A novel Inc-PCF promotes the proliferation of TGF-β1-activated epithelial cells by targeting miR-344a-5p to regulate map3k11 in pulmonary fibrosis

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Emerging evidence suggests that microRNA (miRNA) and long noncoding RNA (IncRNA) play important roles in disease development. However, the mechanism underlying mRNA interaction with miRNA and IncRNA in idiopathic pulmonary fibrosis (IPF) remains unknown. This study presents a novel Inc-PCF that promotes the proliferation of TGF-β1-activated epithelial cells through the regulation of map3k11 by directly targeting miR-344a-5p during pulmonary fibrogenesis. Bioinformatics and in vitro translation assay were performed to confirm whether or not Inc-PCF is an actual IncRNA. RNA fluorescent in situ hybridization (FISH) and nucleocytoplasmic separation showed that Inc-PCF is mainly expressed in the cytoplasm. Knockdown and knockin of Inc-PCF indicated that Inc-PCF could promote fibrogenesis by regulating the proliferation of epithelial cells activated by TGF-β1 according to the results of xCELLigence real-time cell analysis system, flow cytometry, and western blot analysis. Computational analysis and a dual-luciferase reporter system were used to identify the target gene of miR-344a-5p, whereas RNA pull down, anti-AGO2 RNA immunoprecipitation, and rescue experiments were conducted to confirm the identity of this direct target. Further experiments verified that Inc-PCF promotes the proliferation of activated epithelial cells that were dependent on miR-344a-5p, which exerted its regulatory functions through its target gene map3k11. Finally, adenovirus packaging sh-Inc-PCF was sprayed into rat lung tissues to evaluate the therapeutic effect of Inc-PCF. These findings revealed that Inc-PCF can accelerate pulmonary fibrogenesis by directly targeting miR-344a-5p to regulate map3k11, which may be a potential therapeutic target in IPF.

Cell Death and Disease (2017) 8, e3137; doi:10.1038/cddis.2017.500; published online 26 October 2017

The majority of genomes are transcribed using modern molecular biology techniques, such as deep sequencing; however, only 2% of the transcribed genome codes are attributed to proteins. The remaining part of the transcribed genome is known as noncoding RNA (ncRNA). ncRNAs can be divided into small (<200 nt) ncRNAs, such as microRNAs (miRNAs) and transfer RNAs, and long RNAs (>200 nt), such as long noncoding RNAs (lncRNAs) and ribosomal RNAs. Although miRNAs and IncRNAs play important roles in the development and progression of diseases, the interaction of mRNAs with miRNAs and lncRNAs to form an interrelated regulatory network in diseases remains unknown.

Some IncRNAs regulate the biological function of miRNAs by recruiting the miRNAs as competitive endogenous RNAs (ceRNAs). These IncRNAs generally exhibit the same miRNA response element with the transcripts of miRNAs. For instance, Inc-MD1 'sponges' miR-133 and miR-135 to regulate the expression of MAML1 and MEF2C, thereby activating muscle-specific gene expression. Inc-AF1 is identified as the ceRNA that can regulate autophagic cell death by targeting miR-188-3p and ATG7. Nevertheless, the mechanism underlying mRNA interaction with miRNA and IncRNA in idiopathic pulmonary fibrosis (IPF) remains unknown. IPF is defined as a specific form of chronic and progressive interstitial pulmonary fibrosis of an unknown cause, and this disease leads to the progressive loss of lung function, respiratory failure, and death. To date, lung transplant is the only effective treatment available for IPF. Thus, a comprehensive understanding of the molecular mechanisms of fibrogenesis is required in developing specific therapies toward this disease.

This paper is a follow-up to a previously completed research in our laboratory. In the previous study, we described the differentially expressed miRNAs, IncRNAs, and mRNAs in IPF; however, the molecular mechanisms of these RNAs are poorly elucidated. In the present work, we defined a novel Inc-PCF, its function, and the crosstalk between Inc-PCF and the map3k11 target miR-344a-5p in the regulation of fibrogenesis.

