

IL-1 β -Mediated Repression of microRNA-101 Is Crucial for Inflammation-Promoted Lung Tumorigenesis

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Abstract

Inflammatory stimuli clearly contribute to lung cancer development and progression, but the underlying pathogenic mechanisms are not fully understood. We found that the proinflammatory cytokine IL-1 β is dramatically elevated in the serum of patients with non-small cell lung cancer (NSCLC). *In vitro* studies showed that IL-1 β promoted the proliferation and migration of NSCLC cells. Mechanistically, IL-1 β acted through the COX2-HIF1 α pathway to repress the expression of microRNA-101 (miR-101), a microRNA with an established role in tumor suppression. Lin28B was identified as critical effector target of miR-101 with its repression of Lin28B, a critical aspect of tumor suppression. Overall, IL-1 β upregulated Lin28B by downregulating miR-101. Interestingly, cyclooxygenase-2 inhibition by aspirin or celecoxib abrogated IL-1 β -mediated repression of miR-101 and IL-1 β -mediated activation of Lin28B along with their stimulatory effects on NSCLC cell proliferation and migration. Together, our findings defined an IL-1 β -miR-101-Lin28B pathway as a novel regulatory axis of pathogenic inflammatory signaling in NSCLC. *Cancer Res*; 74(17); 4720–30. ©2014 AACR.

Introduction

Chronic inflammation and infection have been established as key promoting factors of tumorigenesis (1). Epidemiologic data have recognized that chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis, tobacco smoke, air pollutant, pulmonary infections, and occupational dust are associated with non-small cell lung cancer (NSCLC), suggesting that chronic inflammation plays important roles in the pathogenesis of NSCLC (2). Cyclooxygenase-2 (COX2), a member of the COXs that are responsible for the production of inflammatory mediator prostaglandins, has been identified as a key inflammation-associated molecule in response to cigarette smoking, cytokines, and growth factors, and COX2 is known to promote the initiation and progression of NSCLC as

well as other types of human epithelial tumors (3–7). Interestingly, inhibition of COX2 by nonsteroid anti-inflammatory drugs (NSAID) has been shown to be an effective approach in prevention and therapy of NSCLC and several other types of cancers by both epidemiologic and experimental data (8–13). Such findings have confirmed the role of inflammation in the pathogenesis of NSCLC, but the molecular connections between inflammation and NSCLC have remained largely elusive.

Recent studies have added microRNAs (miRNA) as a novel class of inflammation-associated molecules (14). miRNAs are approximately 22 nt, noncoding RNAs that negatively regulate gene expression at posttranscriptional level (15). Mounting evidence supports that miRNAs play important roles at different stages of tumor development, including initiation, tumor progression, invasion, and metastasis, whereas miRNA-based new cancer therapies are in active development (16). Intriguingly, a number of cancer-related miRNAs are regulated by inflammatory signals and consequently link the inflammatory responses to tumorigenesis by regulating their cancer-related targets, among which the most prominent ones are miR-155, miR-21, miR-125b, miR-196, and miR-210 (17, 18). For instance, our recent studies indicate that miR-155, which is ubiquitously induced by inflammation stimuli in breast cancer cells, activates protumorigenic inflammatory STAT3 signaling by targeting *socs1* (19) and promotes glucose utilization in aerobic conditions, that is, the Warburg effect, by upregulating *hexokinase-2* (20). Our findings, along with the reports by other laboratories (17, 18), strongly support miRNAs as an important class of mediators linking inflammation and cancer.

In this study, we found that the proinflammatory cytokine IL-1 β was dramatically elevated in the serum of patients with NSCLC. IL-1 β signaling acts to repress *mir-101*, a well-documented tumor-suppressive miRNA, via the COX2-HIF1 α

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pathway. We showed that IL-1 β promoted the proliferation and migration of NSCLC cells and that repression of *mir-101* represents an important mechanism of its tumor-promoting activity. Recent studies showed that miR-101 is ubiquitously downregulated in many types of cancers and that it represses malignant transformation and cancer progression by negatively regulating a cohort of oncogenes (21–27). Nevertheless, the potential role of miR-101 in inflammation-promoted tumorigenesis remained largely unclear. Our results showed that miR-101 displays a tumor-suppressive role in NSCLC cells; and, mechanistically, we identified *Lin28B* as a novel target of miR-101 that mediates its tumor-suppressive effect in these cells. Previous studies showed that Lin28B inhibits the expression of tumor-suppressive let-7 family miRNAs (28–30). Consistently, we found that IL-1 β , through the miR-101–Lin28B axis, downregulates the let-7 family in NSCLC cells. Interestingly, application of aspirin and celecoxib, the NSAIDs that nonselectively and selectively inhibit COX2, respectively, abrogated IL-1 β -mediated regulation of the miR-101–Lin28B–let-7 axis, and its stimulatory effect on cancer cell proliferation and migration. Thus, our findings identify the IL-1 β –miR101–Lin28B pathway as a novel regulatory axis that links inflammation signaling to the control of cancer cell proliferation and migration in NSCLC, providing new mechanistic insights into inflammation-promoted tumorigenesis.

Materials and Methods

Cell lines and human samples

The human NSCLC cell lines H460 and H1299 and embryonic kidney cell line HEK293T cells were obtained from ATCC (February 2011) and cultured according to their guidelines. All the cell lines were recently authenticated by cellular morphology and the short tandem repeat analysis at Ji-Ying Inc. (Shanghai, China; June 2013) according to the guideline from ATCC (31). For cell treatment, 10 ng/mL IL-1 β (PrimeGene), 1.0 mmol/L aspirin (in dimethylsulfoxide; Sangon Biotech), 25 μ mol/L celecoxib (in dimethylsulfoxide; Pfizer), 1 μ mol/L quinazoline (kinasechem), and 400 μ mol/L CoCl₂ (Sigma) were used in cell culture. NSCLC specimens and paired normal adjacent tissues were collected during surgery from Shanghai Chest Hospital (Shanghai, China). Samples were immediately snap-frozen and stored at –80°C. Specimen collection was approved by the hospital authorities.

Plasmid constructs

Human *Lin28B* and *Cox2* coding sequences were cloned into the p3 \times Flag-CMV-14 expression vector (Sigma) to construct p3 \times Flag-Lin28B and p3 \times Flag-COX2. For reporter pRL-TK-Lin28B 3'-untranslated region (3'-UTR), the human *Lin28B* 3'-UTR was cloned downstream of the *Renilla* luciferase gene in pRL-TK (Promega). Eight nucleotides in *Lin28B* 3'-UTR corresponding to 5' part of miR101 were deleted in the pRL-Lin28B 3'-UTR Mut construct. All constructs were confirmed by DNA sequencing.

