Repression of microRNA-101 by IL-1β signaling mediates inflammation-promoted lung tumorigenesis

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Abstract

It is well known that inflammatory stimuli contribute to the initiation and progression of lung cancer; however, the underlying molecular mechanisms are not well understood. In this study, we found that the pro-inflammatory cytokine IL-1β was dramatically elevated in the serum of non-small cell lung cancer (NSCLC) patients. In vitro studies showed that IL-1β significantly promoted the proliferation and migration of NSCLC cells. Mechanistically, we found that IL-1β acts to repress \textit{mir-101}, a well-documented tumor suppressive miRNA, via the cyclooxygenase-2 (COX-2)/hypoxia-inducible factor 1α (HIF1α) pathway. We went on to further identify \textit{lin-28 homolog B (Lin28B)} as a novel target of miR-101, and provided evidence that repression of \textit{Lin28B} represents an important mechanism of the tumor-suppressive effect of miR-101. As a result, IL-1β up-regulates \textit{Lin28B} in NSCLC cells by down-regulating \textit{mir-101}. Interestingly, inhibition of COX-2 by Aspirin or Celecoxib abrogated IL-1β-mediated regulation of \textit{mir-101} and \textit{Lin28B} expression, and its stimulatory effect on NSCLC cell proliferation and migration. Taken together, our findings identify the IL-1β-miR-101-Lin28B pathway as a novel regulatory axis that links inflammation signaling to the control of tumorigenesis in NSCLC.
Introduction

Chronic inflammation and infection have been established as key promoting factors of tumorigenesis (1). Epidemiological 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 (COX-2), a member of the cyclooxygenases 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 COX-2 is known to promote the initiation and progression of NSCLC as well as other types of human epithelial tumors (3-7). Interestingly, inhibition of COX-2 by non-steroid anti-inflammatory drugs (NSAIDs) has been shown to be an effective approach in prevention and therapy of NSCLC and several other types of cancers by both epidemiological 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 (miRNAs) as a novel class of inflammation-associated molecules (14). miRNAs are ~22 nt, non-coding 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, while 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 pro-tumorigenic inflammatory STAT3 signaling by targeting socs1 (19).
and promotes glucose utilization in aerobic conditions, i.e. the Warburg effect, by up-regulating 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 pro-inflammatory cytokine IL-1β was dramatically elevated in the serum of NSCLC patients. IL-1β signaling acts to repress mir-101, a well-documented tumor suppressive miRNA, via the COX-2/HIF-1α pathway. We showed that IL-1β 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 down-regulated 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-1β, through the miR-101-Lin28B axis, down-regulates let-7 family in NSCLC cells. Interestingly, application of Aspirin and Celecoxib, the NSAIDs that non-selectively and selectively inhibit COX-2, respectively, abrogated IL-1β-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-1β-miR-101-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 non-small cell lung cancer cell lines A549 and H1299 and embryonic kidney cell
line HEK293T cells were obtained from ATCC and cultured according to their guidelines. The cellular morphology of these cell lines is normal in both low and high densities of cultures by microscopy according to the guideline from ATCC (31). NSCLC specimens and paired normal adjacent tissues were collected during surgery from Shanghai Chest Hospital affiliated to Shanghai Jiaotong University. Samples were immediately snap-frozen and stored at -80°C. Specimen collection was approved by the hospital authorities.

**RNA Isolation and Quantitative Reverse Transcription PCR (qRT-PCR)**

RNA isolation and qRT-PCR assays were performed as we described previously (19). The primer sequences for qRT-PCR were provided in supplementary Table S1. The experimental details were provided in Supplementary Data.

**Western Blotting and Immunofluorescence Assays**

Western blotting and Immunofluorescence assays were carried out as we recently described (32). The experimental details and source of antibodies were provided in Supplementary Data.

**Cell Proliferation, Soft Agar Colony Formation, Transwell Migration and Xenograft Assays**

Cell Proliferation, Soft Agar Colony Formation, Transwell Migration and Xenograft Assays were performed as we described previously (19, 20; see Supplementary Data for details).