Results

BC158825 was verified as a novel IncRNA and highly expressed in pulmonary fibrosis. Our laboratory studies revealed the different expression levels of IncRNA in pulmonary fibrosis and normal lung tissues by using microarray analysis, and we also acquired transcript BC158825, which is significantly different and has higher expression than other transcripts. Therefore, we selected BC158825 for further study and renamed it as Inc-PCF.
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In cases, many predicted IncRNAs are not real IncRNAs because these IncRNAs can still encode proteins.12 To verify whether or not Inc-PCF is a real IncRNA, we further analyzed its full-length sequence obtained from the National Center for Biotechnology Information. Data showed that Inc-PCF is a Rattusnorvegicus linear cDNA (IMAGE: 7376599) with a length of 1208 bp (Supplementary Figure 1b). To prove that Inc-PCF cannot encode proteins, we divided the full-length sequence into six parts using the open-reading frame (ORF) finder database. Among the six parts, nine sequences indicated with a blue line were considered to possess protein-coding ability. However, none of these nine sequences contained >200 nucleotides, and the amino acid that corresponded to these sequences could not match any protein in the Pfam database (Supplementary Figure 1c). In addition, the protein-coding potential of Inc-PCF was evaluated by using the coding potential calculator tool (http://cpc.cbi.pku.edu.cn/) and the coding potential assignment tool (http://cpc.cbi.pku.edu.cn/) and the coding potential assignment tool (http://cpc.cbi.pku.edu.cn/). The protein-coding ability scores of Inc-PCF were -0.720785 and 0.157507, which indicated that Inc-PCF was devoid of protein-coding potential.13,14 Lnc-PCF translation activities were measured in an in vitro translation system, which revealed that this gene has no translation activities (Figure 1a). These analyses strongly confirmed the prediction in Inc-PCF microarray and verified that Inc-PCF is an actual IncRNA. Furthermore, the subcellular localization of Inc-PCF was detected by fluorescent in situ hybridization (FISH) and confirmed by quantifying nuclear/cytoplasmic RNA. The results showed that the Inc-PCF transcripts were more localized in the cytoplasm than in the nucleus (Figures 1b and c).

To further verify the microarray analysis result, we investigated the expression levels of Inc-PCF for 28 days at a 7-day interval in bleomycin (BLM)-induced animal model by using quantitative real-time PCR (qRT-PCR). The increasing expression level of Inc-PCF was observed at 14, 21, and 28 days, but the level decreased at 7 days (Figure 1d), which is considered an inflammatory phase and not fibrosis.15 Hydroxyproline (Hyp) is an indicator of pulmonary fibrosis. A significant difference was observed for Hyp level at the given time points compared with that in the sham group (Figure 1e). The highest Hyp expression was observed at 21 and 28 days. Pearson’s correlation coefficient was used to confirm the correlation of Inc-PCF and Hyp. Statistical analysis indicated that Inc-PCF is positively correlated with Hyp, thereby suggesting that Inc-PCF is correlated to the degree of pulmonary fibrosis (Figure 1f).

As the most important regulatory factor among fibrogenic cytokines,16,17 TGF-β1 was used to stimulate the rat lung epithelial-t-antigen negative (RLE-6TN) cells to confirm the results in vitro. Lnc-PCF was upregulated in TGF-β1-induced RLE-6TN cell model as compared with that in the normal group (Figure 1g). These experimental results confirmed the microarray analysis results, which stated that Inc-PCF is a confirmed IncRNA that is highly expressed in pulmonary fibrosis. These data suggested that Inc-PCF may play an important role in pulmonary fibrosis.

The interfering sequence of Inc-PCF (Inc-PCF smart silencer, knockdown Inc-PCF), which targeted six different sections of Inc-PCF, and the recombinant plasmid (pLenti-EF1a-EGFP-F2A-Puro-CMV-MCS) of the overexpressed Inc-PCF (RP-Inc-PCF, knockin Inc-PCF) were designed and transfected to RLE-6TN cells to investigate and confirm the function of Inc-PCF in pulmonary fibrosis. The efficiency of knockdown and knockin Inc-PCF was subsequently evaluated by qRT-PCR. The Inc-PCF smart silencer effectively interfered with Inc-PCF expression (Figure 1h). Meanwhile, RP-Inc-PCF induced high levels of Inc-PCF in RLE-6TN cells (Figure 1i).

Lnc-PCF promoted fibrogenesis in pulmonary fibrosis. During pulmonary fibrosis, the epithelial cells obtain mesenchymal characteristics, including the expression of mesenchymal markers, such as α-smooth muscle actin (α-SMA), vimentin, transcription repressor Snail, and loss of the epithelial cell marker E-cadherin.16,17 The protein levels of these markers were examined after incubation with TGF-β1 for 72 h under knockdown or knockin Inc-PCF by using immunofluorescence staining and western blot analysis to investigate the effect of Inc-PCF on fibrogenesis. Immunofluorescence data showed that the α-SMA level was significantly higher in the cells treated with TGF-β1 alone than in the other groups. Nonetheless, α-SMA expression reached lower levels in the cells treated with Inc-PCF smart silencer +TGF-β1 than in TGF-β1+NC group (Figure 2a). Western blot analysis further confirmed that the effects of Inc-PCF smart silencer on α-SMA expression (Figure 2b). Lnc-PCF smart silencer can reduce the expression levels of vimentin and Snail and increase that of E-cadherin (Figure 2b). By contrast, Inc-PCF overexpression can promote the expression levels of vimentin, Snail, and α-SMA and inhibit E-cadherin expression (Figures 2c and d). These findings indicated that knockin Inc-PCF could promote fibrogenesis, whereas knockdown Inc-PCF could block fibrogenesis in pulmonary fibrosis.

