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Original Article

Doublecortin-like kinase 1 expression is induced by alternative NF-κB signaling in human lung cancer cells

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Abstract

Idiopathic pulmonary fibrosis (IPF), a devastating fibrotic lung disease of unknown etiology, is frequently associated with lung cancer. However, the molecular mechanism underlying this association remains unclear. We analyzed the gene expression profiles of IPF lungs using public datasets and extracted 94 genes that were upregulated in all of them. Among these, we identified *DCLK1*, a well-known cancer stem cell-marker. A recent study revealed that DCLK1 enhances cancer stem cell-like features in lung cancer cells. Therefore, if DCLK1 expression is induced in IPF, it could be a molecular link between IPF and lung cancers. In this study, we confirmed that DCLK1 expression was enhanced in human IPF lungs and in lungs of mice with bleomycin-induced fibrosis. We also found that the human lung cancer H1299 cells expressed DCLK1 when exposed to the conditioned medium derived from the lipopolysaccharide-stimulated murine macrophage-like RAW264.7 cells. Further, this DCLK1-inducing activity was sensitive to heat inactivation and proteinase K treatment. We also revealed that IL17 and lymphotoxin-α induced DCLK1 expression in human lung cancer H1299 cells. Moreover, *RELB* silencing, but not *RELA* silencing, blocked the induction of DCLK1 expression by conditioned medium. Hence, the inhibition of alternative NF-κB signaling may be useful to prevent cancer development in IPF lungs.

Key Words: Doublecortin-like kinase 1, Idiopathic pulmonary fibrosis, IL17, Lymphotoxin-α, NF-κB, RELA, RELB

Abbreviations: BIF, bleomycin-induced pulmonary fibrosis; DCLK1, Doublecortin-like kinase 1; IPF, idiopathic pulmonary fibrosis; LPS, lipopolysaccharide; LTA, lymphotoxin-α; PBS, phosphate-buffered saline; qRT-PCR, quantitative reverse transcription-PCR; UIP, usual interstitial pneumonia.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive fibrotic lung disease of unknown etiology[1-3]. IPF shows pathological features of usual interstitial pneumonia (UIP) and is diagnosed using high-resolution computer tomography and lung biopsy after excluding UIP of known causes. In the IPF lung, increased parenchymal fibrosis and destruction of alveolar architecture result in lung function deterioration and ultimately respiratory failure. The incidence of lung cancer is high in IPF patients[4]. The IPF lung exhibits genetic and epigenetic alterations that are associated with lung cancer. Therefore, common pathways may play a pathogenic role in both these diseases, or the genetic and epigenetic changes caused by IPF may contribute to cancer development[5].

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To identify the molecular link between IPF and lung cancer, we analyzed three microarray gene expression datasets obtained from public repositories of patients with IPF. *DCLK1* was upregulated in all three datasets. Its product, doublecortin-like kinase 1 (DCLK1), was initially identified as a brain protein with two doublecortin (DCX) domains and a serine/threonine protein kinase domain[6, 7]. DCLK1 attaches to microtubules through the DCX domains and regulates microtubule-polymerization[8]. DCLK1 is involved in neuronal migration, axon transport, growth cone regulation, dendritic remodeling, and brain development[9–12]. Outside the brain, DCLK1 was first believed to be a marker of quiescent gastrointestinal stem cells and later reported in postmitotic tuft cells, pancreatic progenitor cells and colon stem cells[13–16]. Moreover, experiments using transgenic and knock-in mice revealed that DCLK1-expressing cells initiate cancer and DCLK1 is a marker of colon cancer stem cells[17, 18]. Pancreatic progenitor cells express DCLK1 and generate cancer-initiating cells[19, 20]. In non-small cell lung cancer, DCLK1 protein expression correlates with poor prognosis[21, 22]. microRNAs that target and down-regulate *DCLK1* function as tumor suppressors[21, 22]. More importantly, a recent study demonstrated that DCLK1 confers cancer stem cell-like properties to lung adenocarcinoma cells[23]. Therefore, although there is no direct evidence supporting the implication of DCLK1 in IPF-associated oncogenesis, DCLK1 may be a molecular link between IPF and lung cancers.

In this study, we addressed two questions: whether DCLK1 expression is induced in IPF lungs, and if this is the case, what molecular mechanism underlies the induction. First, we confirmed that the protein expression of DCLK1 was enhanced in human IPF lungs and in a mouse model of bleomycin-induced pulmonary fibrosis (BIF). Second, we revealed that DCLK1 expression is mediated through alternative NF-κB signaling. Hence, our study outcomes suggest that inhibition of alternative NF-κB signaling could prevent cancer development in IPF lungs.

