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ORIGINAL ARTICLE

Regulation of *LOXL2* and *SERPINH1* by antitumor *microRNA-29a* in lung cancer with idiopathic pulmonary fibrosis

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Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive lung disease that is refractory to treatment and carries a high mortality rate. IPF is frequently associated with lung cancer. Identification of molecular targets involved in both diseases may elucidate novel molecular mechanisms contributing to their pathology. Recent studies of microRNA (miRNA) expression signatures showed that *microRNA-29a* (*miR-29a*) was downregulated in IPF and lung cancer. The aim of this study was to investigate the functional significance of *miR-29a* in lung cancer cells (A549 and EBC-1) and lung fibroblasts (MRC-5) and to identify molecular targets modulated by *miR-29a* in these cells. We confirmed the downregulation of *miR-29a* in clinical specimens of IPF and lung cancer. Restoration of *miR-29a* suppressed cancer cell aggressiveness and fibroblast migration. A combination of gene expression data and *in silico* analysis showed that a total of 24 genes were putative targets of *miR-29a*. Among them, lysyl oxidase-like 2 (*LOXL2*) and serpin peptidase inhibitor clade H, member 1 (*SERPINH1*) were direct targets of *miR-29a* by luciferase reporter assays. The functions of *LOXL2* and *SERPINH1* contribute significantly to collagen biosynthesis. Overexpression of LOXL2 and SERPINH1 was observed in clinical specimens of lung cancer and fibrotic lesions. Downregulation of *miR-29a* caused overexpression of *LOXL2* and *SERPINH1* in lung cancer and IPF, suggesting that these genes are involved in the pathogenesis of these two diseases.
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INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive parenchymal lung disease characterized by chronic tissue damage and aberrant wound healing and is associated with severe pathologic changes in the structure of the alveoli.[1] There are limited effective therapeutic options for IPF and patients inevitably succumb to the disease.[2,3] Although pirfenidone and nintedanib reduce the rate at which forced vital capacity declines, their impact on long-term mortality remains unclear.

Another pulmonary disease, lung cancer is the leading cause of cancer-related death in developed countries.[4] Approximately 80% of lung cancers are classified histopathologically as non-small cell lung cancers (NSCLC), and the majority of advanced NSCLC patients dying in less than 5 years despite the use of various combination chemotherapy, chemoradiotherapy or molecularly targeted therapy.[5] Interestingly, NSCLC and IPF share common risk factors, such as smoking, environmental or professional exposure, viral infections, and chronic tissue injury.[6,7] Indeed, the risk of developing lung cancer is seven times higher in patients with IPF.[8] These findings suggest that there are common driver genes and molecular pathways in the two diseases.

The discovery of RNAs that do not code for proteins has provided new directions in the study of human pathogenesis. In this regard, microRNAs (miRNAs) are particularly important. miRNAs are small non-coding RNAs that have pivotal roles in the regulated expression of protein coding/non-coding RNAs. Regulation of gene expression is achieved by repressing the translation of mRNAs or cleaving RNA transcripts in a sequence-specific manner.[9,10] Thus, one miRNA species can regulate the amount of a specific mRNA in human cells.[10,11] Aberrantly expressed miRNAs can disrupt the regulated RNA networks in normal cells, thereby promoting pathologic development and cancer. Many studies have shown that miRNAs are aberrantly expressed in lung cancers and in IPF.[12–16] We hypothesized that the identification of aberrantly expressed miRNAs is an important step toward elucidating miRNA-regulated molecular targets and miRNA-mediated pathogenic RNA networks that contribute to lung cancers and pulmonary fibrosis.

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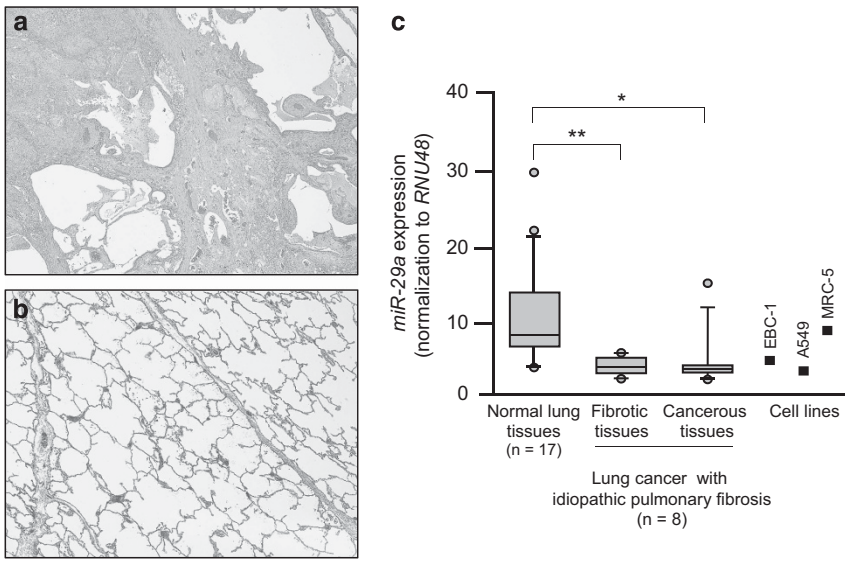