Lnc-PCF promoted the proliferation of epithelial cell activated with TGF-β1. Pulmonary fibrosis is a neoproliferative disorder of the lung and exhibits uncontrolled proliferation that is similar to the cell growth in cancer.20,21 Thus, we investigated the involvement of Inc-PCF in controlling cell proliferation during fibrogenesis. Growth curves showed that the Inc-PCF smart silencer inhibited the proliferation of the cells activated by TGF-β1 (Figure 3a). Western blot analysis performed on RLE-6TN cells showed that the levels of proliferating cell nuclear antigen (PCNA) protein were lower in the Inc-PCF smart silencer+TGF-β1 group than in TGF-β1+NC group (Figure 3b). Moreover, Inc-PCF overexpression significantly promoted cell proliferation and PCNA expression (Figures 3c and d). To further understand the role of Inc-PCF, we selected the cell cycle that was closely associated with cell proliferation to study the function of Inc-PCF during fibrogenesis.22,23 Flow cytometric analysis revealed that Inc-PCF smart silencer significantly increased the percentage of cells in the G1/G0 phase and decreased that in the S and G2/M phases as compared with
those in the TGF-β1-treated cells (Figures 3e and f). However, overexpressed lnc-PCF reduced the percentage of cells in the G1/G0 phase and increased that in the S and G2/M phases as compared with those in BP group (Figures 3g and h). These data indicated that interfering lnc-PCF expression could induce cell cycle arrest at the G1/G0 phase.

To confirm the above finding, we further evaluated the cell cycle relative proteins of cyclin E and cyclin B by western blot analysis. The Inc-PCF knockdown downregulated the expression of cyclin E, which is essential to allow the cells to pass through the G1/S checkpoint, and upregulated the expression of cyclin B, which was degraded after the G2/M phase (Figure 3i). The Inc-PCF knockdown can inhibit cyclin E activity and cyclin B degradation, which might inhibit the cells from passing through the checkpoint at the G1/S phase. In addition, the effect of Inc-PCF overexpression on RLE-6TN cell cycle was assessed. Inc-PCF overexpression could promote the cell cycle process in RLE-6TN cells (Figure 3j). All these results suggested that Inc-PCF can promote the proliferation of epithelial cells activated by TGF-β1 in pulmonary fibrosis.

**Lnc-PCF directly targeted miR-344a-5p.** Targeting miRNA is one of the regulatory functions of IncRNA. Therefore, the target miRNA for Inc-PCF was first predicted based on TargetScan, miRanda data and miRbase. Lnc-PCF possesses many predicted target miRNAs that are possibly the regulatory factors of Inc-PCF. Thus, the bioinformatics of lnc-PCF and predicted target miRNAs was used to evaluate their affinity to limit the selection. The selection rules are as follows: (1) in a 2D structure that contains the binding site position of 3’-UTR or full-length sequence of IncRNA, miRNA, and Inc-PCF should be completely paired. The binding type
includes 8mer, 7mer-m8, 7mer-al, 6mer, offset 6mer, and imperfect (nonstandard, mismatch or deficiency in G/U pair). Except for imperfect type, other types possess approximately the total number of paired nucleotides. Seed match is not required if the binding type is imperfect. (2) Many red bars that mark the AU weight located on both sides of the seed sequence are preferable. (3) Binding sites that are close to both sides are suitable. MiR-344a-5p, miR-138-5p, miR-370-3p, and miR-484 were selected for the study according to their relative high affinity between lnc-PCF and miRNAs (Supplementary Figure 2). These miRNAs at different time points were evaluated through qRT-PCR acquired from rat pulmonary tissues and RLE-6TN cells to investigate the association of lnc-PCF with the expression trend of these four miRNAs. On one hand, the expression trend of miR-138-5p, miR-370-3p, and miR-484 was not ideal. On the other hand, miR-344a-5p exhibited an opposing expression trend to lnc-PCF in vivo and in vitro (Figures 4a and b), thereby indicating its potential as a target for lnc-PCF. Thus, miR-344a-5p was selected for further study.

To investigate whether or not miR-344a-5p is targeted by lnc-PCF, we conducted dual-luciferase report system to construct a plasmid vector with full-length lnc-PCF containing wild-type (WT) and mutant-type (MT) 3′-UTR, which was behind two luciferases, namely, firefly and Renilla. The luciferase genetic testing report showed that miR-344a-5p overexpression suppressed the luciferase activity of the WT reporter vector but not that of the mutant reporter vector (Figure 4c). This result suggested that miR-344a-5p is a target gene for lnc-PCF.