**Statistical analysis**

All results were presented as the mean ± standard error of the mean (s.e.m). A Student's *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 *) considered significant (*** *P*<0.001, ** *P*<0.01).
Results

IL-1β 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 pro-inflammatory cytokines, including IL-1β, IL-4, IL-6, TGF-β and TNF-α, in the sera from NSCLC patients (n=37) and healthy donors (n=37) by ELISA. We found that the levels of IL-1β from patient sera appeared 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-1β in NSCLC. To examine whether IL-1β plays a role in NSCLC cells, we treated two NSCLC cell lines A549 and H1299 with IL-1β. Interestingly, we found that IL-1β treatment significantly enhanced cell proliferation and transwell migration in both cell lines (Supplementary Fig. S2A), suggesting that IL-1β exerts a tumor-promoting effect in NSCLC cells.

Down-regulation of mir-101 is critical for the tumor-promoting effect of IL-1β 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-1β affects miRNA expression in NSCLC cells. To this end, we examined the expression levels of 39 cancer-related miRNAs in IL-1β-treated A549 cells. Our qRT-PCR assays showed that the levels of 13 miRNAs were significantly altered (by >1.5-fold) by IL-1β treatment (Fig. 1B), including 7 up-regulated miRNAs (miR-155, miR-125b, miR-140, miR-198, miR-124, miR-196 and miR-103) and 6 down-regulated 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-1β-treated cells (Fig. 1B), indicating that IL-1β broadly regulates the expression of cancer-related miRNAs in NSCLC cells. Among these, mir-155 is the most dramatically up-regulated by IL-1β, consistent with previous findings by us and other
laboratories that miR-155 is a key mediator in inflammation-associated tumorigenesis (18-20). By contrast, we found that miR-101 was the most reduced in IL-1β-treated cells (Fig. 1B), indicating repression of mir-101 by IL-1β in A549 cells. Consistently, we found that IL-1β 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-1β levels (n=16) were significantly lower than those from patients with lower levels of serum IL-1β (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-1β on both cell proliferation and migration in IL-1β-treated NSCLC cells (Fig. 1D; Supplementary Fig. S2C and S2D). These results together indicate that IL-1β down-regulates mir-101 expression in NSCLC cells, and that mir-101 down-regulation represents an important mechanism underlying the tumor-promoting activity of IL-1β.

**IL-1β down-regulates mir-101 via a COX-2/HIF-1α pathway**

We next asked how IL-1β down-regulates mir-101 in NSCLC cells. Our qRT-PCR assays showed that IL-1β treatment decreased both the primary and mature forms of miR-101 in A549 cells, suggesting that IL-1β regulates mir-101 expression at the transcriptional level (Supplementary Fig. 3A). A previous study reported that HIF-1α is a transcriptional repressor for mir-101 in prostate cancer cells (23). We thus asked whether HIF-1α also acts to regulate mir-101 in NSCLC cells. To this end, we first examined whether CoCl2, a potent HIF-1α activator (33), affects mir-101 expression in A549 cells. Our immunostaining assays showed that CoCl2 treatment significantly increased the nuclear localization of HIF-1α in A549 cells (Supplementary Fig. S3B), indicative of HIF-1α activation. qRT-PCR analyses showed that both pri-miR-101 and mature miR-101 levels were significantly decreased in CoCl2-treated cells (Supplementary Fig. S3C), supporting that HIF-1α acts as a transcriptional repressor for mir-101 in NSCLC cells. We next examined whether HIF-1α is involved in the IL-1β-mediated repression of mir-101 in NSCLC cells. Interestingly, we
found that IL-1β treatment dramatically increased both HIF-1α protein level and its nuclear localization in A549 cells (Supplementary Fig. S3B and S3D), consistent with previous finding that IL-1β can activate HIF-1α (34). As expected, RNAi knockdown of HIF-1α in IL-1β-treated A549 cells completely abolished the impact of IL-1β on mir-101 (Supplementary Fig. 3E), strongly supporting that HIF-1α is essential to IL-1β-mediated down-regulation of mir-101.

It has been previously shown that IL-1β activates HIF-1α through the NF-kB/COX-2 pathway (34, 35). We thus examined whether COX-2 is required for the IL-1β/HIF-1α-mediated repression of mir-101 in NSCLC cells. We found that ectopic Flag-COX-2 in A549 cells strongly reduced the level of miR-101 (Supplementary Fig. S3F), whereas knockdown of Cox-2 significantly increased mir-101 expression (Supplementary Fig. S3G), indicating that COX-2 negatively regulates mir-101 in NSCLC cells. Consistently, our western blot assays confirmed that IL-1β treatment significantly increased both COX-2 and HIF-1α protein levels (Supplementary Fig. 3H, right). Importantly, knockdown of Cox-2 in IL-1β-treated A549 cells strongly attenuated the inhibitory effect of IL-1β on mir-101 expression (Supplementary Fig. 3H, left) with a concomitant decrease in COX-2 and HIF-1α protein levels (right, lane 4), indicating that COX-2 is essential to IL-1β-mediated repression of mir-101. Taken together, these results indicate that IL-1β down-regulates mir-101 via a COX-2/HIF-1α pathway in NSCLC cells.