RNA oligonucleotide and cell transfection

miR101 mimics, anti-miR101, and small interfering RNA (siRNA) and their cognate control RNAs were purchased from

Ribobio (the sequences are listed in Supplementary Table S1). Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Normally, for transfection of the RNA oligonucleotides, 50 nmol/L of siRNA or miRNA mimics and 100 nmol/L of antisense oligonucleotides were used. For restoring *mir-101* expression in IL-1 β -treated NSCLC cells, lower dosages of miR-101 mimics (0.5–2.5 nmol/L) were used. For plasmid, 4 μ g of DNA was used in a 6-well plate. G418 (200 μ g/mL) was added 24 hours after transfection. In the rescue experiment, 1 μ g of plasmid was used in a 6-well plate.

Proinflammatory cytokine levels in serum

Preoperative serum samples from NSCLC were obtained at the time of surgery from Shanghai Chest Hospital. Healthy serum samples were collected from healthy donors in same hospital. The levels of proinflammatory cytokines, including IL-1 β , IL-4, IL-6, TNF α , and TGF β , were measured using ELISA assay kits from R&D Systems.

Luciferase reporter assay

Luciferase reporter assays were carried out as we described previously (19). Luciferase activity was measured using the Dual luciferase Reporter Assay System (Promega) and normalized to respective controls.

RNA isolation and qRT-PCR assays

The assays were performed as we described previously (19). Total RNAs were extracted from cells or tissues with TRIzol reagent (Invitrogen). The levels of miRNA and mRNA were quantified by quantitative reverse transcription PCR (qRT-PCR) using SYBR Green (Takara), with U6 small nuclear RNA and *GAPDH* as internal normalized references, respectively. The qRT-PCR results were analyzed and shown as relative miRNA or mRNA levels of the C_T (cycle threshold) values, which were then converted as fold change. The primer sequences for qRT-PCR are provided in Supplementary Table S1.

Western blotting and immunofluorescence assays

The assays were carried out as we recently described (32). Antibodies for Lin28B, COX2, and hypoxia-inducible factor-1 α (HIF1 α) were obtained from Cell Signaling Technology, antibody for proliferating cell nuclear antigen (PCNA) was from Abcam, and antibody for β -actin was from Sigma. The band intensities in Western blotting were quantified with the LAS4000 Image Analyzer. Cy3 or 488-conjugated donkey anti-rabbit IgG (Sigma) was used as secondary antibody to visualize Lin28B and HIF1 α in immunofluorescence assays. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories).

Immunohistochemistry

Immunohistochemistry of patient tissue sections was performed as described recently (20). The dewaxed 5- μ m sections were subjected to an antigen-demasking procedure of brief, high temperature heating of the sections immersed in citrate buffer (10 mmol/L, pH 6.0). Endogenous peroxidases were blocked

with 0.3% hydrogen peroxide, and nonspecific binding was blocked with 5% normal goat serum and 2% BSA in phosphate-buffered saline (PBS). Sections were then incubated for 2 hours at room temperature with rabbit monoclonal anti-Lin28B antibody (1:50; Cell Signaling Technology). After washing with PBS, sections were incubated with biotinylated secondary antibody, followed by a further incubation with the streptavidin-horseradish peroxidase complex. The sections were then immersed in DAB for 5 to 10 minutes, counterstained with 10% Mayer hematoxylin, dehydrated, and mounted in crystal mount.

Cell proliferation, soft agar colony formation, and Transwell migration assays

These assays were performed as we described previously (19, 20). In brief, cells were transfected with respective RNA oligonucleotide and/or plasmid DNA. Twenty-four hours after transfection, equal numbers of viable cells were seeded in 96-well plates for cell proliferation assay. Cell growth was determined by using MTT assay. In soft agar colony formation assays, approximately 8,000 viable cells in 1.5 mL RPMI-1640 medium with 0.4% soft agar were layered onto 1% solidified agar in RPMI-1640 medium in a 6-well plate. The plates were incubated at 37°C in a humidified incubator with 5% CO₂ for 14 to 21 days, the cells were stained with 0.01% crystal violet, and colony foci were counted using a dissecting microscope. Experiments were carried out in triplicate. The cell migration assays were performed in a 24-well Transwell plate with 8- μ m polyethylene terephthalate membrane filters (Falcon cell culture insert; Becton Dickinson) separating the lower and upper culture chambers. Approximately 8,000 cells were plated in the upper chamber per well in 300 μ L of serum-free RPMI-1640. The bottom chamber contained 500 μ L of RPMI-1640 with 10% FBS. Cells were allowed to migrate for 8 hours. After the incubation time, the filter was removed, and nonmigrating cells on the upper side of the filter were detached using a cotton swab. Filters were fixed with 4% formaldehyde for 15 minutes, cells located in the lower filter were stained with 0.1% crystal violet for 20 minutes, and three random fields were counted. Quantification of the results is presented as mean \pm SD.

Xenograft assays in nude mice

All animal work was performed as we described previously (1) in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication nos. 80-23, revised 1996) and in accordance with the institutional ethical guidelines for animal experimentation. Twenty-four hours after transfection of RNA oligonucleotide and/or plasmid DNA, approximately 2×10^6 cells were suspended in 100 μ L of RPMI-1640 and then injected subcutaneously into male BALB/c athymic nude mouse at 6 to 8 weeks of age. Six mice were included in one experimental group. Tumor growth rates were examined every 5 days for 25 days. Tumor growth rates were analyzed by measuring tumor length (*L*) and width (*W*) and calculating the volume with the formula $LW^2/2$.

Statistical analysis

All results were presented as the mean \pm standard error of the mean (SEM). A Student *t* test was performed to compare

the differences between treated groups relative to their paired controls. One-way ANOVA was used to analyze tumor growth data. *P* values are indicated in the text and figures above the two groups compared with a value <0.05 (denoted by asterisks) considered significant (***, *P* < 0.001; **, *P* < 0.01).