RNA pull down, anti-AGO2 RNA immunoprecipitation (RIP), and rescue experiments were conducted to further verify the direct targeted relationship between Inc-PCF and miR-344a-5p. RNA pull-down was performed to detect the endogenous miR-344a-5p associated with lnc-PCF using transcribed biotin-labeled lnc-PCF in vitro. qPCR analysis revealed that miR-344a-5p was significantly enriched in miR-344a-5p as compared with that in non-targeting miR-708-3p (Figure 4d). Moreover, we induced anti-AGO2 RIP and transiently overexpressed miR-344a-5p in RLE-6TN cells. The endogenous lncRNA-ATB pulled down by AGO2 was specifically enriched in the miR-344a-5p-transfected cells (Figure 4e). To investigate the association of lnc-PCF with the expression trend of miR-344a-5p, we evaluated the level of miR-344a-5p at different time points through RT-PCR acquired from rat pulmonary tissues and TGF-β1-treated RLE-6TN cells. The miR-344a-5p overexpression (mimic) resulted in decreased lnc-PCF expression, and the suppression of miR-344a-5p (inhibitor) significantly enhanced the lnc-PCF expression as compared with that in the TGF-β1+NC group (Figure 4f). In addition, the miR-344a-5p expression levels increased when lnc-PCF was knocked down (Figure 4g) but was reduced by the knockin of lnc-PCF (Figure 4h). All these results revealed the direct targeted association between lnc-PCF and miR-344a-5p.

Lnc-PCF promoted the proliferation of epithelial cells dependent on miR-344a-5p. Given that Inc-PCF was confirmed to directly target miR-344a-5p, experiments were designed to verify whether or not Inc-PCF promotes the
proliferation of activated epithelial cells dependent on miR-344a-5p. The miR-344a-5p mimic and inhibitor were transfected into RLE-6TN cells. Cell proliferation profiles were determined by flow cytometry, western blot analysis, and real-time cell analysis system. Data showed that due to the upregulated miR-344a-5p (mimic) expression, the cells accumulated in G0/G1, and the percentage of cell accumulation in the S and G2/M phases is lower than that in the control (Figure 5a). Western blot analysis showed that the miR-344a-5p overexpression (mimic) could reduce cyclin E expression.
and increase cyclin B expression (Figure 5b). Cyclin E downregulation can prevent the cells from passing through the G0/G1 checkpoint and increase the expression of cyclin B, which was not degraded during G2/M because of G0/G1 retardation. miR-344a-5p mimic could reduce the growth of activated epithelial cells (Figure 5c) and PCNA (Figure 5d). By contrast, miR-344a-5p inhibitor increased the S and G2/M phase proportion, decreased the G0/G1 phase ratio, downregulated cyclin E, and upregulated cyclin B and PCNA, thereby activating epithelial cell proliferation (Figures 5e-h). We further used a rescue experiment (lnc-PCF overexpression + miR-344a-5p mimic) to detect whether or not the effect of lnc-PCF is dependent on miR-344a-5p. As shown in Figure 5i, the miR-344a-5p mimic rescued the function of lnc-PCF in lung fibrosis and the expression of E-cadherin expression, which was inhibited by lnc-PCF overexpression, and reduced the expression of α-SMA, vimentin, and Snail, which were promoted by Inc-PCF overexpression. All of these findings revealed that Inc-PCF promotes cell proliferation that is dependent on miR-344a-5p by directly targeting miR-344a-5p.

