CANCER BIOLOGY & THERAPY
2019, VOL. 20, NO. 4, 537–545
https://doi.org/10.1080/15384047.2018.1537999

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RESEARCH PAPER

Knockdown of FOXP1 promotes the development of lung adenocarcinoma

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ABSTRACT

Lung cancer is one of the most common cancers in the world, which accounts for about 27% of all cancer deaths. However, the mechanisms underlying the pathogenesis of lung cancer cells remain largely elusive. In this study, we examined the role of the Forkhead box protein P1 (*FOXP1*) in lung cancer development. Our Oncomine analysis shows that *FOXP1* is downregulated in lung adenocarcinoma compared with normal lung tissue. Knockdown of *FOXP1* promotes the growth and invasion of PC9 and A549 cells by regulating genes of chemokine signaling molecules, including *CCR1, ADCY5, GNG7, VAV3*, and *PLCB1*. Simultaneous knockdown of *CCR1* and *FOXP1* attenuated *FOXP1* knockdown-induced increase of lung cancer cell growth. Finally, knockdown of *FOXP1* in PC9 cells promotes the tumorigenesis via CCR1 signaling in xenograft mouse model. Taken together, our data suggest that *FOXP1* plays important roles in preventing lung adenocarcinoma development via suppressing chemokine signaling pathways.

ARTICLE HISTORY
Received 1 June 2018
Revised 11 September 2018
Accepted 15 October 2018

KEYWORDS
lung cancer; FOXP1; proliferation; chemokine signaling

Introduction

Lung cancer is the leading cause of cancer-related mortality in the world. In 2014, more than 150,000 deaths were reported in the United States.[1] There are two major subtypes of lung cancer, non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). Approximately 85% of lung cancers are NSCLC, which is composed of adenocarcinoma (40%), squamous-cell carcinoma (25–30%), and large-cell carcinoma (10–15%).[2] Some frequently mutated genes, such as *TP53, Kras, EGFR PTEN, FGFR, KEAP1* and *ALK*, have been investigated previously,[3–7] benefiting the treatment of subsets of NSCLC patient. However, the prognosis of NSCLC patients is still poor due to the complexity of the mechanisms underlying the tumorigenesis of lung cancer. Better understanding of the mechanisms will help to improve the therapeutic outcomes.

The Forkhead box protein P1 (*FOXP1*) belongs to a family of winged-helix transcription factors that are involved in a broad range of functions, such as cell cycle progression, proliferation, differentiation, and apoptosis.[8] *FOXP1* was originally cloned from mouse B cell leukemia cell line and subsequently been found to be expressed in many different types of tissues in human and in a variety of cancers,[9,10] where *FOXP1* can serve as a tumor suppressor or oncogene in different cancers.[11–14] Shu et al. showed that *FOXP1* are highly expressed in the developing airway epithelium and is crucial for the lung development.[15] The expression of *FOXP1* has been shown to be downregulated in lung adenocarcinoma and high expression of *FOXP1* is associated with improved survival in NSCLC patients.[13] However, the role of *FOXP1* in lung cancer cells remains elusive.

In this study, we demonstrated that the expression of *FOXP1* was decreased in the human lung adenocarcinoma compared with normal lung tissue. Moreover, knockdown of *FOXP1* promoted the growth of lung cancer cells PC9 and A549 via inhibiting apoptosis. The migration and invasion of lung cancer cells were enhanced after the *FOXP1* knockdown. Furthermore, RNA-sequence analysis revealed that chemokine signaling genes, including *CCR1, ADCY5, GNG7, VAV3*, and *PLCB1*, were upregulated in PC9 and A549 cells transfected with *FOXP1* siRNA. Concomitant knockdown of *CCR1* with *FOXP1* attenuated *FOXP1* knockdown-induced increase of lung cancer cell proliferation. Finally, knockdown of *FOXP1* in PC9 cells promotes the tumorigenesis in xenograft mouse model. Taken together, our data suggest that *FOXP1* serves as a tumor suppressor of lung adenocarcinoma via downregulating chemokine signaling pathways.