Materials and Methods

Antibodies and reagents

Antibodies and reagents were obtained from commercial sources; rabbit monoclonal anti-DCAMKL1 (DCLK1) (ab-109029) (Abcam); rabbit monoclonal anti-NF-κB p65 (RelA) (C22B4; 4764) (Cell Signaling Technology); mouse monoclonal anti-IL17 (G-4; sc-374218), mouse monoclonal anti-TNFβ (LTA) (E-6; sc-28345) and mouse monoclonal anti-RELB (D-4; sc-48366) (Santa Cruz Biotechnology); rabbit anti-α-tubulin (PM054) and rabbit anti-β-actin (PM053) (Medical and Biological Laboratories Co); rabbit polyclonal anti-histone H1.2 (19649-1-AP) (Proteintech Group Inc.); peroxidase-conjugated goat anti-mouse (0855550) (MP Biomedicals); bleomycin sulfate (B3972) (Tokyo Chemical Industry Co. Ltd.); lipopolysaccharide (LPS) (20389-04) and RNase A (318-06391) (Nippon Gene); recombinant murine IL17A (210-17) and recombinant human α (300-01B) (PeproTech); DNase I (4716728001) (Merck); JSH-23 (15036), GYY4137 (13345), N-acetyl-5-Aminosalicylic Acid (N-Ac-5ASA) (27618) (Cayman Chemical); and Proteinase K (840107277-62) (Boehringer Mannheim).

Cell cultures

H1299 and RAW264.7 cells were cultured in Dulbecco's Modified Eagle Medium (4.5 g/L glucose) (Nacalai Tesque) containing 10% fetal bovine serum and 15 mM Hepes-NaOH pH 7.4 under 5% CO_2 at 37°C. Cell authentication was performed using the short tandem repeat analysis by JCRB Cell Bank (National Institutes of Biomedical Innovation, Health and Nutrition) for H1299 cells. RAW264.7 cells were a gift from Dr. Kohei Miyazono (The University of Tokyo). Mycoplasma contamination was tested using an e-Myco Mycoplasma PCR Detection Kit (iNtRON Biotechnology).

Preparation of conditioned medium

RAW264.7 and H1299 cells were plated at 4 x 10^6 cells/plate and 2 x10^6 cells/plate in 10-cm plates with or without 0.1 mg/L LPS, respectively. After 24 h, the medium was collected and used as the conditioned medium. To destroy DNA, RNA, and proteins, 1 mL of the conditioned medium was incubated with 100 mg/L DNase I, 100 mg/L RNase A, and 50 mg/L Proteinase K for 30 min at 37 °C. For heat inactivation, the medium was incubated at 96 °C for 30 min.

Quantitative reverse transcription-PCR (qRT-PCR)

Total RNA was extracted using TRI reagent and converted to cDNA by ReverTra Ace® (Toyobo Life Science). qRT-PCR was performed using SYBR Green (Roche) and ABI7500 Real-Time PCR system (Applied Biosystems). The primers used were as follows; 5'-ttcaacacaggccccaag-3' and 5'-tatcaagagcggtggttgc-3' for mouse *Dclk1*; 5'-aactttggcattgtggaagg-3' and 5'-acacattgggggtaggaaca-3' for mouse glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). The quantities of *Gapdh* and *Dclk1* from mouse lungs were obtained by the standard curve method. The quantity of *Dclk1* was calibrated by that of *Gapdh* for each sample. The calibrated value for

Table 1. Summary of three microarray datasets

	GSE10667 (2008)	GSE24206 (2011)	GSE53845 (2013)
Patients	Male 49 Female 25 Total 74 *23 IPF samples were used for microarray analysis. 8 samples indicate acute exacerbations.	Male 8 Female 3 Total 11	Male 33 Female 7 Total 40
Age	65.9+/-9.4	63.4+/-5.4	70 (50–87)
Ever Smoker	78%	Not described	68%
Treatment	47 untreated	Not described	Not described
Sampling	Not described	Biopsy 6 Explant 5	Biopsy 11 Explant 29
Platform for microarray	Agilent-014850 Whole Human Genome Microarray 4 x 44K G4112F	Affymetrix Human Genome U133 Plus 2.0 Array	Agilent-014850 Whole Human Genome Microarray 4 x 44K G4112F

each sample from BIF lungs was divided by the mean value obtained from control lungs and described as the relative mRNA amount. Statistical analyses were performed with a two-sided Student's *t*-test using R ver. 4.0.2 with RStudio ver. 1.3.959 (https://rstudio.com/).

RNA interference

Human *DCLK1* silencing experiments were performed using Lipofectamine RNAiMAX (Life Technologies) with siRNA *DCLK1* (s17586) (Ambion), siRNA *RELA* (sc-29410) (Santa Cruz Biotechnology), siRNA *RELB* (sc-36402) (Santa Cruz Biotechnology) and Silencer Select Negative Control No. 2 siRNA (Ambion).

Animal experiments

Animal experiments were approved by the Tokyo Medical and Dental University Animal Care and Use Committee (A2018-191C2) and performed according to guidelines set by the committee. 10-week-old Male C57BL/6 mice were purchased from CLEA Japan, Inc. Bleomycin solution (2 g/L in phosphate-buffered saline (PBS)) was intratracheally injected at 5 mg/kg. Control mice were injected with PBS. Mice were sacrificed on day 14.