Figure 1 Comparison of expression levels of *miR-29a* in clinical specimens and cell lines. (**a**) Typical specimen of lung cancer with idiopathic pulmonary fibrosis (IPF). (**b**) Normal lung tissue. (**c**) Expression levels of *miR-29a* in lung cancer, IPF, normal lung tissues and cell lines. $*P<0.05$, $**P<0.005$. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

Table 1A Characteristics of the patients

Lung cancer	n	*(%)*
Total number	8	
Median age (range)	73.5 (69–89)	
Gender		
Male	5	(62.5)
Female	3	(37.5)
Tissue types		
Adeno	4	(50.0)
Sq	4	(50.0)
Pathological tumor stage		
IA	3	(37.5)
IB	2	(25.0)
IIA	0	
IIB	1	(12.5)
IIIA	2	(25.0)
IIIB	0	
Differentiation		
Well	1	(12.5)
Moderately	6	(75.0)
Poorly	1	(12.5)
Pleural invasion		
(+)	3	(37.5)
(−)	5	(62.5)
Venous invasion		
(+)	0	
(−)	8	(100.0)
Lymphatic invasion		
(+)	4	(50.0)
(−)	4	(50.0)
Recurrence		
(+)	6	(75.0)
(−)	2	(25.0)

Table 1B Normal lung tissue

Normal lung tissue	n
Total number	17
Median age (range)	69 (50–88)
Gender	
Male	17

Based on this proposal, we have sequentially identified tumor-suppressive miRNAs and novel miRNA-mediated RNA networks in lung cancer cells by establishing miRNA expression signatures. For example, *miR-1* and *miR-133a* inhibit cancer cell migration and invasion through targeting colonin-1, which is involved in a variety of cellular processes. Restoration of *miR-206* suppressed cancer cell aggressiveness via its regulation of *EGFR* and *c-MET* oncogenic signaling.[17] More recently, we demonstrated that the *miR-29* family is significantly downregulated in several types of cancers, including lung squamous cell carcinoma cells. These miRNAs act as antitumor miRNAs through their regulation of several extracellular matrix (ECM) components.[18–23] In cancer cells, aberrantly expressed ECM-mediated oncogenic signals trigger cancer cell migration and invasion and promote metastasis.[24,25] Fibrosis is enhanced by excess stiffness of ECM components, and overexpression of ECM components replaces functional tissue and disrupts organ architecture.[26,27] These observations suggest that the *miR-29*-family may also have important roles in the pathogenesis of pulmonary fibrosis.

In this study, we focused on *miR-29a* and investigated its functional significance in lung cancer and IPF. We attempted to identify the genes targeted by *miR-29a* and how they contributed to pathologic changes. The discovery of *miR-29a*-regulated genes provides new insight into the potential molecular pathogenesis of these two diseases.

MATERIALS AND METHODS

Clinical specimens and RNA extraction

A total of eight specimens of lung cancer with IPF and 17 normal lung tissues that obtained from lung cancer patients who underwent surgery at Kagoshima