Results

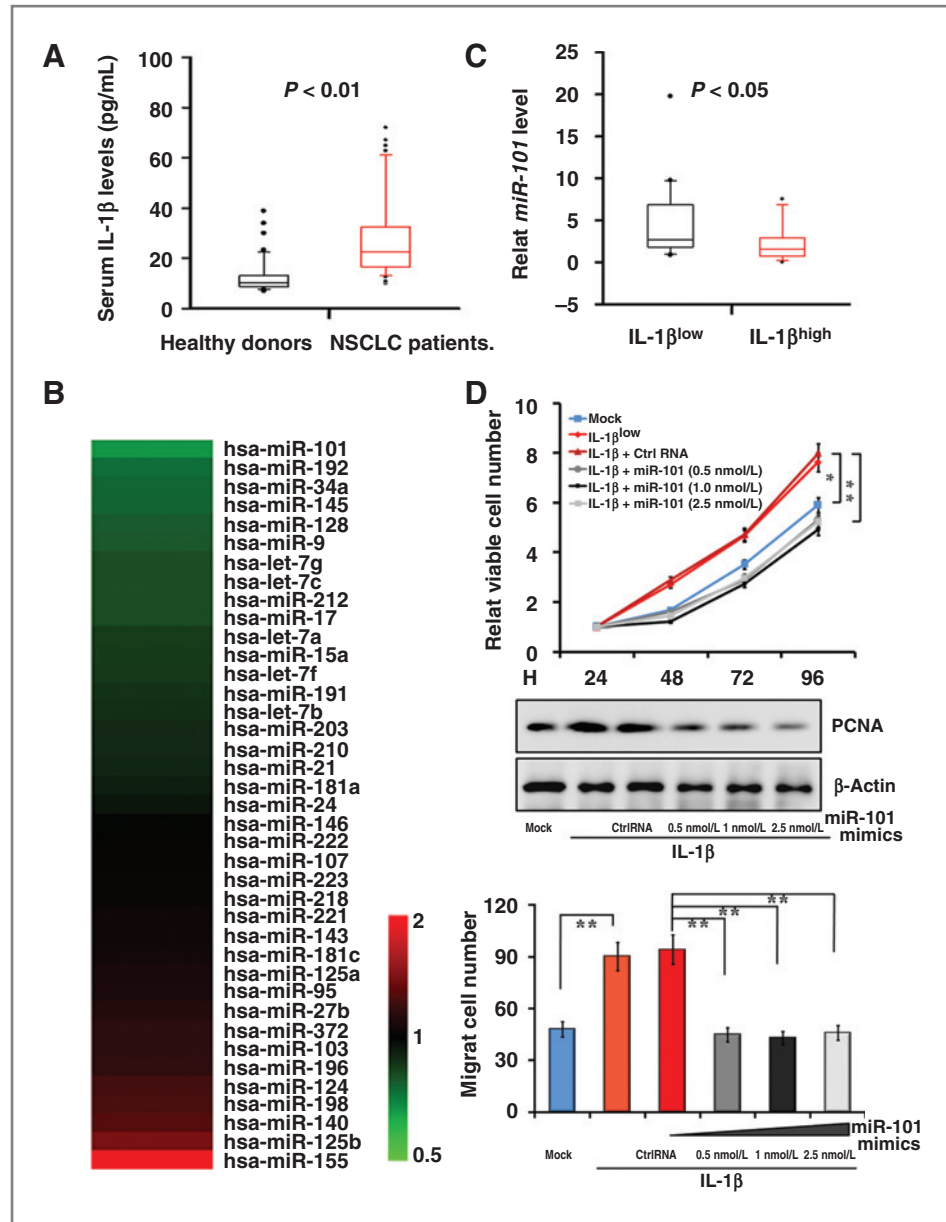
IL-1 β is elevated in NSCLC patients and exerts a tumor-promoting effect in NSCLC cells

To investigate the molecular mechanisms underlying inflammation-associated lung tumorigenesis, we first determined the levels of proinflammatory cytokines, including IL-1 β , IL-4, IL-6, TGF β , and TNF α , in the sera from patients with NSCLC (*n* = 37) and healthy donors (*n* = 37) by ELISA. We found that the levels of IL-1 β from patient sera seemed to be dramatically elevated in comparison with those from healthy controls (Fig. 1A), but there was no significant difference of the serum levels of other tested cytokines between the groups of patients and healthy donors (Supplementary Fig. S1), suggesting a potential role of IL-1 β in NSCLC. To examine whether IL-1 β plays a role in NSCLC cells, we treated two NSCLC cell lines H460 and H1299 with IL-1 β . Interestingly, we found that IL-1 β treatment significantly enhanced cell proliferation and Transwell migration in both cell lines (Supplementary Fig. S2A), suggesting that IL-1 β exerts a tumor-promoting effect in NSCLC cells.

Downregulation of *mir-101* is critical for the tumor-promoting effect of IL-1 β in NSCLC cells

Given that miRNAs have been documented as a new class of mediators linking inflammation and tumorigenesis (17, 18), we next asked whether IL-1 β affects miRNA expression in NSCLC cells. To this end, we examined the expression levels of 39 cancer-related miRNAs in IL-1 β -treated H460 cells. Our qRT-PCR assays showed that the levels of 13 miRNAs were significantly altered (by >1.5 -fold) by IL-1 β treatment (Fig. 1B), including seven upregulated miRNAs (miR-155, miR-125b, miR-140, miR-198, miR-124, miR-196, and miR-103) and six downregulated miRNAs (miR-101, miR-192, miR-34a, miR-145, miR-128, and miR-9). In addition, the levels of many other tested miRNAs were modestly altered in IL-1 β -treated cells (Fig. 1B), indicating that IL-1 β broadly regulates the expression of cancer-related miRNAs in NSCLC cells. Among these, *mir-155* is the most dramatically upregulated by IL-1 β , consistent with previous findings by us and other laboratories that miR-155 is a key mediator in inflammation-associated tumorigenesis (18–20). In contrast, we found that miR-101 was the most reduced in IL-1 β -treated cells (Fig. 1B), indicating repression of *mir-101* by IL-1 β in H460 cells. Consistently, we found that IL-1 β treatment similarly reduced miR-101 level in H1299 cells (Supplementary Fig. S2B). Moreover, the levels of miR-101 in NSCLC tumors from patients with higher serum IL-1 β levels (*n* = 16) were significantly lower than those from patients with lower levels of serum IL-1 β (*n* = 15; Fig. 1C). Interestingly, restoration of *mir-101* expression by transfection of miR-101 mimics with a dosage as low as 0.5 nmol/L completely overrode the impact of IL-1 β on both cell proliferation and migration in IL-1 β -treated NSCLC cells (Fig. 1D; Supplementary Fig. S2C and S2D). These results together indicate that IL-1 β

Figure 1. Downregulating *mir-101* is involved in the tumor-promoting activity of IL-1 β in NSCLC cells. A, comparison of IL-1 β levels in serum from a set of 37 healthy donors and 37 patients with NSCLC. B, the effect of IL-1 β treatment on expression of 39 cancer-related miRNAs in H460 cells. Heatmap represents of differentially expressed miRNAs 24 hours after IL-1 β treatment, with the upregulated miRNAs in red and downregulated ones in green. C, comparison of miR-101 levels between NSCLC tumors from patients with higher (red) and lower serum concentrations of IL-1 β (black). A set of 31 primary NSCLC tumor specimens were divided into two groups, that is, higher IL-1 β group ($n = 16$) and lower IL-1 β group ($n = 15$). D, restoring *mir-101* expression overrode the tumor-promoting effect of IL-1 β in H460 cells. Top, MTT assays; bottom, Transwell cell migration assays. The average values \pm SD of three separate experiments were plotted. *, $P < 0.05$; **, $P < 0.01$. Results shown are representative of three independent experiments.



downregulates *mir-101* expression in NSCLC cells, and that *mir-101* downregulation represents an important mechanism underlying the tumor-promoting activity of IL-1 β .