Histochemistry

The study design of using human pathology archives has been approved by the IRB of Hamamatsu University School of Medicine (20-011 for HS). IPF was clinically diagnosed and pathologically confirmed by two pathologists. Paraffin-embedded sections were immunostained with anti-DCAMKL1 (DCLK1) (ab-109029) (Abcam) after antigen retrieval and then colorization was performed using 3,3'-diaminobenzidine and the Envision+ system (DakoCytomation). Mouse lungs were fixed by intratracheal injections of 4% (w/v) paraformaldehyde in PBS. Specimens were embedded in paraffin and cut into 10-μm thick sections. Sections were incubated with anti-DCLK1 antibody (1 : 100) at 4 °C overnight. Visualization was performed using R.T.U. Universal Elite ABC Kit (Vector Laboratories).

Immunoblotting

Immunoblotting was performed using peroxidase-conjugated secondary antibodies, ClearTrans® nitrocellulose membrane, 0.2 μm (Wako Pure Chemical Industries, Ltd.), Amerscham™ ECL™ Western Blotting Detection Reagents (GE Healthcare UK Ltd.), Chemi-Lumi One Ultra (Nacalai Tesque), and FUJI MEDICAL X-RAY FILM, Super RX (Fujifilm).

Results

DCLK1 gene expression is enhanced in human IPF lungs

We began this study with the analysis of three independent microarray datasets (GSE10667, GSE24206, and GSE53845) obtained from human IPF lungs. We selected 94 genes that were enhanced in all three datasets (Table 1 and Table 2)[24–26]. The major groups consisted of extracellular matrix-related genes encoding collagens, periostin, and metalloproteinases (26 out of 94) and cytokine/growth factor-related genes encoding CXCL2, IGF1, TGFβ-binding protein, receptors for IL13 and IL17 (12 out of 94) (Table 1). Cell adhesion molecules, transcription factors, cytoskeleton regulators, and metabolic enzymes were also listed. In addition, *DCLK1*, a well-known cancer stem cell marker, was enhanced in all three datasets.

Table 2. The genes enhanced in human IPF lung

Gene Name	Description	Gene Name	Description	Gene Name	Description
ALDH1A3	Aldehyde dehydrogenase 1 family member A3	EPHA3	EPH receptor A3	PAMR1	Peptidase domain containing associated with muscle regeneration 1
AQP5	Aquaporin 5	FBLN2	Fibulin 2	PCDH7	Protocadherin 7
ASPN	Asporin	FERMT1	Fermitin family member 1	PDLIM4	PDZ and LIM domain 4
BCL11A	B-cell CLL/lymphoma 11A	FHL2	Four and a half LIM domains 2	PGM2L1	Phosphoglucomutase 2 like 1
BHLHE22	Basic helix-loop-helix family member e22	FNDC1	Fibronectin type III domain containing 1	PLN	Phospholamban
BICC1	BicC family RNA binding protein 1	FRMD6	FERM domain containing 6	POSTN	Periostin
C12orf75	Chromosome 12 open reading frame 75	GOLM1	Golgi membrane protein 1	PROM2	Prominin 2
CCDC80	Coiled-coil domain containing 80	HS6ST2	Heparan sulfate 6-O-sulfotransferase 2	PRSS2	Protease, serine 2
CD24	CD24 molecule	IGDCC4	Immunoglobulin superfamily DCC subclass member 4	PSD3	Pleckstrin and Sec7 domain containing 3
CDCA7	Cell division cycle associated 7	IGF1	Insulin like growth factor 1	ROBO1	Roundabout guidance receptor 1
CDH3	Cadherin 3	IGFBP4	Insulin like growth factor binding protein 4	S100A2	S100 calcium binding protein A2
CFB	Complement factor B	IL13RA2	Interleukin 13 receptor subunit alpha 2	SCG5	Secretogranin V
CFH	Complement factor H	IL17RD	Interleukin 17 receptor D	SERPIND1	Serpin family D member 1
CLIC6	Chloride intracellular channel 6	ITGB8	Integrin subunit beta 8	SFRP2	Secreted frizzled related protein 2
COL10A1	Collagen type X alpha 1 chain	KCNMA1	Potassium calcium-activated channel subfamily M alpha 1	SFRP4	Secreted frizzled related protein 4
COL14A1	Collagen type XIV alpha 1 chain	KIAA1211	KIAA1211	SLAMF7	SLAM family member 7
COL15A1	Collagen type XV alpha 1 chain	KRT17	Keratin 17	SLC4A11	Solute carrier family 4 member 11
COL1A1	Collagen type I alpha 1 chain	LAMP5	Lysosomal associated membrane protein family member 5	SPATA18	Spermatogenesis associated 18
COL1A2	Collagen type I alpha 2 chain	LDLRAD4	Low density lipoprotein receptor class A domain containing 4	ST6GALNAC1	ST6 N-acetylgalactosaminide alpha-2,6-sialyltransferase 1
COL3A1	Collagen type III alpha 1 chain	LRRC17	Leucine rich repeat containing 17	SULF1	Sulfatase 1
COL8A2	Collagen type VIII alpha 2 chain	LRRN1	Leucine rich repeat neuronal 1	SYNPO2	Synaptopodin 2
COMP	Cartilage oligomeric matrix protein	LTBP1	Latent transforming growth factor beta binding protein 1	TDO2	Tryptophan 2,3-dioxygenase
CPXM2	Carboxypeptidase X, M14 family member 2	MDK	Midkine (neurite growth-promoting factor 2)	THBS2	Thrombospondin 2
CTHRC1	Collagen triple helix repeat containing 1	MFAP2	Microfibrillar associated protein 2	THY1	Thy-1 cell surface antigen
CTSE	Cathepsin E	MMP10	Matrix metallopeptidase 10	TMEM176B	Transmembrane protein 176B
CTSK	Cathepsin K	MMP16	Matrix metallopeptidase 16	TP63	Tumor protein p63
CXCL12	C-X-C motif chemokine ligand 12	MMP7	Matrix metallopeptidase 7	TRIM2	tripartite motif containing 2
CXCL13	C-X-C motif chemokine ligand 13	MOXD1	Monooxygenase DBH like 1	TSHZ2	Teashirt zinc finger homeobox 2
CXCL14	C-X-C motif chemokine ligand 14	MXRA5	Matrix remodeling associated 5	TSPAN11	Tetraspanin 11
DCLK1	Doublecortin like kinase 1	NHS	NHS actin remodeling regulator	VSIG1	V-set and immunoglobulin domain containing 1
DIO2	Deiodinase, iodothyronine, type II	NTN1	Netrin 1	ZNF521	Zinc finger protein 521
ECM2	Extracellular matrix protein 2				