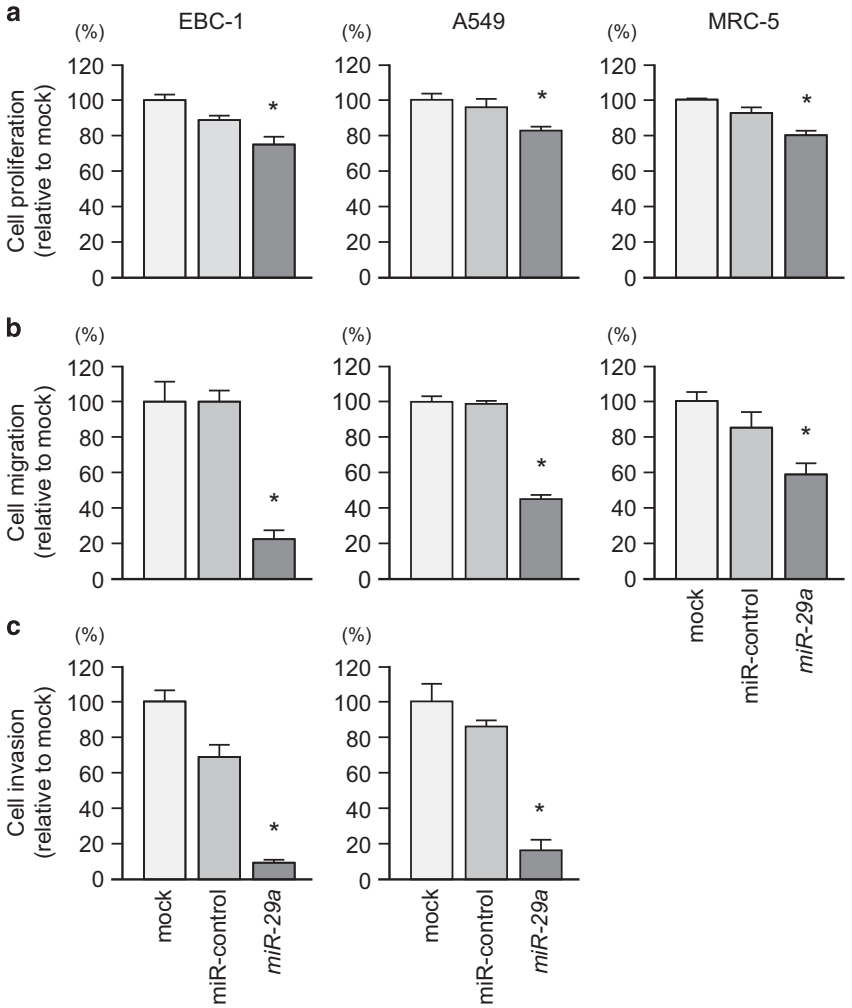

Figure 2 Effects of *miR-29a* transfection on EBC-1, A549 and MRC-5 cells. (**a**) Cell proliferation was determined with XTT assays 96 h after transfection with 10 nM *miR-29a*, miR-control or mock. (**b**) Cell migration activity was determined by migration assay 48 h after transfection. (**c**) Cell invasion activity was determined by Matrigel invasion assay 72 h after transfection. $*P<0.05$.

University Hospital (Kagoshima, Japan) from 2010 to 2013. IPF was diagnosed according to the official ATS/ERS/JRS/ALAT statement.[2] Formalin-fixed paraffin-embedded tissue sections were used for quantitative real-time RT-PCR analysis and immunohistochemical staining.

Samples were staged according to the TNM classification for lung cancer and were histologically graded.[28] Our studies were approved by the Institutional Review Board of Kagoshima University (IRB number 25–180). Prior written informed consent and approval were provided by each patient.

Total RNA (including miRNA) was extracted from tissues as previously described.[18,29] The RNA integrity was assessed using the RNA 6000 Nano Assay Kit and a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Cell culture

The EBC-1 and A549 human lung cancer cell lines and MRC-5 fibroblast cell line were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). The cell lines were maintained in RPMI 1640 medium or Eagle's minimum essential medium supplemented with 10% fetal bovine serum.

Quantitative real-time PCR analysis

Quantitative Taqman real-time PCR analysis was used to evaluate lysyl oxidase-like 2 (*LOXL2*) and serpin peptidase inhibitor clade H, member 1 (*SERPINH1*) mRNA expression levels. Primers and probes were assay-on-demand gene expression products: *LOXL2* (P/N: Hs00158757_m1, Applied Biosystems, Foster City, CA, USA), *SERPINH1* (P/N: Hs01060397_m1, Applied Biosystems). Stem-loop RT-PCR for *miR-29a* (P/N: 002112, Applied Biosystems) was used to quantify the expression levels of miRNAs according to the manufacturer's protocol. Quantitative Taqman real-time PCR relative to human *GAPDH* (P/N: Hs02758991_m1; Applied Biosystems) and *RNU48* (P/N: 440888; Applied Biosystems) as the internal controls were performed to assess the expression levels of mRNA and miRNAs in the sample, using the delta–delta Ct method.

Restoration and inhibition of *miR-29a*

Transfection was performed as described previously.[17,29] We used Pre-*miR-29a* miRNA precursors (P/N: AM 17100; Applied Biosystems), *miR-29a* inhibitor (P/N: AM 4464084; Applied Biosystems) and negative-control miRNA (P/N: AM 17111; Applied Biosystems).

Cell proliferation, migration and invasion assays

Cell proliferation, migration and invasion assays were performed as described before.[18] All experiments were performed in triplicate.

Table 2 Downregulated genes in miR-29a transfectant

			miR-29a transfectant Log2 ratio					
Entrez gene ID	*Gene symbol*	*Description*	*EBC-1*	*A549*	*MRC-5*	*miR-29a conserved site*	*miR-29a poorly conserved site*	*GSE 19188 (fold-change)*
4017	*LOXL2*	Lysyl oxidase-like 2	−4.05	−3.35	−1.93	1	1	2.56
871	*SERPINH1*	Serpin peptidase inhibitor, clade H (heat shock protein 47), member 1, (collagen-binding protein 1)	−2.57	−1.35	−1.90	1	0	1.78
57761	*TRIB3*	Tribbles homolog 3 (Drosophila)	−2.40	−3.43	−1.84	0	1	1.72
55920	*RCC2*	Regulator of chromosome condensation 2	−2.11	−2.05	−1.24	1	0	1.86
140576	*S100A16*	S100 calcium-binding protein A16	−2.10	−1.65	−1.61	0	2	1.30
51129	*ANGPTL4*	Angiopoietin-like 4	−2.03	−1.46	−1.39	0	1	1.69
83986	*ITFG3*	Integrin alpha FG-GAP repeat containing 3	−1.85	−2.24	−1.51	0	1	1.21
642	*BLMH*	Bleomycin hydrolase	−1.85	−2.76	−1.32	1	0	1.53
8140	*SLC7A5*	Solute carrier family 7 (amino acid transporter light chain, L system), member 5	−1.71	−1.40	−2.49	0	1	2.64
51256	*TBC1D7*	TBC1 domain family, member 7	−1.67	−2.11	−1.36	1	0	2.01
114904	*C1QTNF6*	C1q and tumor necrosis factor related protein 6	−1.52	−1.88	−1.00	2	2	1.76
3915	*LAMC1*	Laminin, gamma 1 (formerly LAMB2)	−1.49	−1.08	−1.27	1	0	1.61
4605	*MYBL2*	v-myb myeloblastosis viral oncogene homolog (avian)-like 2	−1.42	−1.22	−1.95	1	0	8.02
58505	*OSTC*	Oligosaccharyltransferase complex subunit	−1.35	−2.28	−1.15	1	0	1.54
4150	*MAZ*	MYC-associated zinc finger protein (purine-binding transcription factor)	−1.35	−1.11	−1.25	1	0	1.51
6611	*SMS*	Spermine synthase	−1.34	−1.58	−1.28	0	1	1.56
80727	*TTYH3*	Tweety homolog 3 (Drosophila)	−1.31	−1.25	−1.22	0	1	1.60
388969	*C2orf68*	Chromosome 2 open-reading frame 68	−1.31	−2.40	−1.23	0	2	1.30
79017	*GGCT*	Gamma-glutamylcyclotransferase	−1.23	−1.39	−1.29	1	0	2.46
9871	*SEC24D*	SEC24 family, member D (S. cerevisiae)	−1.23	−3.73	−1.03	0	1	1.37
84733	*CBX2*	Chromobox homolog 2	−1.21	−1.10	−1.18	1	0	3.97
26521	*TIMM8B*	Translocase of inner mitochondrial membrane 8 homolog B (yeast)	−1.12	−1.51	−1.65	1	0	2.17
1021	*CDK6*	Cyclin-dependent kinase 6	−1.11	−2.01	−1.06	3	0	1.73
5480	*PPIC*	Peptidylprolyl isomerase C (cyclophilin C)	−1.07	−2.05	−1.42	1	0	1.14