Results

FOXP1 is downregulated in lung adenocarcinoma

To examine the role of FOXP1 in lung adenocarcinoma, we compared the expression of *FOXP1* in normal lung tissue and lung adenocarcinoma obtained from four studies in Oncomine microarray database.[16–19] As shown in Figure 1, the expression of *FOXP1*was dramatically downregulated in lung adenocarcinoma compared with normal lung tissue from different datasets, indicating that *FOXP1* plays important roles in lung adenocarcinoma. Furthermore, the expression of *FOXP1* was further reduced in

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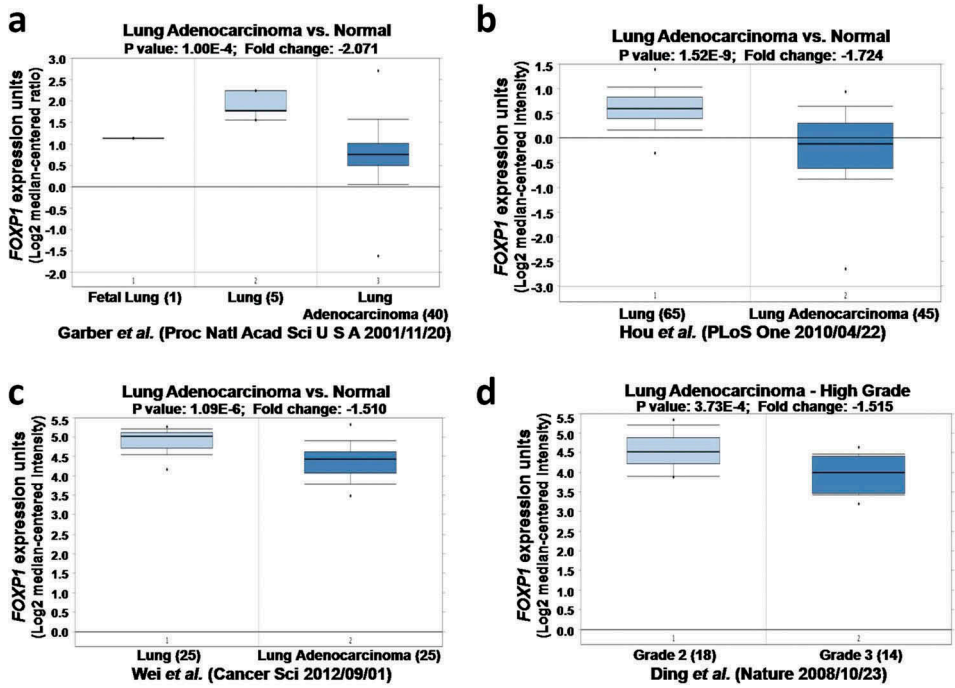

Figure 1. The expression of FOXP1 in normal lung tissues and lung cancer by Oncomine analysis. FOXP1 expression is decreased in lung cancer compared with normal lung. a-d figures were from different Oncomine databases.

grade 3 than grade 2 of lung adenocarcinoma (Figure 1d), indicating that the expression of *FOXP1*correlates with the severity of lung adenocarcinoma.

Knockdown of *FOXP1* promotes growth and invasion of lung cancer cells

Cancer cell proliferation is an important step in cancer development. First of all, we examined the effect of *FOXP1* on lung cancer cell growth by using the siRNA specific for *FOXP1*. As shown in Figure 2, after the *FOXP1* siRNA transfection, the *FOXP1* expression was significantly reduced in both PC9 and A549 cells compared with that in control siRNA-transfected cells as evidenced by the western blot analysis, indicating the successful knockdown of *FOXP1* in these cells. Furthermore, MTT assay demonstrated that the cell growth of PC9 and A549 cells was significantly enhanced beginning at 24 hrs after the knock-down of *FOXP1*, suggesting that *FOXP1* exhibits inhibitory effect on lung cancer cell growth (Figure 2b & 2e). Furthermore, flow cytometer analysis demonstrated that the apoptosis of lung cancer cells was significantly decreased after the knockdown of *FOXP1* (Figure 2c & 2f). Next, we examined the effect of *FOXP1* on invasion of lung cancer cells by employing the Boyden chamber invasion assay. As shown in Figure 2g & h, after *FOXP1* siRNA treatment, the number of cells penetrated through the Matrigel was dramatically increased in both PC9 and A549 cells transfected with *FOXP1* siRNA. These results indicate that knockdown of *FOXP1* promotes the growth and invasion of lung cancer cells.