IL-1 β downregulates *mir-101* via a COX2–HIF1 α pathway

We next asked how IL-1 β downregulates *mir-101* in NSCLC cells. Our qRT-PCR assays showed that IL-1 β treatment decreased both the primary and mature forms of miR-101 in H460 cells, suggesting that IL-1 β regulates *mir-101* expression at the transcriptional level (Supplementary Fig. S3A). A previous study reported that HIF1 α is a transcriptional repressor for *mir-101* in prostate cancer cells (23). We thus asked whether HIF1 α also acts to regulate *mir-101* in NSCLC cells. To this end, we first examined whether CoCl $_2$, a potent HIF1 α activator (33), affects *mir-101* expression in H460 cells. Our immunos-

taining assays showed that CoCl $_2$ treatment significantly increased the nuclear localization of HIF1 α in H460 cells (Supplementary Fig. S3B), indicative of HIF1 α activation. qRT-PCR analyses showed that both pri-miR-101 and mature miR-101 levels were significantly decreased in CoCl $_2$ -treated cells (Supplementary Fig. S3C), supporting that HIF1 α acts as a transcriptional repressor for *mir-101* in NSCLC cells. We next examined whether HIF1 α is involved in the IL-1 β -mediated repression of *mir-101* in NSCLC cells. Interestingly, we found that IL-1 β treatment dramatically increased both HIF1 α protein level and its nuclear localization in H460 cells (Supplementary Fig. S3B and S3D), consistent with previous finding that IL-1 β can activate HIF1 α in another NSCLC cell line A549 (34). As expected, RNAi knockdown of *HIF1 α* in IL-1 β -treated H460 cells completely abolished the impact of IL-1 β on *mir-101*

the mutant reporter was barely affected (Fig. 2B), suggesting *Lin28B* as a target of miR-101. To further corroborate this, we ectopically expressed miR-101 in H1299 cells, which express a low endogenous level of miR-101 (Supplementary Fig. S5A), using a vector coexpressing *mir-101* and GFP and examined the endogenous *Lin28B* expression by immunofluorescent staining. We found that the level of Lin28B protein was greatly reduced in cells with a strong GFP signal and miR-101 overexpression (Fig. 2C, top) but unaltered in control cells (bottom). In contrast, inhibition of miR-101 by anti-miR-101 in H460 cells, which show a higher endogenous *mir-101* expression (Supplementary Fig. S5A), led to enhanced *Lin28B* expression (Fig. 2D). These results together indicate that *Lin28B* is a direct target of miR-101.

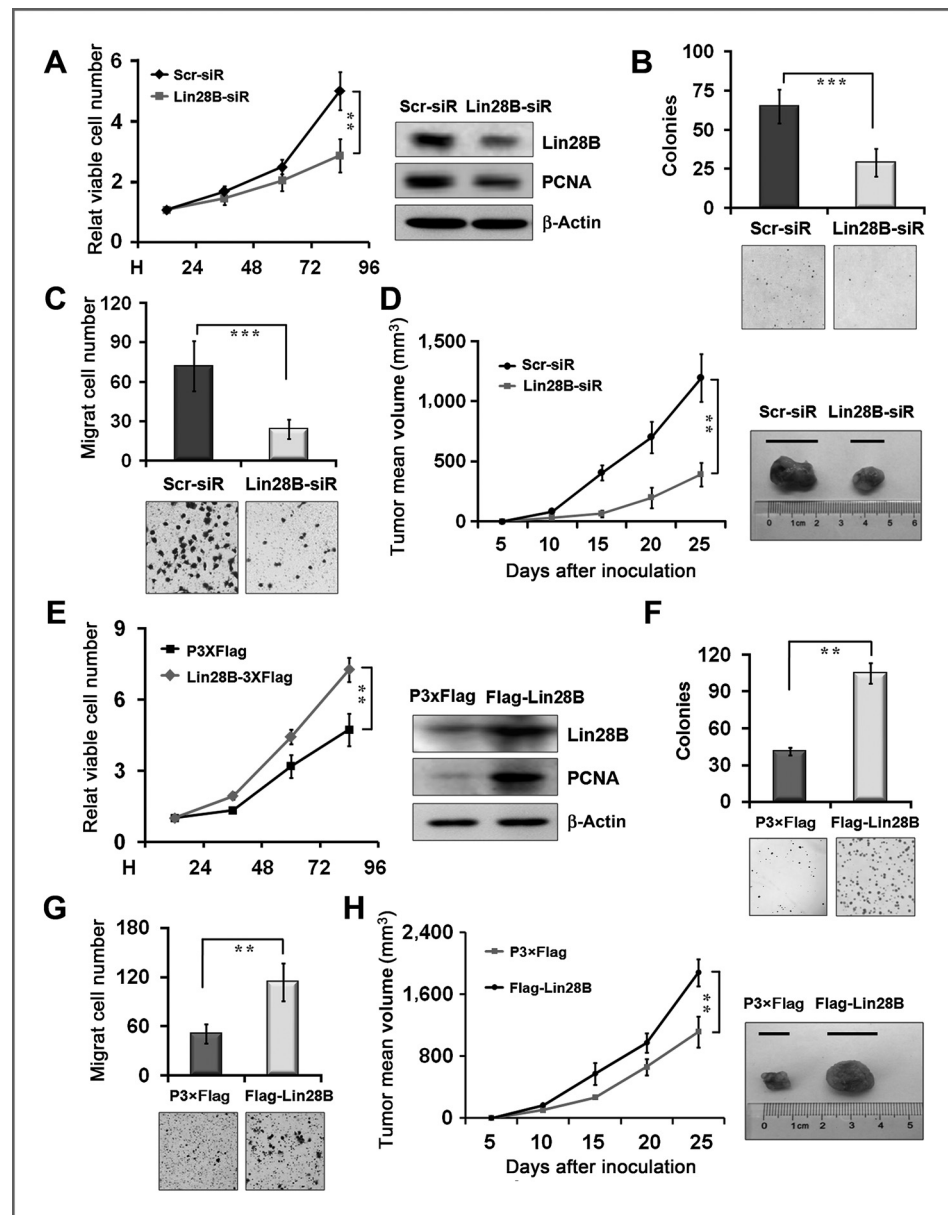
As expected, transfection of miR-101 mimics into H1299 cells significantly increased the levels of all tested let-7 miRNAs