Identification of putative target genes of *miR-29a*

TargetScan database (http://www.targetscan.org) was used to identify *miR-29a* target genes. In addition, we performed gene expression analysis using *miR-29a*-transfected cells (EBC-1, A549 and MRC-5). Oligo-microarray human 60Kv (Agilent Technologies) was used for gene expression studies. To investigate the expression status of candidate *miR-29a* target genes in NSCLC clinical specimens, we analyzed gene expression profiles in the gene expression omnibus database (accession number: GSE 19188). Microarray procedures and data mining methods were described previously.[18,29]

Western analysis

Expression of LOXL2 and SEPINH1 protein *in vitro* was assessed using western analysis as described previously.[17,29] Immunoblotting was done with anti-LOXL2 antibody (1:1000; ab96233; Abcam, Cambridge, UK) or with anti-SERPINH1 antibody (1:500; sc-5293; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-GAPDH antibody (1:10000; MAB374; Chemicon, Temecula, CA, USA).

Plasmid construction and dual-luciferase reporter assays

Partial wild-type sequences of the 3′-untranslated region (UTR) of *LOXL2* and *SERPINH1* or those with a deleted *miR-29a* target site (positions 555–561 or 757–763 of *LOXL2* 3′-UTR and 57–63 of *SERPINH1* 3′-UTR) were inserted between the XhoI and PmeI restriction sites in the 3′-UTR of the hRluc gene in the psiCHECK-2 vector (C8021; Promega, Madison, WI, USA). Dual-luciferase reporter assay was performed as described previously.[17,29]

Immunohistochemistry

Formalin-fixed paraffin-embedded tissue sections were treated as previously described.[17] Anti-LOXL2 rabbit polyclonal antibody (ab96233; Abcam) and anti-SERPINH1 mouse monoclonal antibody (sc-5293; Santa Cruz Biotechnology) were used.

Statistical analysis

All data are shown as mean ± s.e. Statistical comparison was made by analysis of variance, followed by Bonferroni's *post hoc* analysis. Expert StatView software, version 4 (SAS Institute, Cary, NC, USA), was used in these analyses.

RESULTS

Expression levels of *miR-29a* in lung cancer, IPF and normal lung tissues

The expression level of *miR-29a* was evaluated by quantitative real-time PCR methods by using formalin-fixed paraffin-embedded specimens of lung cancer lesions, fibrotic lesions and normal lung tissues. Representative formalin-fixed paraffin-embedded specimens are shown in Figures 1a and b. The patient backgrounds and clinicopathological characteristics are summarized in Tables 1A and 1B. The expression level of *miR-29a* was significantly reduced in lung cancer and fibrotic lesions compared with normal lung tissues (Figure 1c).

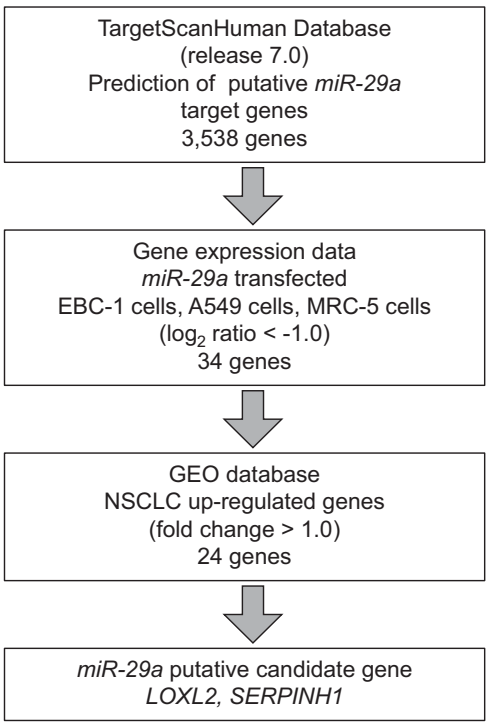

Figure 3 Flow chart illustrating the strategy for identification of *miR-29a* target genes. In total, 3538 genes were identified as putative targets of *miR-29a* according to the TargetScan database. We merged the expression analysis data of downregulated genes in *miR-29a*-transfected EBC-1, A549 and MRC-5 cells (Log_2 ratio < -1.0). Upregulated genes were determined according to the gene expression data set of lung NSCLC clinical specimens according to the GEO database (accession no. GSE 19188). From this selection, 24 candidate genes were identified as putative targets of the *miR-29a*.