Chemokine signaling pathway is involved in *FOXP1*-mediated tumor suppressing effects

To reveal the mechanism underlying *FOXP1*-mediated suppression of lung cancer cells, we performed RNA-Seq analysis in PC9 cells transfected with control or *FOXP1* siRNA. As shown in the heatmap, the gene expression profiles of PC9 cells transfected with F*OXP1* siRNA clustered together and were distinguishable from that of control siRNA-treated cells (Figure 3a). The GO enrichment analysis demonstrated that the differentially expressed genes were categorized into 17 functional categories (Figure 3b). Of particular interest is the chemokine signaling pathway, which is known to play important roles in non-small cell lung cancer progression.[20,21] Genes related to chemokine signaling pathway are dramatically altered by the knock down of *FOXP1*, such as *CCR1, ADCY5, GNG7, VAV3*, and *PLCB1*. The changes of these genes were further validated by quantitative RT-PCR experiments in both A549 and PC9 cells (Figure 3c & 3d). These results indicate that multiple genes downstream of *FOXP1* might be involved in *FOXP1*-mediated suppressing of lung tumor cell growth and invasion.

CCR1 is required for *FOXP1* sirna-induced lung cancer cell growth and invasion

Chemokine (C-C motif) receptor 1 (*CCR1*) has been shown to promote cancer cell proliferation and invasion of cancer cells, including lung cancer cell lines.[22] Of important note, *CCR1* also was identified to be regulated by *FOXP1* in colon cancer cell lines.[23] As such, we attempted to determine the role of *CCR1* in *FOXP1* siRNA-induced growth and invasion of lung cancer cells, we transfected A549 and PC9 cells with *CCR1*