**IL-1β represses let-7 family via down-regulating mir-101**

We noted that IL-1β-mediated repression of miR-101 was accompanied with a moderate reduction of let-7 family miRNAs (1.2- to 1.4-fold) (Fig. 1B), which raised an intriguing possibility that there might be a functional connection between IL-1β-mediated repression of mir-101 and let-7 miRNAs. To further examine this possibility, we transfected miR-101 or let-7 mimics into IL-1β-treated A549 cells and determined the levels of these miRNAs using qRT-PCR assays. Strikingly, we found that ectopic expression of mir-101 completely rescued the repression of let-7
miRNAs by IL-1β (Supplementary Fig. S4A), whereas transfection of let-7 mimics barely affected miR-101 levels in IL-1β-treated cells (data not shown). These results together indicate that down-regulation of mir-101 is important for IL-1β-mediated repression of let-7 family.

**Lin28B is a target of miR-101 and miR-101 up-regulates let-7 family via targeting Lin28B**

We next asked how miR-101 regulates let-7 miRNAs. Interestingly, using computational prediction programs (36, 37), we found that Lin28B, encoding a homolog of Lin28 proteins that inhibit the processing of pri-let-7 transcripts into mature miRNAs (28-30), was a putative target of miR-101 (Fig. 2A, top). To experimentally test whether miR-101 regulates Lin28B, we cloned the wild-type Lin28B 3’UTR or a mutant version with deletion of the 8-bp sequence complementary to the 5’ part of miR-101 (Lin28B-Mut) downstream of the Renilla luciferase cDNA in pRL-TK vector (Fig. 2A, bottom), and transfected the reporters into 293T cells along with synthetic control RNA or miR-101 mimics. We found that co-transfection of miR-101 mimics significantly decreased the luciferase activity of the wild-type reporter, whereas 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 co-expressing 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 A549 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), while the effect of miR-101 was completely abolished when a miR-101-resistant
form of Lin28B was co-expressed in these cells. These results indicate that miR-101 up-regulates 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 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 pro-tumorigenic role of Lin28B in NSCLC cells. In support of this notion, ectopic expression of Flag-Lin28B in A549 cells, which show a lower endogenous Lin28B expression level (Supplementary Fig. S5B), significantly promoted cell proliferation, soft-agar colony formation, transwell cell migration as well as tumor growth in nude mice (Fig. 3E-3H). 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 A549 cells (Fig. 3E, right). These results together demonstrate that Lin28B plays a pro-tumorigenic role in NSCLC cells.

We next examined whether down-regulation 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 A549 cells significantly increased cell proliferation, soft-agar colony formation, transwell cell migration, as well as xenograft tumor growth in nude mice (Supplementary Fig. S5C and S6), while transfection of miR-101 mimics into H1299 cells significantly decreased the cell growth, migration and tumorigenicity (Fig. 4A-4D). Importantly, the anti-tumorigenic effect of miR-101 was dramatically attenuated by co-transfection of a
miR-101-resistant form of Lin28B (Fig. 4A-4D). 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 NSCLC patients, 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 down-regulated in NSCLC tumors compared to normal tissues (Fig. 5A), while Lin28B expression was significantly up-regulated (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's 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 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 NSCLC patients.

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

Given our above results showing that IL-1β down-regulates mir-101 (Fig. 1B) and that Lin28B is a target of miR-101 (Fig. 2), we next examined whether Lin28B is regulated by IL-1β. We found that both Lin28B protein and mRNA levels were significantly elevated in IL-1β-treated A549 cells (Supplementary Fig. S7A). As a previous report shows that inflammatory cytokines promote Lin28B transcription through activating its transcriptional activator NF-κB (38), we thus asked whether NF-κB is required for IL-1β-induced Lin28B expression. Interestingly, we found that Quinazoline (QNZ), a NF-κB inhibitor (39), completely blocked both IL-1β-induced Lin28B mRNA and protein
expression (Supplementary Fig. S7A), supporting that IL-1β indeed promotes Lin28B transcription through NF-κB. Intriguingly, transfection of miR-101 mimics strongly reduced Lin28B protein level but marginally changed its mRNA level in IL-1β-treated cells (Supplementary Fig. S7A). These results together indicate that both activation of NF-κB and repression of mir-101 are required for IL-1β-induced Lin28B expression in NSCLC cells.