Effects of *miR-29a* restoration on the proliferation, migration and invasion of NSCLC cell lines and a fibroblast cell line

To evaluate the functional roles of *miR-29a*, we carried out gain-of-function studies using mature miRNA transfection into two NSCLC cell lines (EBC-1 and A549) and a fibroblast cell line (MRC-5).

The XTT assays revealed inhibition of cell proliferation in EBC-1, A549 and MRC-5 cells transfected with *miR-29a* in comparison with mock- or control-transfected cells (Figure 2a). The wound-healing assays showed significant inhibition of cell migration activities in *miR-29a* transfectants (EBC-1, A549 and MRC-5) compared with that in mock- or miR-control-transfected cells (Figure 2b). Moreover, Matrigel invasion assays demonstrated that miR-29a transfection significantly inhibited cell invasion. The number of invading cells was significantly reduced in *miR-29a* transfected EBC-1 and A549 cells (Figure 2c). In this assay, we omitted examination of MRC-5 cells because the ability for invasion of this cell line is extremely low.

In contrast to restoration study, inhibition of *miR-29a* enhanced EBC-1 cell proliferation, migration and invasion (Supplementary Figure 1).

Identification of putative *miR-29a* regulated genes in lung cancer and fibroblast cells

To identify *miR-29a*-regulated genes, we carried out a combination of *in silico* analysis and genome-wide gene expression analysis of *miR-29a* transfectant cells (EBC-1, A549 and MRC-5). By using TargetScan database analysis, a total of 3538 genes were identified as putative *miR-29a*-regulated genes, that is, those that had putative target sites for *miR-29a* in their 3′-UTRs. We undertook genome-wide gene expression analysis using EBC-1, A549 and MRC-5 cells. Among the 3538 genes, we selected a total of 34 genes that were commonly down-regulated in EBC-1, A549 and MRC-5 cells (average log_2 ratio < -1.0) following *miR-29a* transfection compared with the expression levels in mock cells. Finally, we investigated the expression status of those 34 genes in lung cancer specimens by using the gene expression omnibus database (accession number: GSE 19188). From this selection, 24 candidate genes were identified as targets of *miR-29a* (Table 2).

Extracellular matrix has a key role in the progression of cancer and fibrosis (24–27). Therefore, we focused on the ECM-related genes.

Among these candidate genes, we chose *LOXL2* and *SERPINH1* genes that code for collagen cross-linking enzymes and those genes were subjected to further analyses. Our method for selecting *miR-29a* target genes is shown in Figure 3.

Direct regulation of *LOXL2* and *SERPINH1* by *miR-29a* in lung cancer cells and fibroblasts

We conducted qRT-PCR and western blotting to confirm that restoration of *miR-29a* resulted in downregulation of *LOXL2* and *SERPINH1* in EBC-1, A549 and MRC-5 cells. The mRNA and protein expression levels of *LOXL2* and *SERPINH1* were significantly repressed in *miR-29a* transfectants compared with mock or miR-control transfectants in EBC-1, A549 and MRC-5 (Figures 4a and b). In contrast to restoration study, inhibition of *miR-29a* resulted in upregulation of *LOXL2* and *SERPINH1* in these cells (Supplementary Figure 2).

Next, we performed dual-luciferase reporter assays in A549 cells to confirm whether *LOXL2* and *SERPINH1* mRNAs were directly regulated by *miR-29a*. The TargetScan database predicted that putative *miR-29a*-binding sites existed in the 3′-UTRs of *LOXL2* (positions 555–561 and 757–763; Figure 5a) and *SERPINH1* (position 57–63; Figure 5b). We used vectors encoding either the partial wild-type sequence of the 3′-UTR of *LOXL2* or *SERPINH1* mRNA, including the predicted *miR-29a* target sites, or deletion vectors lacking the *miR-29a* target sites. We found that the luminescence intensities were significantly reduced by transfection with *miR-29a* and vectors carrying the wild-type 3′-UTR of *LOXL2* and *SERPINH1*, whereas transfection with deletion vectors blocked the decrease in luminescence (Figure 5). These data suggested that *miR-29a* bound directly to specific sites in the 3′-UTRs of *LOXL2* and *SERPINH1* mRNA.

Expression of LOXL2 and SERPINH1 in clinical specimens of lung cancer with IPF

We used immunohistochemical staining to examine the expression status of LOXL2 and SERPINH1 in clinical specimens of lung cancer with associated IPF (Figures 6a and b). LOXL2 was strongly expressed in cancer cells and fibroblasts (Figures 6c and d). Similar over-expression of SERPINH1 was observed in cancer cells and fibroblasts (Figures 6e and f).