We employed three DNA array data available from National Center for Biotechnology information to identify the genes up-regulated in human IPF lungs. 94 genes increased 2-fold in all the three data sets. Genes related to extracellular matrix and to cytokines and growth factors are shown in green and blue, respectively.

DCLK1 protein expression is enhanced in human IPF lungs

To confirm the IPF-associated enhancement of DCLK1 at the protein level, we immunostained DCLK1 in human IPF lung and found that the DCLK1 expression was augmented in the alveolar epithelial cells (**Fig. 1**).

DCLK1 protein and *Dclk1* gene expression are enhanced in mouse BIF lung

Mouse BIF is widely used as a model of IPF[27–31]. We next examined whether DCLK1 protein expression increased in the BIF lung. Immunofluorescence and immunoblotting of the BIF lung demonstrated the increased protein level of DCLK1 (**Fig. 2A and Fig. 2B**). As shown by qRT-PCR, the level of *Dclk1* mRNA also significantly increased (**Fig. 2C**).

Conditioned medium from LPS-treated murine macrophage-like RAW264.7 cells induces DCLK1 expression in human lung cancer H1299 cells

The above findings support the hypothesis that DCLK1 expression is enhanced in human IPF lung as well as in mouse BIF lung through the upregulated gene transcription. In IPF, fibrosis is regarded as an outcome of aberrant wound healing in response to recurrent lung injury[1, 32]. Macrophages play key roles in tissue repair and fibrosis[33, 34]. Pulmonary macrophages have attracted attention as potential therapeutic targets in IPF[35]. Therefore, we speculated that cytokines derived from pulmonary macrophages may induce DCLK1 expression in IPF. To determine the molecular mechanism underlying induction of DCLK1, we exposed human lung adenocarcinoma H1299 cells to conditioned medium from