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

Given that IL-1β down-regulates mir-101 by up-regulating Cox-2 (Supplementary Fig. S3), we next asked whether COX-2 is important for IL-1β-induced Lin28B expression. We found that knockdown of Cox-2 largely abolished the impact of IL-1β on Lin28B expression (Supplementary Fig. S7B), indicating that IL-1β signaling modulates Lin28B expression via COX-2 in NSCLC cells. We further used Aspirin and Celecoxib, which respectively inhibit COX-2 non-selectively and selectively (40), to verify the role of COX-2 in the IL-1β-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-1β in A549 cells (Fig. 6A and 6B). Consistently, both Aspirin and Celecoxib significantly attenuated the impact of IL-1β on expression of let-7 miRNAs in these cells (Fig. 6C and 6D). As expected, we found that both drugs significantly reduced the stimulatory effect of IL-1β on proliferation and migration of A549 cells (Fig. 6E). These results together indicate that Aspirin and Celecoxib could reverse the effect of IL-1β on the miR-101-Lin28B-let-7 regulatory axis, and antagonize the tumor-promoting effect of IL-1β in NSCLC cells.

Intriguingly, we found that Aspirin and Celecoxib, the inhibitors of COX-2, actually reduced COX-2 protein levels in IL-1β-treated cells (Supplementary Fig. S7C; lane 2 vs lane 3). Given a previous study showing that Cox-2 itself is a target of miR-101 (21), we reasoned that the NSAIDs-mediated reduction of COX-2 protein might be due to the prevention of IL-1β-mediated
down-regulation of miR-101. Indeed, inhibition of miR-101 by anti-miR-101 completely restored COX-2 levels in IL-1β-treated cells despite the presence of Aspirin or Celecoxib (Supplementary Fig. S7C; lane 5), suggesting the existence of a double-negative regulatory loop between COX-2 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-1β, a pro-inflammatory cytokine, is dramatically up-regulated in NSCLC patients. Functional studies show that IL-1β acts to down-regulate tumor suppressive miR-101 and let-7 miRNAs and up-regulate pro-tumorigenic Cox-2 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 Cox-2, Lin28B and let-7 miRNAs (Fig. 6F), revealing a novel molecular mechanism for miRNAs in inflammation-associated tumorigenesis.

Our data show that down-regulation of mir-101 by IL-1β is critical for its tumor-promoting activity. Intriguingly, we found that Lin28B is a novel target of miR-101 and that IL-1β represses mir-101, thus resulting in up-regulation of Lin28B expression at the post-transcriptional level.
Moreover, we found that IL-1β also promotes Lin28B transcription by activating its transcriptional activator NFκB, suggesting that IL-1β up-regulates 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). Additionally, our results show that COX-2, a key promoting factor of inflammation-associated tumorigenesis (3), acts to repress mir-101 expression. Interestingly, Cox-2 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 COX-2, 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 anti-tumor 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), Cox-2 in colon cancer (21), and Mistf 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 pro-tumorigenic 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 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 anti-tumor activity in NSCLC cells (Fig. 6F).

Additionally, epidemiological 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 NASIDs may exert anticancer activities in both cyclooxygenase-dependent and cyclooxygenase-independent manners, including suppressing \( E2F1 \), \( Mcl-1 \) and survivin and activating the eIF2\( \alpha \) 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\( \beta \)-mediated down-regulation 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 COX-2-miR-101-Lin28B-let-7 pathway connecting inflammation and NSCLC (Fig. 6F). Given that pulmonary inflammation and the lung matrix remodelling 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. Additionally, as COX-2, Lin-28B, 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), rationale intervention of this pathway may provide novel preventive or therapeutic strategies to reduce the health burden of NSCLC.

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**Figure legends**

**Figure 1** Down-regulating mir-101 is involved in the tumor-promoting activity of IL-1β in Non-small cell lung cancer (NSCLC) cells.

(A) Comparison of IL-1β levels in serum from a set of 37 healthy donors and 37 NSCLC patients.

(B) The effect of IL-1β treatment on expression of 39 cancer-related miRNAs in A549 cells. Heatmap represents of differentially expressed miRNAs 24 h after IL-1β treatment, with the up-regulated miRNAs in red and down-regulated ones in green.