DISCUSSION

Our present study showed that expression of *miR-29a* was significantly reduced in lung cancer tissues and in fibrotic lesions of IPF and that restoration of *miR-29a* significantly suppressed proliferation,

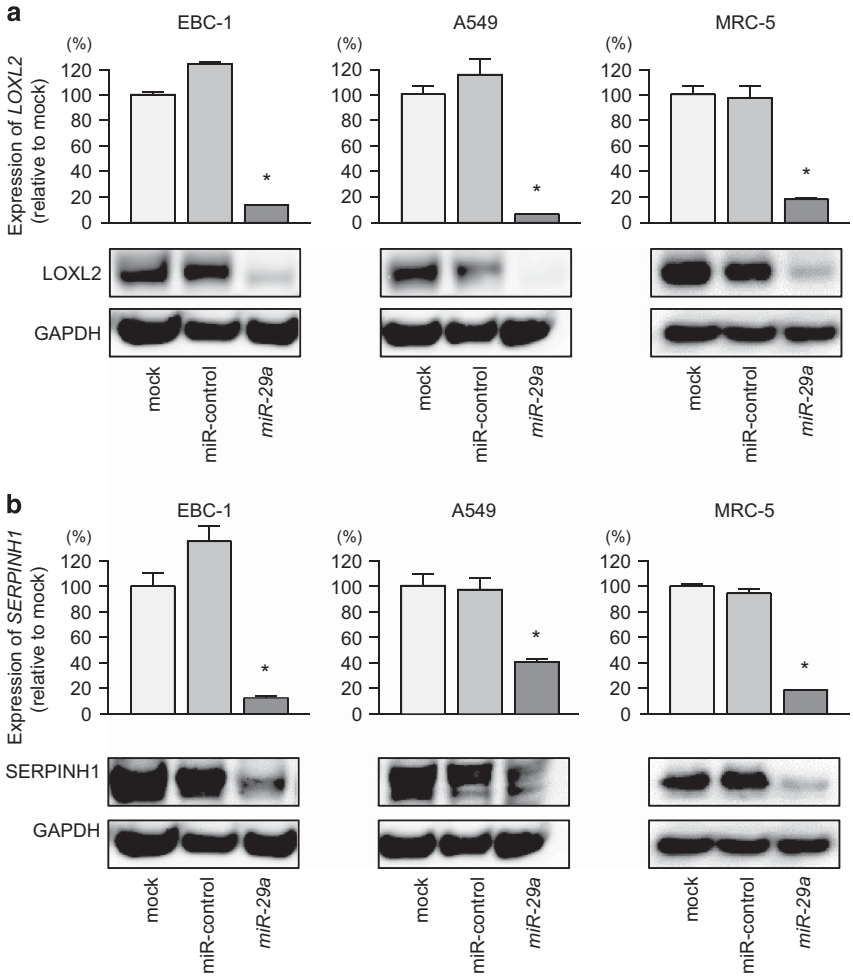

Figure 4 Effects of *miR-29a* transfection on *LOXL2* and *SERPINH1* expression in EBC-1, A549 and MRC-5 cells. (**a**, bar graph) *LOXL2* mRNA expression 72 h after transfection with *miR-29a*. *GAPDH* was used as an internal control. (**a**, blot) *LOXL2* protein expression 72 h after transfection with *miR-29a*. GAPDH was used as a loading control. (**b**, bar graph) *SERPINH1* mRNA expression 72 h after transfection with *miR-29a*. (**b**, blot) *SERPINH1* protein expression 72 h after transfection with *miR-29a*. $*P<0.0001$.

migration and invasion in cancer cells and fibroblasts. Those data suggested that *miR-29a* and *miR-29a*-regulated genes were deeply involved in these diseases. Our recent studies of cancer cells showed that all members of the *miR-29*-family were downregulated in several types of cancer tissues and that restoration of these miRNAs inhibited cancer cell aggressiveness through targeting collagen biosynthesis genes, laminins and integrins.[30] Most types of cells require ECM-integrin-mediated signaling pathways for proliferation, migration, invasion and survival.[31,32] Aberrant expression of ECM-related genes disturbs tightly regulated integrin-meditated signaling and these phenomena promote cancer cell aggressiveness.[30] Thus, elucidation of tumor-suppressive miRNAs that regulate novel ECM-related pathways should point to new therapies for cancer.

Recent studies have indicated that dysregulated miRNAs contribute to the disruption of normal cellular RNA networks, promoting the development of cancer cells and associated pathology. We have identified novel miRNA-regulated cancer pathways based on the aberrant expression of miRNAs.[17,18,33] In this study, we identified *miR-29a*-regulated genes that participate in the pathogenesis of lung cancer and IPF.