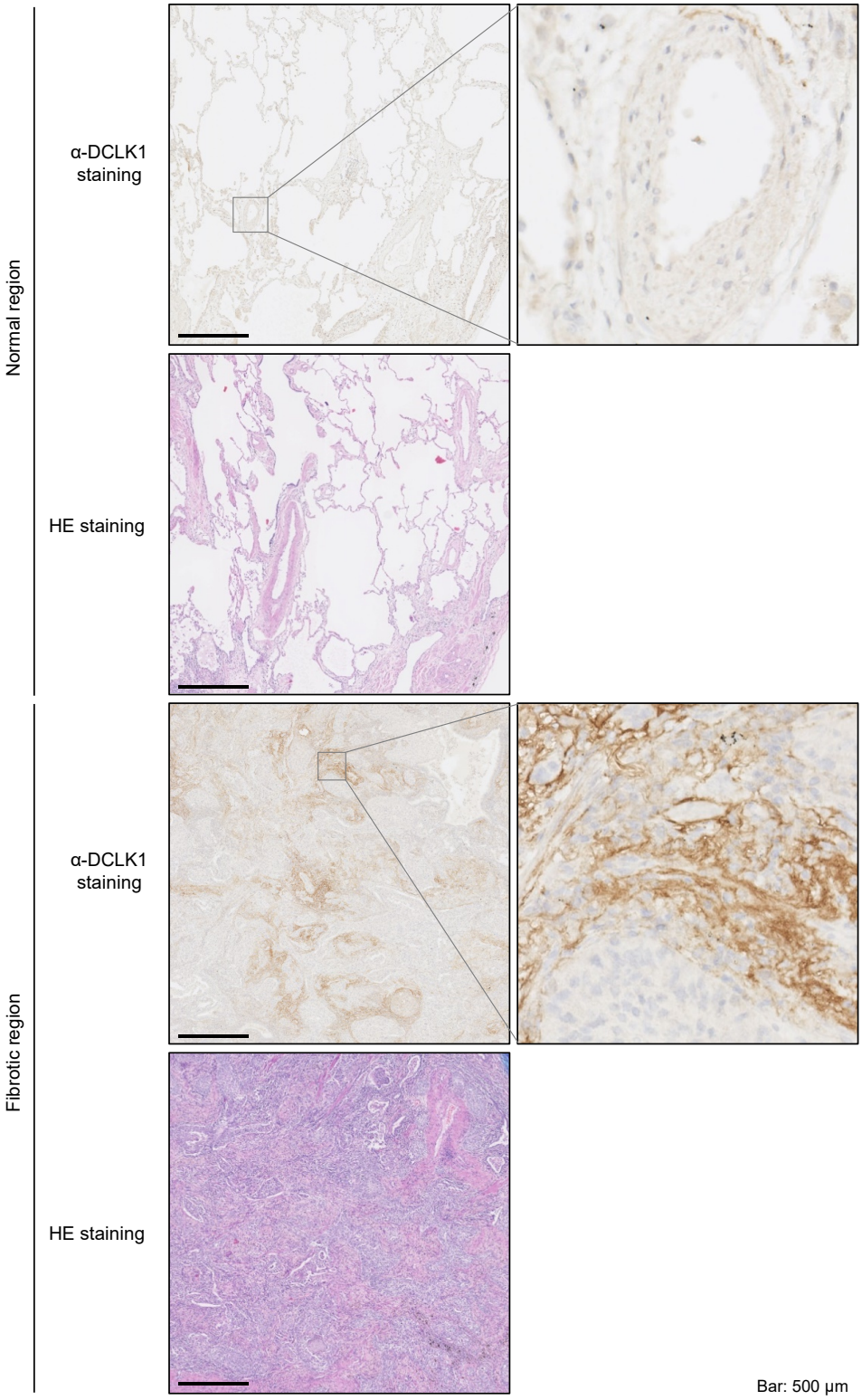
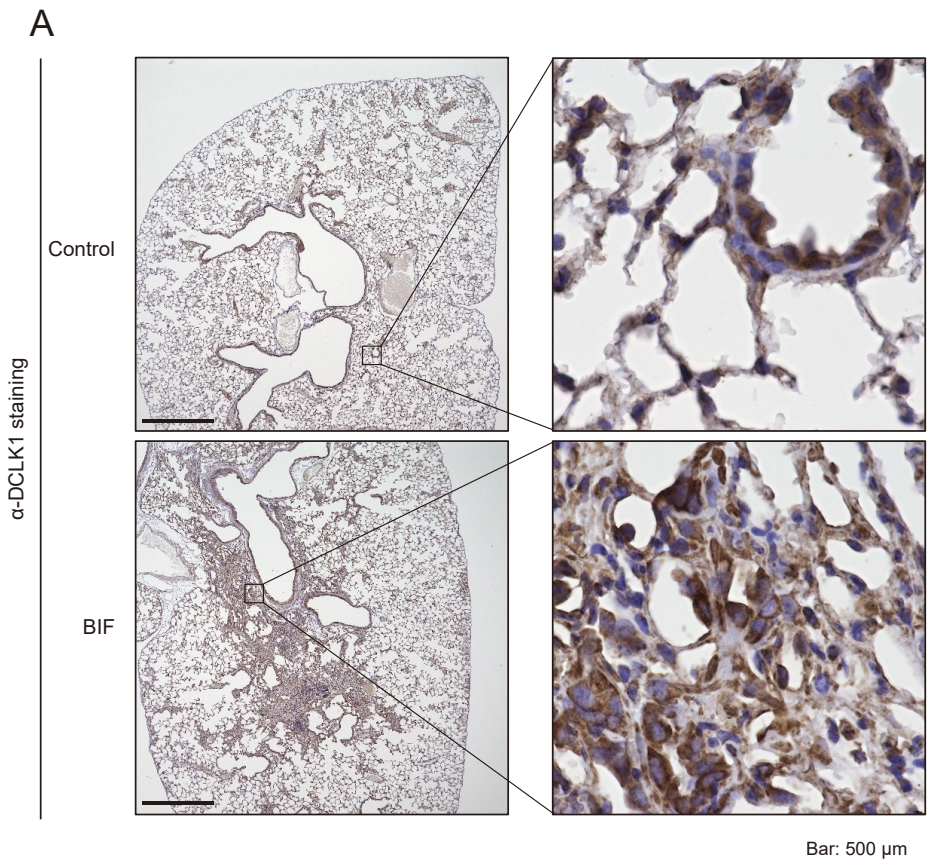
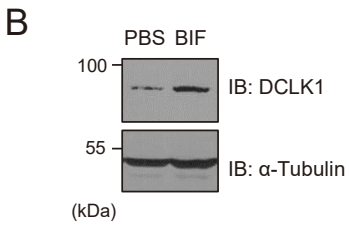


