802–812.
8. Fung HC, Tang WC, Yuan Y, Zhang L, Li Y, Liu et al. JAK/STAT3 signaling is required for TGF-beta-induced epithelial-mesenchymal transition in lung cancer cells. Int J Oncol 2014; 44: 802–812.
9. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281–297.
10. Bhattacharyya SN, Habermacher R, Martine U, Cossi EL, Filipowicz W. Stress-induced reversal of microRNA repression and mRNA P-body localization in human cells. Cold Spring Harb Symp Quant Biol 2006; 71: 513–521.
11. Ambros V. MicroRNA pathways in flies and worms: growth, death, fasting, stress, and timing. Cell 2003; 113: 673–676.
12. Georgantas RW 3rd, Streicher K, Luo X, Greenleea L, Zhu W, Liu et al. MicroRNA-206 induces G1 arrest in melanoma by inhibition of CDK4 and Cyclin D. Pigment Cell Melanoma Res 2014; 27: 275–286.
13. Yuan Y, Shen Y, Yue L, Fan H. miR-140 suppresses tumor growth and metastasis of non-small cell lung cancer by targeting insulin-like growth factor 1 receptor. PLoS One 2013; 8: e52904.
14. Wang PY, Li YJ, Zhang S, Li ZL, Yue X, Nie et al. Regulating Smad3 mRNA growth by ASO inhibiting miRNA expression. Mol Cell Biochem 2010; 339: 163–171.
15. Li YJ, Zhang YX, Wang PY, Chi YL, Zhang C, Ma Y et al. Regression of A549 lung cancer tumors by anti-miR-150 vector. Oncor Rep 2012; 27: 129–134.
16. Pan W, Wang H, Jianwei R, Ye Z. MicroRNA-27a promotes proliferation, migration and invasion by targeting MAP2K4 in human osteosarcoma cells. Cell Physio Biochem 2014; 33: 402–412.
42. Grosshans J, Wieschaus E. A genetic link between morphogenesis and cell division during formation of the ventral furrow in Drosophila. Cell 2000; 101: 523–531.
43. Hegedus Z, Czibula A, Kiss-Toth E. Tribbles: a family of kinase-like proteins with potent signalling regulatory function. Cell Signal 2007; 19: 238–250.
44. Mata J, Curado S, Ephrussi A, Rorth P. Tribbles coordinates mitosis and morphogenesis in Drosophila by regulating string/CDC25 proteolysis. Cell 2000; 101: 511–522.
45. Yokoyama T, Nakamura T. Tribbles in disease: signaling pathways important for cellular function and neoplastic transformation. Cancer Sci 2011; 102: 1115–1122.
46. Lee JM, Dedhar S, Kalluri R, Thompson EW. The epithelial-mesenchymal transition: new insights in signaling, development, and disease. J Cell Biol 2006; 172: 973–981.
47. Xu J, Lamouille S, Derynck R. TGF-beta-induced epithelial to mesenchymal transition. Cell Res 2009; 19: 156–172.
48. Kokudo T, Suzuki Y, Yoshimatsu Y, Yamazaki T, Watabe T, Miyazono K. Snail is required for TGFbeta-induced endothelial-mesenchymal transition of embryonic stem cell-derived endothelial cells. J Cell Sci 2008; 121(Pt 20): 3317–3324.
49. Olmeda D, Jorda M, Penaud H, Fabra A, Cano A. Snail silencing effectively suppress tumour growth and invasiveness. Oncogene 2007; 26: 1862–1874.
50. Zhang S, Zhang C, Li Y, Wang P, Yue Z, Xie S. MiR-98 regulates cisplatin-induced A549 cell death by inhibiting TP53 pathway. Biomed Pharmacother 2011; 65: 436–442.
51. Xie S, Xie N, Li Y, Wang P, Zhang C, Li Q et al. Upregulation of TRB2 induced by miR-98 in the early lesions of large artery of type-2 diabetic rat. Mol Cell Biochem 2012; 361: 305–314.