Identification of target-regulating mechanism in miR-344a-5p, map3k11, and Inc-PCF. miRNAs exert their regulatory functions through specific interactions with their target genes. Therefore, the miR-344a-5p target genes were predicted based on TargetScan, miRanda data, and miRbase. Among the list of miR-344a-5p target genes, we focused on map3k11 because it has relatively higher affinity than other genes and is an important indicator of the master regulatory factor in cell differentiation, proliferation, and individual development.24
Figure 5  Lnc-PCF promoted cell proliferation dependent on miR-344a-5p. (a) The miR-344a-5p mimic (MI) increased the percentage of cells in G0/G1, whereas the percentage of cells decreased in the S and G2/M phases in RLE-6TN cells. RLE-6TN cells were first transfected with 50 nM miR-344a-5p mimic/NC and cocultured with 5 ng/ml TGF-β1 for 72 h. The DNA of RLE-6TN cells was labeled with PI and analyzed by flow cytometry. (b) miR-344a-5p mimic downregulated cyclin E and upregulated cyclin B expression. (c) Proliferation analysis was performed using the RTCA system under the condition of miR-344a-5p mimic transfection. The miR-344a-5p overexpression evidently retarded cell proliferation in RLE-6TN cells. (d) PCNA protein levels were tested using western blot analysis. The miR-344a-5p mimic decreased the PCNA expression evidently. (e) miR-344a-5p inhibitor (IN) can increase the S and G2/M phase proportion and decrease the G0/G1 phase ratio. RLE-6TN cells were transfected with 50 nM miR-344a-5p inhibitor/NC before the cells were exposed to 5 ng/ml TGF-β1 treatment. (f) miR-344a-5p inhibitor downregulated cyclin E and increased cyclin B protein expression. (g) Proliferation analysis was performed using the RTCA system under the condition of miR-344a-5p inhibitor transfection. Silencing of miR-344a-5p evidently promoted cell proliferation in RLE-6TN cells. (h) PCNA protein level was tested by western blot analysis. The miR-344a-5p inhibitor evidently increased the PCNA expression. (i and j) miR-344a-5p mimic could rescue E-cadherin expression inhibited by lnc-PCF overexpression and decrease α-SMA, vimentin, and snail, which were promoted by lnc-PCF overexpression.
as a target gene of miR-344a-5p. The luciferase activity of the WT 3′-UTR–map3k11 was significantly decreased in cells transfected with miR-344a-5p mimics, whereas miR-344a-5p mimics could not inhibit the luciferase activities of the MU 3′-UTR–map3k11 (Figures 6a, 1–4). Thus, map3k11 is the target gene of miR-344a-5p.

In addition, cells were transfected with Inc-PCF WT/MT to confirm that Inc-PCF could protect map3k11 by competitively binding with miR-344a-5p (Figure 6a, 5–10). The miR-344a-5p mimic and inhibitor were transfected into pulmonary epithelial cells activated with TGF-β1 to further confirm the target relation between map3k11 and miR-344a-5p. Data showed that the miR-344a-5p mimic could inhibit map3k11 expression, whereas the miR-344a-5p inhibitor could promote map3k11 expression as compared with that in the TGF-β1-activated group (Figures 6b and c).

The map3k11 levels were inversely correlated with miR-344a-5p expression. Rescue experiments further supported the evidence that miR-344a-5p directly regulates map3k11 expression. Knockdown or knockin of Inc-PCF could significantly downregulate or upregulate the expression of map3k11, which was rescued by cotransfection with the miR-344a-5p inhibitor or mimic (Figures 6d–g). These results indicated that miR-344a-5p directly binds with map3k11, which consequently downregulates its expression level. This occurrence may imply that Inc-PCF competitively binds with miR-344a-5p to protect map3k11 from being degraded by miR-344a-5p, which results in the promotion of cell proliferation. In conclusion, the profibrotic function of Inc-PCF could be mediated by targeting map3k11 via miR-344a-5p.

**Therapeutic value of Inc-PCF in vivo.** An interfered sequence of Inc-PCF (sh-Inc-PCF) was synthesized,
packaged in adenovirus, and sprayed across rat lung tissues by using a Penn-Century MicroSprayer (Penn-Century Inc., Wyndmoor, PA, USA) to determine the potential of lnc-PCF as a therapeutic target in vivo. Fibrosis was evaluated using hematoxylin and eosin staining (H&E). The rats in the sh-lnc-PCF group presented a continuous bronchial mucous membrane structure with a more intact wall than those in the BLM group. The alveoli in the sh-lnc-PCF group showed clearer hollow cavities with thinner alveolar walls than those in the BLM group. In addition, the lung mesenchyme in the sh-lnc-PCF group displayed few collagen fibers, thereby indicating that the hallmark of the fibroblastic foci was distinctly decreased (Figure 7a). The BLM group had significantly increased lnc-PCF level compared with that in the sham group. Furthermore, sh-lnc-PCF could inhibit lnc-PCF expression in the sh-lnc-PCF group compared with that in the BLM group (Figure 7b).

To further investigate the antifibrotic action of sh-lnc-PCF in vivo, we tested the expression levels of E-cadherin, SP-C, α-SMA, collagen III, and vimentin, which are indicators of pulmonary fibrosis, after sh-lnc-PCF spraying in vivo. The sh-lnc-PCF could decrease the expression levels of α-SMA, collagen III, and vimentin, but increase those of E-cadherin and SP-C in the sh-lnc-PCF group as compared with those in the BLM group (Figures 7c–e).