Figure 1. DCLK1 is enhanced in human IPF lung
Immunohistochemical staining of DCLK1 (α-DCLK1 staining) and hematoxylin and eosin (HE staining) stained images of normal human region and fibrotic regions. DCLK1 signal is enhanced in fibrotic region in IPF lung. Scale bar, 500 μm.





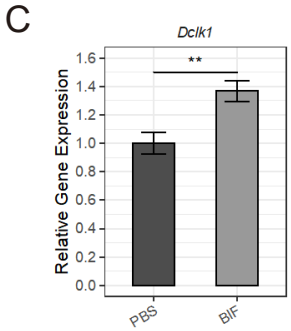


Figure 2. DCLK1 is enhanced in mouse BIF lung

(A) Immunohistochemical staining of DCLK1 in mouse control lung and BIF lung. Scale bar, 50 μm.

(B) Immunoblotting of DCLK1 in mouse control lung and BIF lung. Each lane contained 30 μg of total protein. *α*-Tubulin was used as a loading control.

(C) qRT-PCR was performed to detect *Dclk1* mRNA. *Gapdh* was used as a reference. Statistical analysis was performed as described in the Materials and Methods. The value for PBS-injected lung (control) was set to 1.0. Data are mean +/- SD. **, $p<0.01$. Two independent experiments were performed.

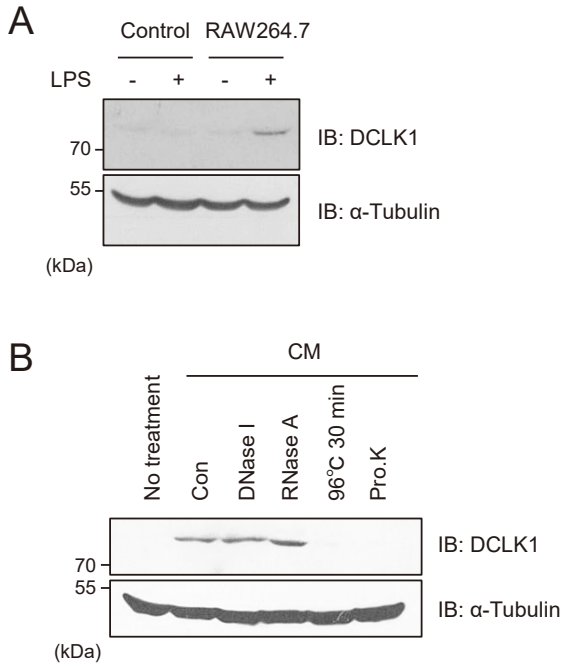


Figure 3. The conditioned medium of LPS-treated RAW264.7 cells induces DCLK1 expression in human lung cancer H1299 cells

(A) H1299 cells were exposed to the conditioned medium, which was prepared as described in Methods, for 24 h. Whole cell lysates were immunoblotted with anti-DCLK1 and anti-*α*-tubulin antibodies. The conditioned medium obtained from H1299 cells was used as a control (Control). The medium from LPS-treated RAW264.7 cells induced DCLK1 expression (RAW264.7 +), whereas the medium from untreated RAW264.7 cells did not (RAW264.7 -). Three independent experiments were performed and the representative results are shown.

(B) The conditioned medium from LPS-treated RAW264.7 cells (CM) was treated with DNase I (DNase I), ribonuclease (RNase A), heat (96 ºC for 30 min), and proteinase K (Proteinase K). Heat treatment and proteinase K abolished the activity of the conditioned medium to induce DCLK1.