(Supplementary Fig. S4B, left) with a concomitant decrease in Lin28B proteins (right), whereas the effect of miR-101 was completely abolished when a miR-101-resistant form of *Lin28B* was coexpressed in these cells. These results indicate that miR-101 upregulates expression of the let-7 family through the direct targeting of *Lin28B* in NSCLC cells.

miR-101 suppresses NSCLC tumorigenesis via targeting *Lin28B*

As previous studies showed that miR-101 represses malignant transformation and cancer progression by negatively regulating a number of oncogenes, such as *Mcl-1* and *Stmn1*, etc. (21–27), it would thus be critical to determine whether the targeting of *Lin28B* by miR-101 plays an important role in NSCLC. To this end, we first examined the function of *Lin28B* in NSCLC cells. We found that knockdown of *Lin28B* in H1299

Figure 3. *Lin28B* plays an oncogenic role in NSCLC cells. A–D, RNAi knockdown of *Lin28B* in H1299 cells decreased the cell proliferation (A), soft-agar colony formation assays (B), Transwell cell migration (C), and xenograft tumor growth in nude mice (D). A, left, MTT assays; right, Western blot analyses of Lin28B and PCNA proteins. B, top, quantitative results of soft agar foci per field; bottom, representative images. C, top, quantitative results of migratory cells per field; bottom, representative images. D, left, the time course of xenograft tumor growth (mean \pm SD; $n = 6$ per group); right, representative tumors 25 days after inoculation xenograft assay in nude mice. E–H, ectopic expression of *Lin28B* in H460 cells promoted the cell proliferation (E), soft-agar colony formation assays (F), Transwell cell migration (G), and xenograft tumor growth in nude mice (H). The average values \pm SD of three separate experiments are plotted. **, $P < 0.01$; ***, $P < 0.001$. Results shown are representative of three independent experiments.



cells, which harbor a higher endogenous level of Lin28B protein (Supplementary Fig. S5B), significantly reduced cell proliferation (Fig. 3A), anchorage-independent growth (Fig. 3B), Transwell cell migration (Fig. 3C), and xenograft tumor growth in nude mice (Fig. 3D), indicating a protumorigenic role of Lin28B in NSCLC cells. In support of this notion, ectopic expression of Flag-Lin28B in H460 cells, which show a lower endogenous *Lin28B* expression level (Supplementary Fig. S5B), significantly promoted cell proliferation, soft-agar colony formation, Transwell cell migration, and tumor growth in nude mice (Fig. 3E–H). Western blot analyses confirmed that the expression of the proliferation marker PCNA was reduced by *Lin28B* knockdown in H1299 cells (Fig. 3A, right) and enhanced by *Lin28B* overexpression in H460 cells (Fig. 3E, right). These results together demonstrate that *Lin28B* plays a protumorigenic role in NSCLC cells.

We next examined whether downregulation of *Lin28B* by miR-101 plays a critical role in NSCLC cells. Consistent with the well-known tumor-suppressive role of miR-101, we found that inhibition of miR-101 function by anti-miR-101 in H460 cells significantly increased cell proliferation, soft-agar colony formation, Transwell cell migration, and xenograft tumor growth in nude mice (Supplementary Figs. S5C and S6), whereas transfection of miR-101 mimics into

H1299 cells significantly decreased the cell growth, migration, and tumorigenicity (Fig. 4A–D). Importantly, the anti-tumorigenic effect of miR-101 was dramatically attenuated by cotransfection of a miR-101-resistant form of *Lin28B* (Fig. 4A–D). Collectively, our results suggest that targeting *Lin28B* represents an important mechanism underlying the tumor-suppressive function of miR-101 in NSCLC.

Negative correlation between miR-101 and *Lin28B* expression in NSCLC patients

To test the clinical relevance of miR-101-mediated regulation of *Lin28B* in patients with NSCLC, we examined the levels of miR-101 and *Lin28B* mRNA in 27 sets of NSCLC primary tumor and paired normal tissue specimens. Our qRT-PCR assays showed that *mir-101* was significantly downregulated in NSCLC tumors compared with normal tissues (Fig. 5A), whereas *Lin28B* expression was significantly upregulated (Fig. 5B). Moreover, we found that tumors from stage II/III patients tended to express more Lin28B and less miR-101 (Fig. 5C). Using Pearson correlation analysis, we obtained a significant inverse correlation between miR-101 and *Lin28B* mRNA levels in tumor specimens ($R = -0.561$; $P < 0.01$; Fig. 5D). Consistently, we found that NSCLC tumors with high miR-101 levels showed weak Lin28B