Discussion

In contrast to the role of miRNA, the function of lncRNA is significantly unknown. Emerging evidence suggests that the changes in IncRNAs are associated with the development of various diseases. Several studies reported that IncRNA can act as ceRNA for miRNA and might be involved in physiological and pathological processes. Kallen et al. reported that the increased expression of paternally imprinted H19 IncRNA may act as a sponge for let-7, thereby explaining the downregulation of this miRNA in non-small-cell lung cancer types. However, whether IncRNAs interact with miRNAs to form an interrelated regulatory network in IPF remains unknown. In the current study, we presented a novel, highly expressed Inc-PCF that can promote the proliferation of activated epithelial cells by competitively binding to miR-344a-5p-targeted map3k11 in pulmonary fibrosis (Figure 8). Although targeting the liver-specific miR-122 by using an antisense-based approach is not related to IPF, this method is currently under human trials for the treatment of the hepatitis C virus. Our findings suggested that Inc-PCF is a significant cause of IPF and can be used as a potential therapeutic target through different approaches, such as antisense oligonucleotides and short interfering RNAs.

The present study is a follow-up to a prior work in our laboratory, which revealed the differential expression of IncRNAs, analyzed the relationship between IncRNA and protein-coding gene, and identified the role of IncRNA as ceRNA in pulmonary fibrosis. In the current study, Inc-PCF was identified as a highly expressed IncRNA associated with IPF development. Our result is consistent with several reports, which posited that the abnormally expressed IncRNAs are closely related to several important fibrotic
diseases and can influence the expression and intracellular distribution of specific proteins.\textsuperscript{32–34} IncRNAs exhibit a distinct expression pattern both in time and space and are highly expressed in specific cells where they are activated.\textsuperscript{35} The number of foreseen IncRNA transcripts may even exceed the number of protein-coding mRNAs in mammals; thus, a growing body of evidence involved in IncRNA-based studies focuses on the pathogenesis of pulmonary fibrosis.\textsuperscript{36,37} Although the ability to predict IncRNA was facilitated with genomic sequencing and bioinformatics analyses, this assumptive gene must be verified. In the present study, we combined microarray analysis and gene data bank and identified the sequence, ORF, locations on the chromosome, and translation activities of this gene \textit{in vitro}. We concluded that BC158825 is a sense IncRNA, which is referred to as Inc-PCF. Our results showed that Inc-PCF is highly expressed \textit{in vivo} and \textit{in vitro}, which is consistent with our microarray analysis.

IPF is a severe disease that is characterized with myofibroblast proliferation. The transition of epithelial cells into myofibroblast is one of the main sources of myofibroblasts. In this process, epithelial cells lose their epithelial phenotype, acquire myofibroblast-like properties, and exhibit decreased cell adhesion and increased motility. This process is accompanied by an increase in pulmonary fibrosis markers, such as $\alpha$-SMA, collagen, vimentin, and Snail, and has also gained considerable research attention in the past few years as a potential contributor to pulmonary fibrosis.\textsuperscript{38–40} Our data showed that Inc-PCF could stimulate the proliferation of myofibroblast derived from epithelial transition by facilitating the driving cell cycle progression. Nevertheless, the mechanism of Inc-PCF to regulate the transformation of epithelial cells into myofibroblast remains unknown. Different types of RNAs communicate with one another via their targeted miRNAs. This regulatory mode is involved in oncogenesis and cancer progression.\textsuperscript{41–44} However, investigations on this regulatory mode were performed almost exclusively in non-small-cell lung cancer in respiratory diseases.\textsuperscript{30} Therefore, we further investigated the ability of Inc-PCF to regulate mRNA through miRNA in pulmonary fibrosis. In this study, IncRNA-miRNA, miRNA–mRNA, and mRNA–IncRNA interactions were thoroughly surveyed, identified, and detected. Systematic and integrative analyses of different RNA molecules with potential cross-talk may significantly contribute in determining the complex mechanisms underlying pulmonary fibrosis. Test screening and data analysis results showed that miR-344a-5p

![Lnc-PCF promotes pulmonary fibrosis](image-url)
is one of the directly targeted miRNAs by Inc-PCF, and map3k11 is a target gene of miR-344a-5p. Lnc-PCF can regulate map3k11 by targeting miR-344a-5p to promote the proliferation of activated epithelial cells, which results in pulmonary fibrosis.

According to a clinical perspective, ncRNA targeted as a novel therapeutic approach will require a thorough understanding of their function and mechanism of action. In addition, the changes in the expression levels of miRNA and IncRNA can be used as biomarkers for disease strategy and/or assessment of drug action. In general, our study on the regulation of the activated epithelial cell proliferation in pulmonary fibrosis by Inc-PCF and miR-344a-5p-targeted map3k11 introduces a new approach to examine the complex post-transcriptional regulatory networks and present new therapeutic approaches to pulmonary fibrosis.