Basically, the function of LOXL2 is covalent cross-linking of collagen and/or elastin in the ECM.[34,35] Recent studies of cancer cells showed that aberrant expression of LOXL2 was correlated with disease progression and aggressiveness in several types of cancers.[35] Our present data indicated that overexpression of LOXL2 enhanced cancer cell migration and invasion in several cancers.[18,36,37] SERPINH1 (also known as HSP47) is a 47 kDa collagen-binding glycoprotein localized in the endoplasmic reticulum (ER) and as a collagen-specific molecular chaperon.[38] Many studies have reported that SERPINH1 is overexpressed in cancers and fibrotic diseases.[39–41] To our surprise, our past studies demonstrated that *LOXL2* was involved in cancer cell metastasis and directly regulated by the *miR-29*-family in renal cell carcinoma, head and neck cancer and lung squamous cell carcinoma.[18,36,37] Moreover, *SERPINH1* was regulated by *miR-29a* in cervical cancer.[23] Our present data showed that both *LOXL2* and *SERPINH1* were directly regulated by *miR-29a* in lung cancer cells and fibroblasts. These findings indicated that direct regulation of *LOXL2* and *SERPINH1* by *miR-29a* is widespread and indispensable for the normal function of the cells.

Fibrosis is caused by excess accumulation of ECM components, such as collagens, fibronectins, elastin and alpha-smooth muscle

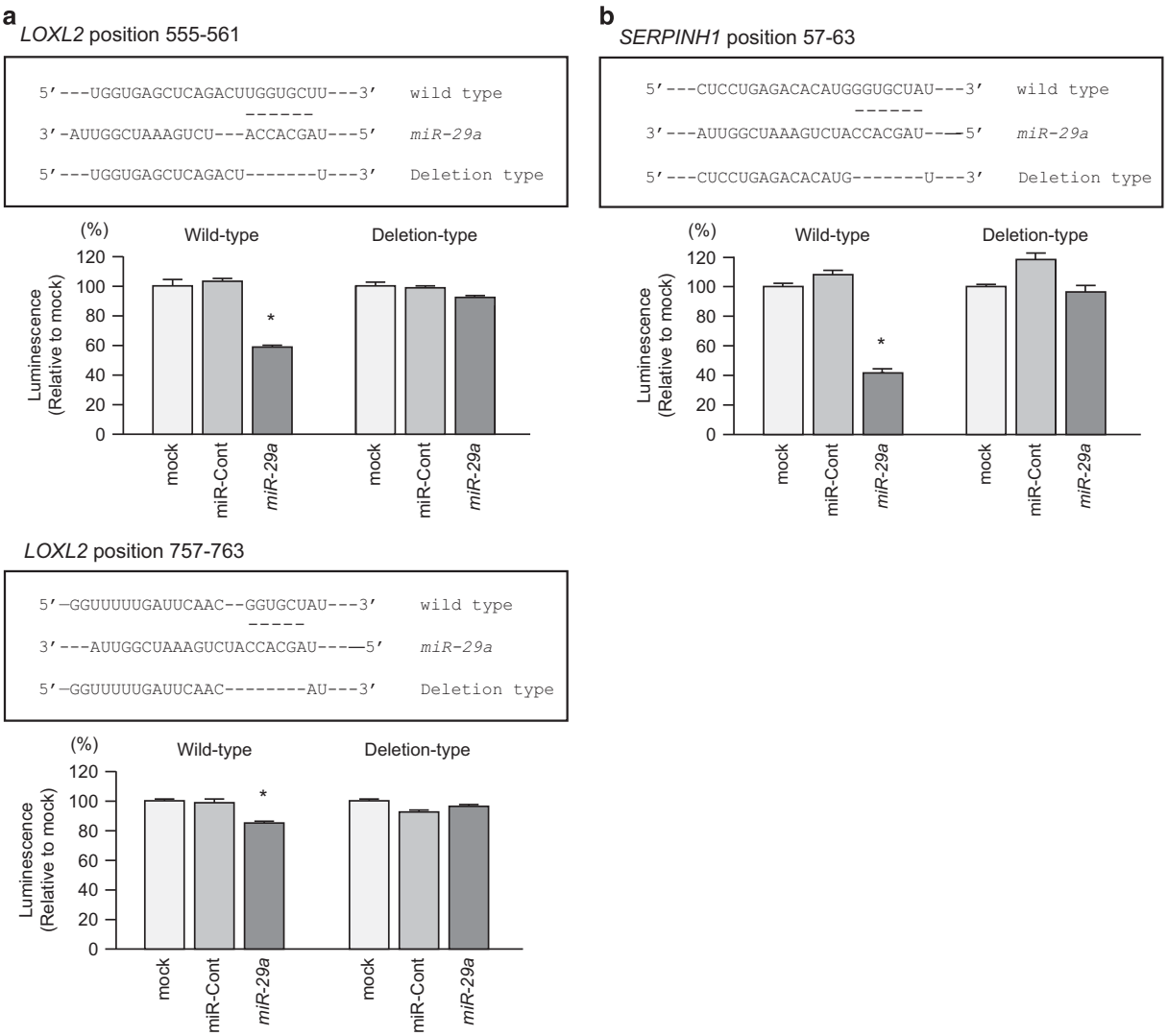

Figure 5 Direct regulation of *LOXL2* and *SERPINH1* by *miR-29a* in A549 cells. (**a**) The *miR-29a*-binding site in the 3′-UTR of *LOXL2* mRNA. Luciferase reporter assays used vectors that included (WT) or lacked (DEL) wild-type sequences of the putative *miR-29a* target sites at position 555–561 and 757–763. *Renilla* luciferase values were normalized to firefly luciferase values. (**b**) The *miR-29a*-binding site in the 3′-UTR of *SERPINH1* mRNA. Luciferase reporter assays used vectors that included (WT) or lacked (DEL) wild-type sequences of the putative *miR-29a* target sites at position 57–63. *$P<0.001$.