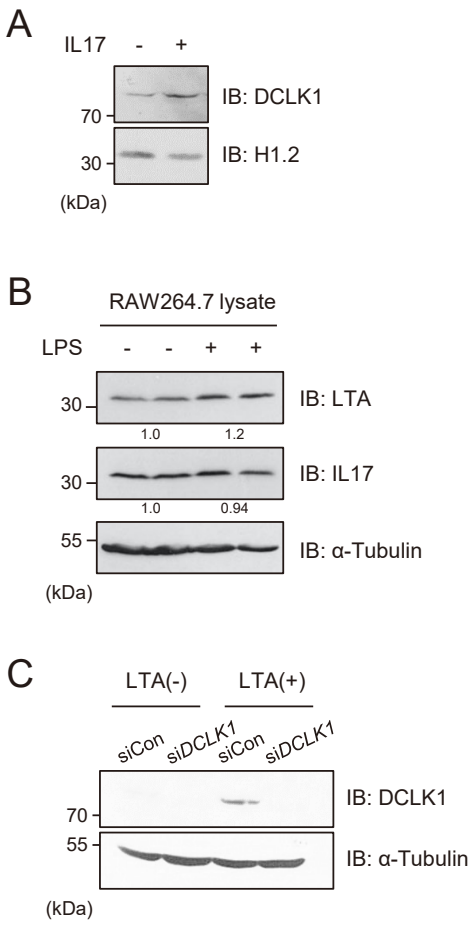


Figure 4. IL17 and LTA induce DCLK1 expression in human lung cancer H1299 cells, but LPS enhanced LTA expression but not IL17 expression in RAW264.7 cells

(A) H1299 cells were cultured in the medium with or without 100 ng/L IL17. After 24 h, cells were harvested and whole cell lysates were immunoblotted. Histone H1.2 was used as a reference protein.

(B) RAW264.7 cells were exposed to 0.1 mg/L LPS for 24 h and the whole cells lysates were immunoblotted by anti-LTA and anti-IL17 antibodies. The value for H1299 cells transfected with siControl was set to 1.0.

(C) H1299 cells were plated at $1x10^6$ cells/dish in 6-cm dishes and transfected with either control siRNA or siRNA against *DCLK1*. After 24 h, the cells were replated at $2x10^5$ cells/well in a 12-well plate. Six hours later, the medium was changed to medium with or without 5 μg/L LTA. After 24 h, whole cell lysates were immunoblotted. The signal detected with anti-DCLK1 antibody disappeared by *DCLK1* silencing, which supports that the antibody detected induced DCLK1 protein. Two independent experiments were performed for (B), (C), and (D).

murine macrophage-like RAW264.7 cells. The medium from LPS-stimulated RAW264.7 cells induced DCLK1 expression, whereas the control medium from H1299 cells did not (Fig. 3A). Treatment with heat or proteinase K abolished the DCLK1-inducing activity of the conditioned medium, whereas treatment with ribonuclease or deoxyribonuclease had no effect (Fig. 3B). Therefore, we concluded that this activity was mediated by protein(s).

LTA enhances DCLK1 expression in human lung cancer H1299 cells

In the pancreas, IL17 derived from immune cells is reported to induce DCLK1 expression and impart stemness to cancer cells[36]. IL17RB, a member of the IL17 receptor family, is a marker for human colorectal cancer stems expressing DCLK1[37]. Gene encoding IL17RD, another member of the IL17 receptor family, which forms a dimer with IL17RA and mediates IL17 signaling, was listed among the genes upregulated in IPF lung (Table 1)[38]. Therefore, we exposed human lung

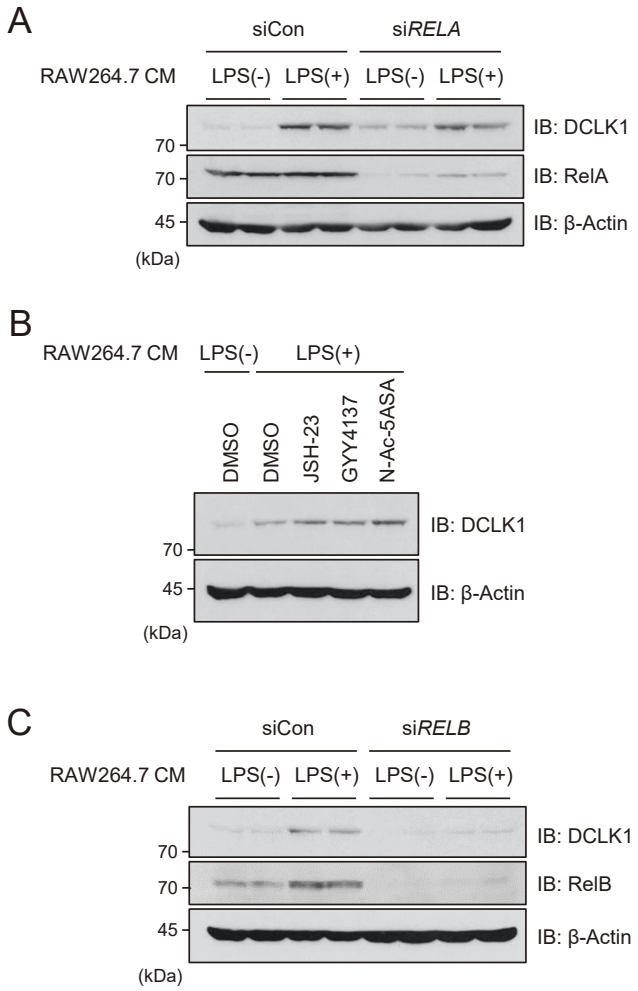


Figure 5. *RELB* silencing blocks the induction of DCLK1 in H1299 cells

(A) and (C) H1299 cells were transfected with either control siRNA, siRNA *RELA, or* siRNA *RELB*. After 48 h, H1299 cells were treated with the conditioned medium derived from RAW264.7 cells as described in Figure 3A. The validation of *RELA* and *RELB* silencing was evaluated by the immunoblotting.