Materials and Methods

Animal model and treatment. Sprague–Dawley rats with a mean weight of 200 g were obtained from the Green Leaf Experimental Animal Center (Yantai, China) and maintained according to the regulation approved by the Institutional Animal Ethics Committee of Binzhou Medical University. Pulmonary fibrosis was induced with a single intratracheal instillation of BLM dissolved in saline as previously described. The normal group was administered with an equivalent volume of saline. Lung tissues were collected on 7, 14, 21, and 28 days following BLM treatment.

The interfered sequence of Inc-PCF was packaged in the adenovirus. The treatment of adenovirus packaging interfered with the sequence of Inc-PCF was as follows: the rats were randomly divided into the following four groups (10 rats each): sham, BLM-treated (BLM), BLM-empty vector, and BLM+sh-Inc-PCF. The adenovirus packaging interfered with Inc-PCF sequence was sprayed into the rat lung tissues by using a Penn-Century MicroSprayer (Penn-Century Inc.). All rats were killed on day 28. Lung tissues sections were collected and immediately frozen in liquid nitrogen for further studies.

Cell culture. RLE-6TN cell line was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and maintained in DMEM medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum and 80%.

RNA transfection. Lnc-PCF smart silencer, miR-344a-5p mimic, and miR-344a-5p inhibitor were purchased from RiboBio Co., Ltd. (Guangzhou, China). The RLE-6TN cells were plated in six-well culture dishes for 2×10⁵ cells per well) until the cell density reached 70–80% prior to small RNA transfection. On the following day, the cells were transfected with 50 nM small RNA accompanied with reagent buffer using a Lipofectamine 2000 (Invitrogen, Carlsbad, CA) transfection kit. After 48 h, the complete medium was removed, and the cells were rinsed using 100 μl of the medium was removed, and 25 μl of miR-344a-5p transfection mixture were added to 200 μl of transfection regents and incubated for 5 min. Afterward, we added 1 μl of transfection reagents and were incubated for 5 min.

Flow cytometry analysis. Approximately 1×10⁵ cells were collected and resuspended in 500 μl of 70% cold ethanol, which was added dropwise under gentle vortexing. The cells were fixed overnight at 4 °C, collected by centrifugation, washed once in PBS, collected again, and stained with propidium iodide (PI) staining solution, which was composed of 20 μg/ml PI, 200 μg/ml RNAse A, and 0.1% Triton X-100 at 37 °C. The DNA contents of the stained cells were analyzed using a flow cytometer.

Dual-luciferase reporter assay. Vector construction: we used the forward-forward primer (5′-GAGGATCTGTTTGGTATGC-3′) and reverse-forward primer (5′-TGTAAAGCAGGCGGACG-3′) to obtain the Inc-PCF sequence. Restriction enzymes, namely, Mla and HindII, were used to sever the Inc-PCF sequence at AAGCTT and ACCGCT, respectively. The digested Inc-PCF was inserted into the rear of the luciferase reporter genes (firefly and Renilla) in pmirGLO-resistant Amp resistance. Plasmid-transfected 293 T cells: 293 T cells were cultivated in a 96-well plate at a density of 2×10⁵well. After 24 h, Inc-PCF and transfection reagents were prepared using 0.2 μg of firefly, 0.01 μg of Renilla, and 0.25 μl of transfection reagents and were incubated for 5 min. MR-344a-5p transfection reagents were prepared at the final density of 100 nM miRNA and 0.25 μl of transfection regents and incubated for 5 min. Afterward, we mixed Inc-PCF and miR-344a-5p with their transfection reagents and incubated the mixtures for 20 min. About 50 μl of the medium was removed, and 25 μl of Inc-PCF transfection mixture and 25 μl of Mr-344a-5p transfection mixture were added to the cultured cells. After transfection for 6 h, the transfection mixture was replaced with a new complete medium. Dual-luciferase reporter assay: after cotransfection for 48 h, the complete medium was removed, and the cells were rinsed using 100 μl of PBS, which was subsequently replaced with 1× passive lysis buffer to lyse the cells. The mixture was shaken for 15 min. Each well was added with 100 μl of Stop&Glo reagent for 2 s to obtain test data under dark condition.

RNA pull-down. Cloning of the Inc-PCF sequence into Pgen-3z vector: the Inc-PCF sequence was ligated into multiple cloning sites located between the T7 promoter (5′-TAATACGACTCACTATAGGG-3′) and SP6 promoter (5′-AATATTGATGACACTATAGAA-3′). Approximately 5 μl of restriction endonuclease was added to 20 μg of plasmid. The total volume was adjusted to 50 μl by using H2O. The mixture was incubated at 37 °C from 2 h to overnight. The linearized SYBR green-based PCR Master Mix Kit (TaKaRa, Shiga, Japan) on a Rotor Gene 3000 RT-PCR system of Corbett Research (Sydney, NSW, Australia). The PCR reaction system for miRNA was as follows: initial denaturation at 95 °C for 20 s and 30 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 10 s. For IncRNA, the reaction system was as follows: initial denaturation at 95 °C for 30 s and 35 cycles of 95 °C for 5 s and 60 °C for 20 s. The threshold cycle was determined after the reactions. The relative miRNA and IncRNA expression levels were calculated based on Ct values and were normalized to the U6 or GAPDH levels of each sample, respectively.