actin. ECM-related genes also contribute to the development of fibrosis.[26,27,42] Recent studies reported that members of the *miR-29*-family have potential roles in the inhibition of ECM synthesis, indicating anti-fibrotic activity.[43,44] Interestingly, the search for *miR-29* family targets by *in silico* analysis revealed that many collagen genes and several ECM component genes were putative targets of *miR-29*. Downregulation of the *miR-29* family was demonstrated in several types of human fibrotic tissues, including IPF,[13,15,45,46] supporting our present findings. It would be important to confirm the downregulation of *miR-29a* in fibroblasts and cancer cells in this study. The *in situ* hybridization to detect the reduction of *miR-29a* signals in these cells will be examined in future studies.

Regulation of *miR-29a* expression was investigated by chromatin immunoprecipitation assay and *in silico* analysis.[47] The promoter region of *miR-29a* contained two putative E-box sites, a Gli-binding site and four nuclear factor-kB-binding sites. These transcription factors bound to these regions and negatively regulated *miR-29a* expression.[47] Moreover, TGF-β inhibited the expression of the *miR-29* family and induced the expression of ECM components.[46,48] We know that TGF-β has a pivotal role in the pathogenesis of IPF by promoting the proliferation, activation and differentiation of epithelial cells and myofibroblasts.[1,49] Therefore, suppression of the *miR-29* family by TGF-β is a possible critical event in the progression of IPF, and inhibition of these pathways might be a therapeutic target of the disease.

In conclusion, downregulation of *miR-29a* was observed in clinical specimens of lung cancer and IPF, and *miR-29a* might contribute to these diseases by targeting *LOXL2* and *SERPINH1*. Overexpression of *LOXL2* and *SERPINH1* was confirmed in clinical specimens of lung cancer with IPF and they enhance the aggressiveness of these diseases. The identification of novel pathways regulated by the downregulation of miRNA may lead to a better understanding of molecular pathogenesis in lung cancer and pulmonary fibrosis.

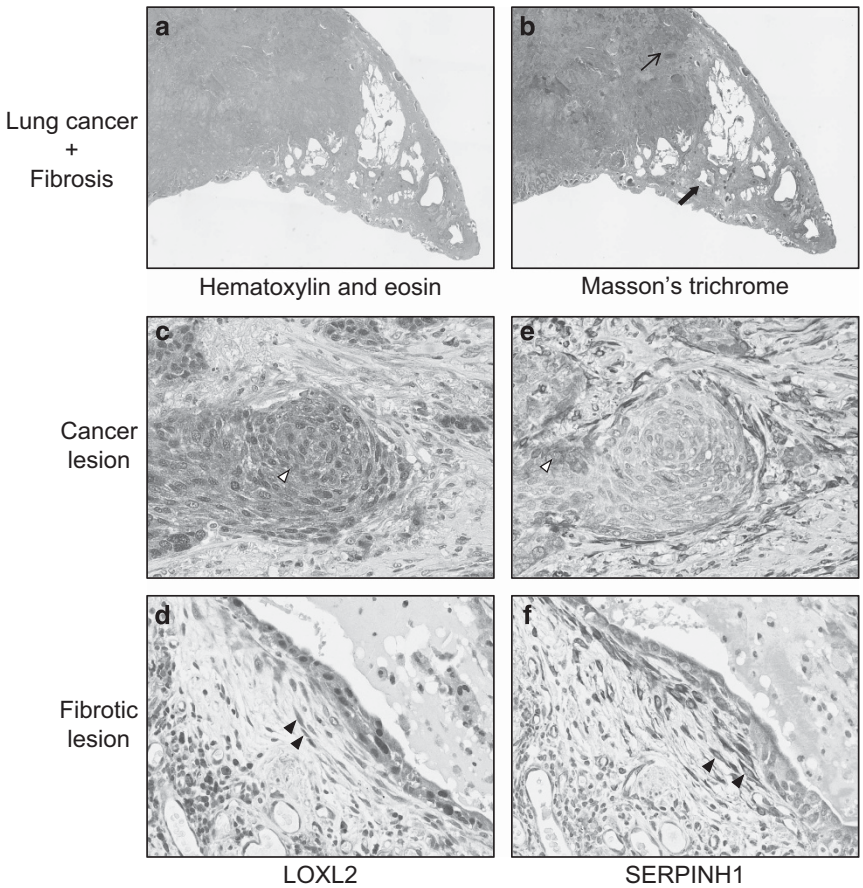

Figure 6 Immunohistochemical staining of LOXL2 and SERPINH1 in clinical specimens of lung cancer with IPF. (**a**) Hematoxylin-eosin staining. (**b**) Masson's trichrome staining. Cancer lesions (thin arrow) and fibrotic lesions (thick arrow) are noted. (**c**, **d**) LOXL2 staining. Cancer cells (open arrowheads) and fibroblasts (arrowheads) are noted. (**e**, **f**) SERPINH1 staining. Cancer cells (open arrowheads) and fibroblasts (arrowheads) are noted. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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