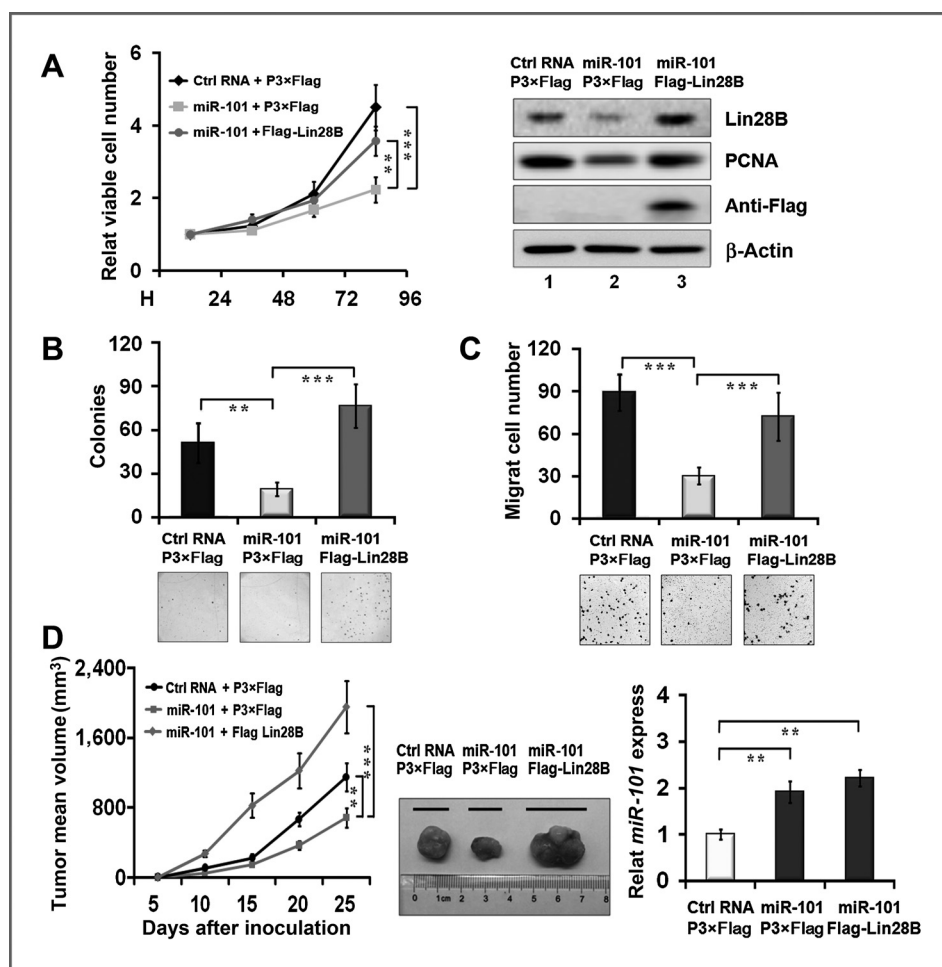


Figure 4. The miR-101–Lin28B axis is of functional importance in regulating tumorigenesis in NSCLC cells. A–D, ectopic expression of *Lin28B* in miR-101 mimics-transfected H1299 cells significantly attenuated the effect of miR-101 on the cell proliferation (A), soft-agar colony formation assays (B), Transwell cell migration (C), and xenograft tumor growth in nude mice (D). The average values \pm SD of three separate experiments are plotted. **, $P < 0.01$; ***, $P < 0.001$. Results shown are representative of three independent experiments.

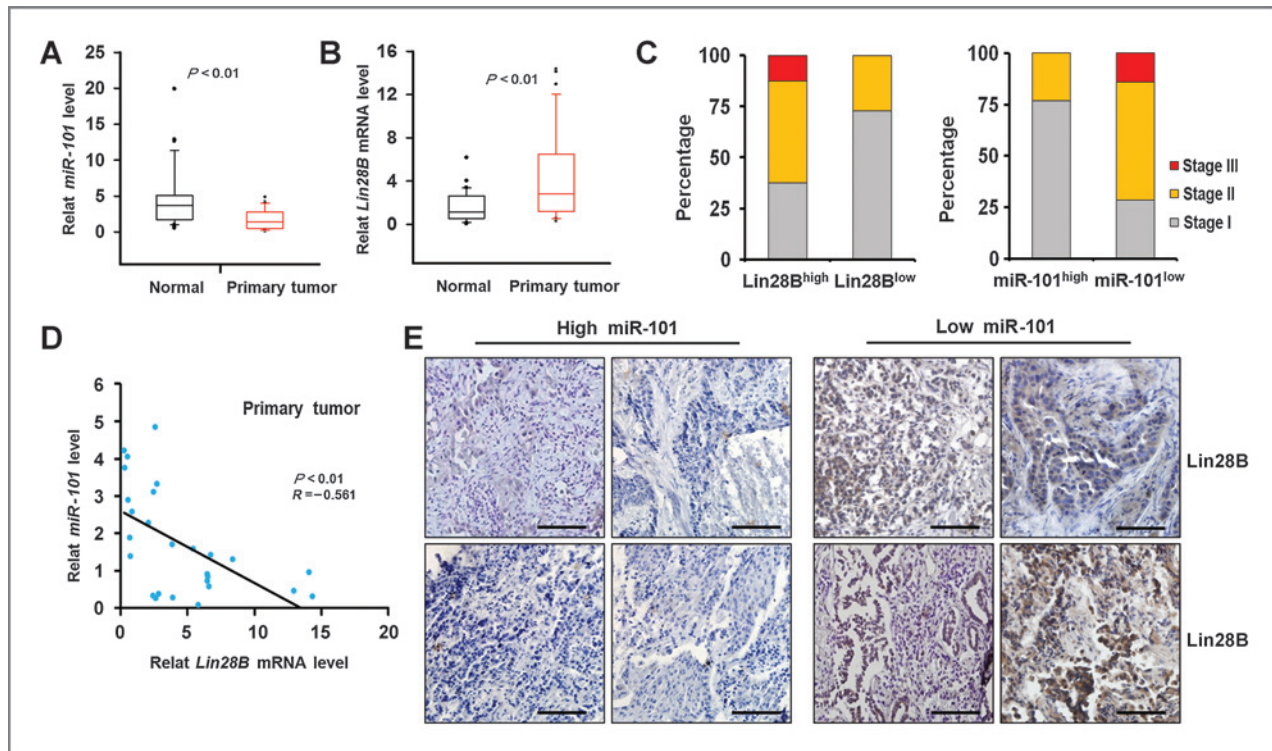


Figure 5. miR-101 and *Lin28B* expression in NSCLC specimens. A and B, comparison of miR-101 (A) and *Lin28B* mRNA levels (B) in NSCLC tumors and paired normal adjacent tissues ($n = 27$). C, comparison of the cancer stage distribution of patients with NSCLC based on *Lin28* (left) and *mir-101* expression levels (right). D, miR101 and *Lin28B* mRNA levels were inversely correlated in NSCLC tumors. E, representative images of *Lin28B* immunohistochemical staining in NSCLC tumors with high (left) or low (right) miR101 levels. The average values \pm SD of three separate experiments are plotted. *, $P < 0.05$; **, $P < 0.01$. Results shown are representative of three independent experiments.

immunohistochemical staining (Fig. 5E, left), whereas tumors with low *mir-101* expression showed strong *Lin28B* staining (right). Collectively, these results strongly suggest that the miR-101-*Lin28B* regulation newly discovered in our study is of clinical relevance in patients with NSCLC.

IL-1 β promotes *Lin28B* expression through two distinct mechanisms

Given our above results showing that IL-1 β downregulates *mir-101* (Fig. 1B) and that *Lin28B* is a target of miR-101 (Fig. 2), we next examined whether *Lin28B* is regulated by IL-1 β . We found that both *Lin28B* protein and mRNA levels were significantly elevated in IL-1 β -treated H460 cells (Supplementary Fig. S7A). As a previous report shows that inflammatory cytokines promote *Lin28B* transcription through activating its transcriptional activator NF- κ B (38), we thus asked whether NF- κ B is required for IL-1 β -induced *Lin28B* expression. Interestingly, we found that quinazoline, a NF- κ B inhibitor (39), completely blocked both IL-1 β -induced *Lin28B* mRNA and protein expression (Supplementary Fig. S7A), supporting that IL-1 β indeed promotes *Lin28B* transcription through NF- κ B. Intriguingly, transfection of miR-101 mimics strongly reduced *Lin28B* protein level but marginally changed its mRNA level in IL-1 β -treated cells (Supplementary Fig. S7A). These results together indicate that both activation of NF- κ B and repression of *mir-101* are required for IL-1 β -induced *Lin28B* expression in NSCLC cells.