Immunofluorescence staining. Approximately 2×10⁵ RLE-6TN cells were cultivated on the sterile slides in a 24-well plate. After small RNA transfection and TGF-β1 treatment, these cells were rinsed with cold phosphate-buffered saline (PBS) for three times and fixed in 4% paraformaldehyde for 1 h. The cell lines were rinsed with PBS for three times, incubated with 0.5% Triton X-100 for 15 min at room temperature, and blocked with 10% normal goat serum for 1 h at 37 °C. Afterward, α-SMA antibody (1 : 300) was added dropwise into each slide at 4 °C overnight. RLE-6TN was rinsed with PBS for three times and incubated with fluorescein-labeled immunoglobulin G (lgG) antibody (1 : 200) α-SMA (FITC-labeled) at 37 °C for 1 h in a wet box. All operating steps were conducted in the dark because fluorescent secondary antibodies were added. After rinsing with PBS for three times, the nuclei were stained with DAPI (Roche Molecular Biochemicals, Basel, Switzerland) for 5 min at room temperature. Immunofluorescence was analyzed under a fluorescence microscope.

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plasmid was purified using the QiAquick Gel Purification Kit. After purification and size check of RNA, the prepared denatured RNA gel was used to confirm whether or not the RNAs were transcribed at the right size. Sufficient cells were cultured and collected at a minimum of 10 cm × 10 cm plate for each pull down. The cell pellet was suspended using 1 ml of ProteaPrep2xwitterionic cell lysis buffer supplemented with protease and phosphatase inhibitor cocktails (1:100), anti-RNase (10 U/μl), panbrocinostat (1:100), and molybdatat (1:100). The tube was then incubated on ice for 40 min and vortexed every 10 min. The mixture was centrifuged at 14,000 g for 15 min at 4 °C, and the supernatant was transferred to a new tube for further use. Afterward, 50 μl of beads were transferred to a fresh tube. The tube was placed on a magnetic separator for 1 min, and the supernatant was removed. After clearing the lysate with activated beads to reduce nonspecific binding, 20 μg of biotinylated RNA was heated to 90 °C for 2 min. The mixture was chilled on ice for 2 min and briefly centrifuged. The total volume was adjusted to 100 μl by using the RNA structure buffer. The mixture was incubated at room temperature for 20 min to allow proper secondary structure formation. About 50 μl of activated avidin magnetic beads were also prepared and were immediately subjected to RNA (20 μg) capture in RNA capture buffer for 30 min at room temperature with gentle agitation. The RNA capture buffer was removed. The RNA-captured beads were washed once with NT2 buffer and incubated with 30 mg pre-cleared cell lysate for 2 h at 4 °C with gentle rotation. The tube was centrifuged and placed on a magnetic separator. The supernatant was removed from the beads and discarded. The RNA-binding protein complexes were washed with NT2 buffer twice, NT2 high-salt buffer (500 mM NaCl) twice, NT2 high-saltbuffer (1 M NaCl) once, NT2 high-saltbuffer (750 mM KSCN) once, and PBS twice. The beads were eluted by incubating the sample with elution buffer for 20 min at 4 °C with frequent agitation, and the elute was collected. The elution step was repeated, and the elutes were pooled together. PCR was used to test the samples.

RIP experiment. RIP experiment was performed using an EZ Magna RIP kit (Millipore, Billerica, MA, USA) following the manufacturer’s protocol. The cells were lysed in complete RIP lysis buffer. The extract was incubated with Ag2 antibody or control IgG (Millipore, Billerica, MA, USA) following the manufacturer’s protocol. The cell lysate with activated beads to reduce nonspecific binding, 20 μg of pre-cleared cell lysate for 2 h at 4 °C with gentle agitation. The samples were added, the mixture was gently mixed by pipetting. If necessary, the mixture was cleared once, and PBS twice. The beads were eluted by incubating the sample with elution buffer for 20 min at 4 °C with frequent agitation, and the elute was collected. The elution step was repeated, and the elutes were pooled together. PCR was used to test the samples.

In vitro translation assay. The T7-BC158825 sample DNA was prepared by PCR using the following primers: T7-T7-BC158825-F: 5'-ACCGCCCTAACTACGTCGCTATAGGGACCTCCACCTGCA TGACCCTGGC-3', T7-BC158825-R: 5'-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
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