(C) Comparison of miR-101 levels between NSCLC tumors from patients with higher (red) and lower serum concentrations of IL-1β (black). A set of 31 primary NSCLC tumor specimens were divided into two groups, i.e. higher IL-1β group (n=16) and lower IL-1β group (n=15).

(D) Restoring mir-101 expression overrode the tumor-promoting effect of IL-1β in A549 cells. Top, MTT assays; bottom, transwell cell migration assays.

The average values ± s.d. of three separate experiments were plotted. *P<0.05, **P<0.01. Results shown are representative of three independent experiments.

**Figure 2** Lin28B is a target of miR-101 in NSCLC cells.

(A) Lin28B is predicted to be a target of miR-101. The seed sequences are shown on the top. The sequences of wild-type and mutated Lin28B 3’UTR-Renilla luciferase reporters are shown on the
bottom.

(B) Lin28B 3’UTR luciferase reporter assays in 293T cells.

(C) Immunofluorescence analyses of the effect of mir-101 overexpression on Lin28B expression in H1299 cells. GFP images (green) and arrowheads indicate cells transfected with mir-101 expression vector pSIF-GFP-miR101 (top) or control pSIF-GFP (bottom). Red, Lin28B protein was immunostained with anti-Lin28B; blue, nuclei were counterstained with DAPI.

(D) qRT-PCR analyses of Lin28B mRNA levels (left) or western blot of Lin28B protein levels (right) in anti-miR-101-transfected A549 cells.

The average values ± s.d. of three separate experiments were plotted. *P<0.05, ***P<0.001. Results shown are representative of three independent experiments.

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±s.d., n=6 per group); right, representative tumors 25 days after inoculation xenograft assay in nude mice.

(E-H) Ectopic expression of Lin28B in A549 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 ± s.d. of three separate experiments were plotted. **P<0.01, ***P<0.001. Results shown are representative of three independent experiments.
**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 ± s.d. of three separate experiments were 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 NSCLC patients based on Lin28 (left) and mir-101 expression levels (right).

(D) miR-101 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 miR-101 levels (right).

The average values ± s.d. of three separate experiments were plotted. *P<0.05, **P<0.01. Results shown are representative of three independent experiments.

**Figure 6** Aspirin and Celecoxib reverse the effect of IL-1β on the miR-101-Lin28B-let-7 regulatory axis, and antagonize the tumor-promoting effect of IL-1β in NSCLC cells

(A) Aspirin and Celecoxib abolished the inhibitory effect of IL-1β on mir-101 in A549 cells.

(B) Aspirin and Celecoxib abolished the stimulatory effect of IL-1β on Lin28B in A549 cells.

(C and D) Aspirin (C) and Celecoxib (D) abolished the inhibitory effect of IL-1β on let-7 miRNAs in A549 cells.

(E) Aspirin and Celecoxib reduced the stimulatory effect of IL-1β on proliferation and migration of
A549 cells.

( F ) Model of miR-101 as a key regulatory node linking inflammation to lung tumorigenesis.

The average values ± s.d. of three separate experiments were plotted. *P<0.05, **P<0.01. Results shown are representative of three independent experiments.
Figure 4

A

![Graph showing cell viability over time](image)

B

![Bar graph showing colony counts](image)

C

![Bar graph showing migration cell numbers](image)

D

![Graph showing tumor growth over time](image)
Figure 5

A summary of the data is presented in the form of a bar chart. The chart shows the distribution of miR-101 expression levels across different stages of cancer, with the percentage of each stage indicated. The stages are categorized as Stage I, Stage II, and Stage III.

In Figure 5B, the relative Lin28B mRNA levels are compared between normal and primary tumor tissues. The box plot indicates a statistically significant difference ($P < 0.01$) between the two groups.

Figure 5C further illustrates this with a scatter plot, showing a strong correlation ($R = 0.561$) between Lin28B expression and the level of miR-101. The correlation is significant at the 0.01 level ($P < 0.01$).

Figure 5D presents a comparison of Lin28B mRNA levels between low miR-101 and high Lin28B cases, with images depicting representative samples for each category.

In Figure 5E, the same comparison is made for high miR-101 and low Lin28B cases, with images illustrating the differences in protein expression.

The overall data supports the hypothesis that miR-101 and Lin28B expression levels are inversely correlated in cancerous tissues, with higher miR-101 levels associated with lower Lin28B expression.
IL-1β-mediated repression of microRNA-101 is crucial for inflammation-promoted lung tumorigenesis

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