Aspirin and celecoxib abrogated the IL-1 β -mediated regulation of miR-101-*Lin28B*-let-7 axis and reversed the tumor-promoting effect of IL-1 β in NSCLC cells

Given that IL-1 β downregulates *mir-101* by upregulating *Cox2* (Supplementary Fig. S3), we next asked whether COX2 is important for IL-1 β -induced *Lin28B* expression. We found that knockdown of *Cox2* largely abolished the impact of IL-1 β on *Lin28B* expression (Supplementary Fig. S7B), indicating that IL-1 β signaling modulates *Lin28B* expression via COX2 in NSCLC cells. We further used aspirin and celecoxib, which respectively inhibit COX2 nonselectively and selectively (40), to verify the role of COX2 in the IL-1 β -mediated regulation of *mir-101* and *Lin28B*. Interestingly, we found that both aspirin and celecoxib significantly reversed the repression of *mir-101* and induction of *Lin28B* by IL-1 β in H460 cells (Fig. 6A and B). Consistently, both aspirin and celecoxib significantly attenuated the impact of IL-1 β on expression of let-7 miRNAs in these cells (Fig. 6C and D). As expected, we found that both drugs significantly reduced the stimulatory effect of IL-1 β on proliferation and migration of H460 cells (Fig. 6E). These results together indicate that aspirin and celecoxib could reverse the effect of IL-1 β on the miR-101-*Lin28B*-let-7 regulatory axis, and antagonize the tumor-promoting effect of IL-1 β in NSCLC cells.

Intriguingly, we found that aspirin and celecoxib, the inhibitors of COX2, actually reduced COX2 protein levels in IL-1 β -treated cells (Supplementary Fig. S7C; lane 2 vs. lane 3). Given a previous study showing that *Cox2* itself is a target of miR-101

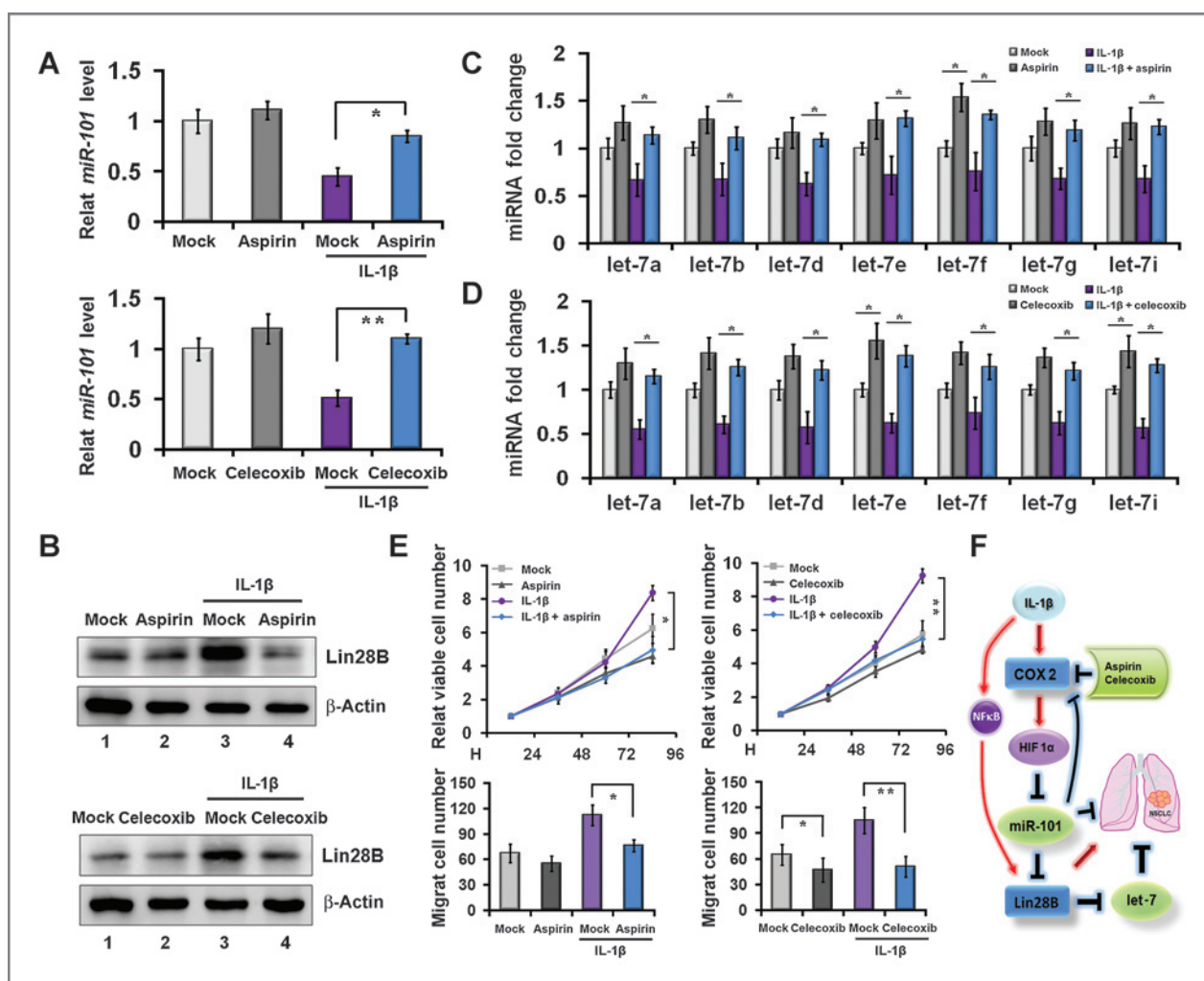


Figure 6. Aspirin and celecoxib reverse the effect of IL-1 β on the miR-101–Lin28B–let-7 regulatory axis and antagonize the tumor-promoting effect of IL-1 β in NSCLC cells. A, aspirin and celecoxib abolished the inhibitory effect of IL-1 β on *mir-101* in H460 cells. B, aspirin and celecoxib abolished the stimulatory effect of IL-1 β on *Lin28B* in H460 cells. Aspirin (C) and celecoxib (D) abolished the inhibitory effect of IL-1 β on proliferation and migration of H460 cells. E, aspirin and celecoxib reduced the stimulatory effect of IL-1 β on proliferation and migration of H460 cells. F, model of miR-101 as a key regulatory node linking inflammation to lung tumorigenesis. The average values \pm SD of three separate experiments are plotted. *, $P < 0.05$; **, $P < 0.01$. Results shown are representative of three independent experiments.