(B) H1299 cells were exposed to the conditioned medium of RAW264.7 cells in the presence of 10 μM JSH-23, 10 μM GYY4137, or 10 mM N-Ac-5ASA.

cancer H1299 cells to 100 μg/L IL17. As expected, IL17 enhanced DCLK1 expression (Fig. 4A). However, IL17 was detected in RAW264.7 cells even without LPS treatment, and LPS did not significantly enhance IL17 expression in RAW264.7 cells (Fig. 4B), indicating that the DCLK1-inducing activity of the conditioned medium of LPS-treated RAW264.7 cells was not attributable to IL17. Considering the implication of other inflammatory cytokines, we scrutinized the public datasets, and found one dataset (GSE19139) describing the upregulation of *Dclk1* in response to lymphotoxin-β receptor signaling in mouse aorta smooth muscle cells[39]. Indeed, 5 μg/L LTA, a ligand of the lymphotoxin-β receptor, induced DCLK1 expression in H1299 cells (Fig. 4C). Moreover, LPS enhanced LTA expression in RAW264.7 cells (Fig. 4B). These findings suggest that LTA, but not IL17, contributes to DCLK1-inducing activity of RAW264.7 cells. It is worth noting that various cytokines are expressed in IPF lungs (Table 2); therefore, it is possible that other cytokines are also involved in the enhancement of DCLK1 expression in IPF lungs.

Alternative NF-κB signaling is involved in the induction of DCLK1

If DCLK1 is involved in cancer development, it is important to block DCLK1 expression in IPF lungs. However, if multiple cytokines induce DCLK1, therapy targeting individual cytokine would not be effective. Therefore, we reasoned that it was rational to inhibit a common signal triggered by cytokines. With this in mind, we silenced *RELA* in H1299 cells. However, *RELA* silencing had no effect (Fig. 5A), and RELA inhibitors did not suppress DCLK1 expression (Fig. 5B). In contrast, *RELB* silencing blocked DCLK1 expression (Fig. 5C). Interestingly, RELB protein expression was enhanced in H1299 cells exposed to the conditioned medium (Fig. 5C). It means that not canonical but alternative NF-κB signaling is involved in the induction of DCLK1.

Discussion

The initial aim of this study was to gain insights into the molecular link between IPF and lung cancer. As RNA-seq data were not available, we used three microarray datasets of human IPF lungs and extracted 94 genes that were upregulated in all of them. Among them, we were interested in DCLK1. As DCLK1 induces tumor development in the mouse pancreas, we hypothesized that DCLK1 also contributes to the tumorigenesis in lung and is involved in the pathogenesis of IPF-associated lung cancer[17-20]. While we were doing this study, Panneerselvam *et al.* reported that DCLK1 induces the expression of not only epithelial mesenchymal transition markers but also stem cell markers and confers human lung cancer cells with tumor-initiating capacity[23]. This report is consistent with our hypothesis and suggests that blocking DCLK1 expression prevents IPF-associated oncogenesis. Therefore, we attempted to identify the molecular mechanism, by which DCLK1 expression is induced.

Pulmonary macrophages secrete various inflammatory cytokines and play important roles in the pathogenesis

of IPF[32, 35]. Further, it is known that IL17 induces DCLK1 expression in pancreatic adenocarcinoma cells[36]. Thus, we speculated that macrophage-derived cytokines may induce DCLK1 expression. We used murine macrophage-like RAW264.7 cells and human lung adenocarcinoma H1299 cells to confirm that the LPS-activated RAW264.7 cells secreted certain factor(s) that stimulate DCLK1 expression in H1299 cells. Moreover, these secreted factors(s) were sensitive to proteinase K and heat treatment. Thus, we proved the implication of inflammatory cytokines.

IL17RB and DCLK1 are markers of colorectal cancer stem cells[37]. *IL17RD* is listed among the genes upregulated in the IPF lung. Hence, we first confirmed that IL17 induced the expression of DCLK1. Unexpectedly, IL17 expression was not enhanced by LPS in RAW264.7 cells. In contrast, LPS enhanced LTA expression in RAW264.7 cells and LTA induced DCLK1 expression in H1299 cells. Therefore, it is reasonable to assume that LTA contributes, to some extent, to DCLK1 expression in H1299 cells exposed to conditioned medium from LPS-stimulated RAW264.7 cells. However, as various cytokines were detected in IPF lungs (**Table 2**), we infer that more than one cytokine co-operatively mediate DCLK1 expression. We noted that inhibition of alternative NF-κB signaling suppressed DCLK1 expression. Therefore, we expect that inhibitors of alternative NF-κB signaling may be useful to prevent cancer development in IPF lungs.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgements

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