(21), we reasoned that the NSAIDs-mediated reduction of COX2 protein might be due to the prevention of IL-1 β -mediated downregulation of miR-101. Indeed, inhibition of miR-101 by anti-miR-101 completely restored COX2 levels in IL-1 β -treated cells despite the presence of aspirin or celecoxib (Supplementary Fig. S7C; lane 5), suggesting the existence of a double-negative regulatory loop between COX2 and miR-101.

Discussion

Despite that the role of inflammation in the initiation and progression of NSCLC has been well recognized (2), the underlying molecular mechanisms are incompletely understood. In the present study, we found that IL-1 β , a proinflammatory cytokine, is dramatically upregulated in patients with NSCLC. Functional studies show that IL-1 β acts to downregulate

tumor-suppressive miR-101 and let-7 miRNAs and upregulate protumorigenic *Cox2* and *Lin28B* expression in NSCLC cells, revealing a novel molecular connection between inflammation and NSCLC and bringing new insights into inflammation-associated pathogenesis of NSCLC.

miR-101 acts as a key mediator linking inflammation and NSCLC

Accumulating data indicate that miRNAs function as central modulators linking inflammation and cancer (17, 18). Our recent studies, along with reports by others, indicate that a number of oncomiRs are induced by inflammatory stimuli in cancer cells, and link inflammation to tumorigenesis by regulating various tumor-suppressive genes (18–20, 41). In contrast, in the present study, we found that *mir-101*, a tumor-suppressive miRNA, is repressed by inflammation signaling

and acts as a key regulatory node linking inflammation to expression control of several cancer-related genes, including *Cox2*, *Lin28B*, and *let-7* miRNAs (Fig. 6F), revealing a novel molecular mechanism for miRNAs in inflammation-associated tumorigenesis.

Our data show that downregulation of *mir-101* by IL-1 β is critical for its tumor-promoting activity. Intriguingly, we found that *Lin28B* is a novel target of miR-101 and that IL-1 β represses *mir-101*, thus resulting in upregulation of *Lin28B* expression at the posttranscriptional level. Moreover, we found that IL-1 β also promotes *Lin28B* transcription by activating its transcriptional activator NF κ B, suggesting that IL-1 β upregulates *Lin28B* through two distinct mechanisms (Fig. 6F). Our findings further support a previous report that *Lin28B* is a key molecule linking inflammation with cell transformation (38). In addition, our results show that COX2, a key promoting factor of inflammation-associated tumorigenesis (3), acts to repress *mir-101* expression. Interestingly, *Cox2* itself is known to be a target of miR-101 (21). Thus, our data suggest the presence of a double-negative feedback loop between miR-101 and COX2, providing an additional layer of regulation between inflammation and cancer. Collectively, our findings strongly support miR-101 as a novel key mediator linking inflammation and cancer.

miR-101 elicits its antitumor activity through targeting *Lin28B*

miR-101 is a well-documented tumor-suppressive miRNA in various types of cancers. For instance, miR-101 has been reported to repress malignant transformation and cancer progression by targeting *Mcl-1*, *Stmn1*, *Junb*, and *Cxcr7* in hepatocellular carcinoma (22, 27), *Mycn* in neuroblastoma (25), *Ezh2* in prostate cancer (23), *Cox2* in colon cancer (21), and *Mitf* and *Ezh2* in melanoma (26). Intriguingly, a recent study found that miR-101 represses TET2 and surprisingly shows an oncogenic potential in malignant hematopoiesis (42). Nevertheless, the precise role of miR-101 in NSCLC remained largely unclear. Our results show that miR-101 also displays an anti-tumor activity in NSCLC. We further show that *Lin28B*, which is known to be protumorigenic in breast and colon cancers (43–45), is a novel target of miR-101, and that miR-101 exerts its tumor-suppressive function in NSCLC cells at least, in part, through repressing *Lin28B*. Moreover, consistent with previous findings that *Lin28B* inhibits the expression of the *let-7* family (28–30), our results show that miR-101 represses *Lin28B* and simultaneously increases the levels of *let-7* miRNAs. Given that *let-7* miRNAs possess tumor-suppressive roles in lung cancer (46), elevation of *let-7* expression by miR-101 may further sustain its antitumor activity in NSCLC cells (Fig. 6F).

In addition, epidemiologic data show that aspirin, celecoxib, and other NSAIDs exert chemopreventive effects on lung cancer

as well as several other types of epithelial tumors (9–11). Recent studies found that NSAIDs may exert anticancer activities in both COX-dependent and -independent manners, including suppressing *E2F1*, *Mcl-1*, and *survivin* and activating the eIF2 α kinase PKR in various cancer cells (12, 47, 48). Nevertheless, whether miRNA regulation is involved in the anticancer actions of NSAIDs remained largely unexplored. Our data indicate that application of aspirin and celecoxib prevents IL-1 β -mediated downregulation of the tumor-suppressive miRNAs miR-101 and *let-7* miRNAs in NSCLC cells, raising the possibility that regulation of miRNAs might constitute a novel mechanism for the chemopreventive effects of NSAIDs.

In summary, our study here reveals a novel COX2–miR-101–*Lin28B*–*let-7* pathway connecting inflammation and NSCLC (Fig. 6F). Given that pulmonary inflammation and the lung matrix remodeling that underlies COPD are important precursors to lung cancer (2), it will be interesting in future studies to identify whether this pathway also contributes to COPD-associated lung tumorigenesis. In addition, as COX2, *Lin28B*, miR-101, and *let-7* are all demonstrated to play critical roles in the tumorigenicity and invasiveness of cancer cells (3, 21–27, 40, 43–46, 49), rational intervention of this pathway may provide novel preventive or therapeutic strategies to reduce the health burden of NSCLC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: D. Li, J.-T. Lou, M.-F. Liu
Development of methodology: L. Wang, L.-F. Zhang, J.-T. Lou
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Wang, L.-F. Zhang, J. Wu, S.-J. Xu, Y.-Y. Xu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Wang, L.-F. Zhang
Writing, review, and/or revision of the manuscript: D. Li, J.-T. Lou, M.-F. Liu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Wang, L.-F. Zhang, J. Wu, S.-J. Xu, Y.-Y. Xu
Study supervision: J.-T. Lou, M.-F. Liu

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