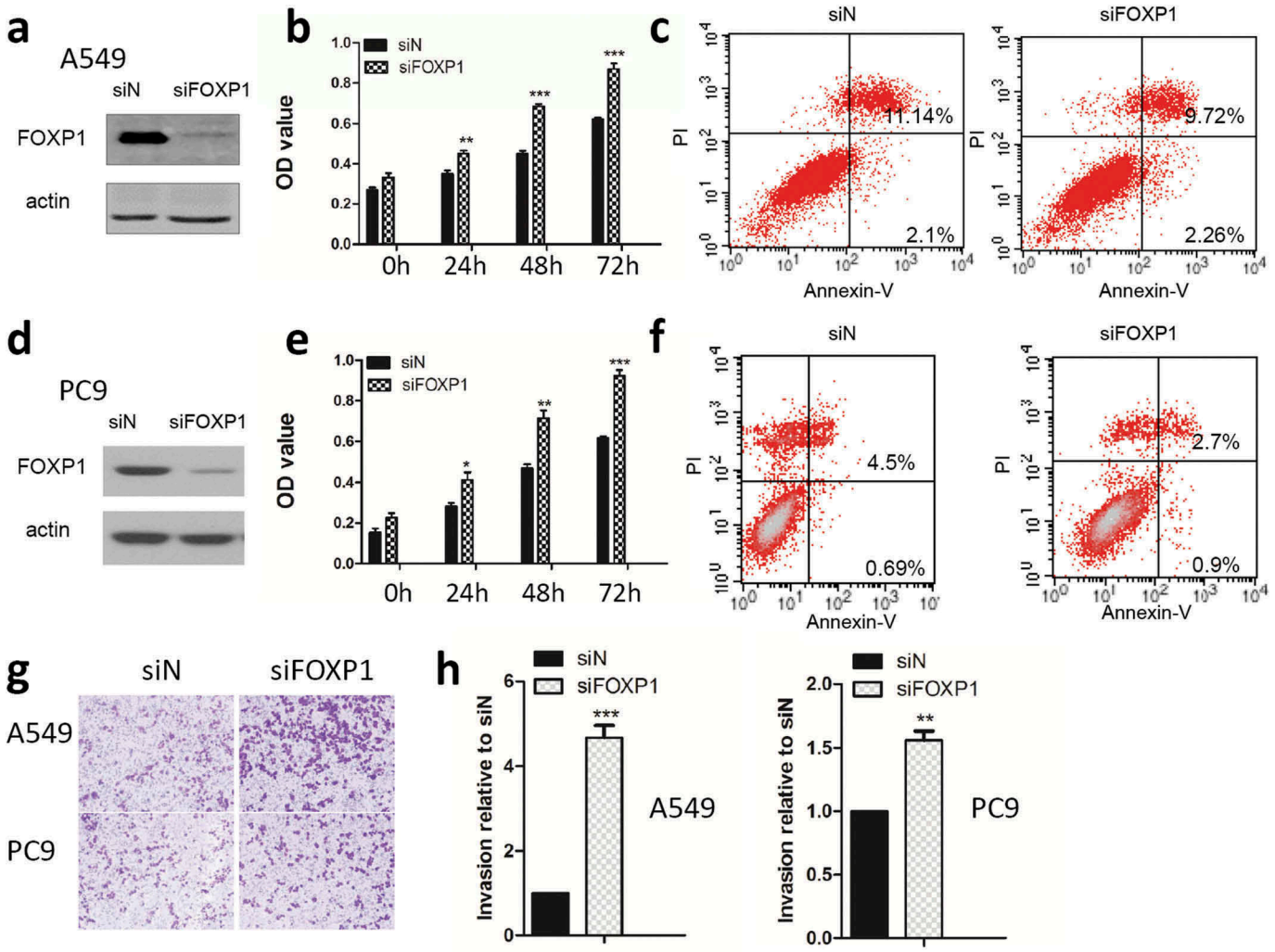

Figure 2. Knockdown of FOXP1 promotes the growth and invasion of lung cancer cells.

Western blot shows the protein expression of *FOXP1* in A549 (a) and PC9 (d) cells 48 hrs after siFOXP1 treatment. Bar graphs show OD values of A549 (b) and PC9 (e) cells at 24h, 48h, and 72h after the knockdown of *FOXP1*. Flow cytometry analysis shows the apoptosis of A549 (c) and PC9 (f) cells 48 hrs after the knockdown of *FOXP1*. Representative pictures (g) and summarized data (h) show that the invasion of A549 and PC9 cells 48 hrs after the knockdown of *FOXP1*. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

siRNA in addition to *FOXP1* siRNA transfection and measured the cancer cell growth and invasion. As shown in Figure 4, knockdown of *CCR1* reduced the growth and invasion of lung cancer cells. Furthermore, co-transfection of siRNAs targeting *CCR1* and *FOXP1* reversed the increase of A549 and PC9 cells growth induced by *FOXP1* siRNA. Similarly, the enhanced invasion of A549 and PC9 cells induced by *FOXP1* siRNA was also dramatically attenuated by the co-transfection of *CCR1* siRNA. In line with the critical role of *CCR1*, overexpression of *CCR1* dramatically decreased the expression of *ADCY5, GNG7*, and *VAV3*. However, the expression of *PLCB1* was not affected by *CCR1* overexpression (Figure 4e). To further explore the mechanism underlying the regulation of CCR1 by FOXP1, we performed luciferase assay. As shown in Figure 4f and g, the CCR1 promoter activity was significantly enhanced by the knockdown of FOXP1. These results indicate that *CCR1*-mediated downstream signaling pathway plays important roles in *FOXP1*-mediated tumor suppression.

Knockdown of *FOXP1* increased the tumor growth

To examine the role of *FOXP1* in lung cancer development in vivo, we utilized xenograft mouse model by injecting PC9 cells transfected control or *FOXP1* and *CCR1* shRNAs into both flanks of nude mice. As shown in Figure 5, 28 days after the injection, the size and weight of tumors induced by *FOXP1* shRNA-transfected PC9 cells were significantly larger than that induced by control shRNA-transfected PC9 cells. Furthermore, PC9 cells co-transfected with *FOXP1* and *CCR1* shRNAs failed to increase the size and weight of tumors compared with control shRNA-transfected PC9 cells. These results further confirm that *FOXP1* suppresses the lung cancer development via *CCR1*-mediated signaling pathway.

Discussion

FOXP1 is a member of the widely expressed *FOXP* subfamily of "forkhead" transcription factors that have a variety of functions in cellular proliferation, differentiation and

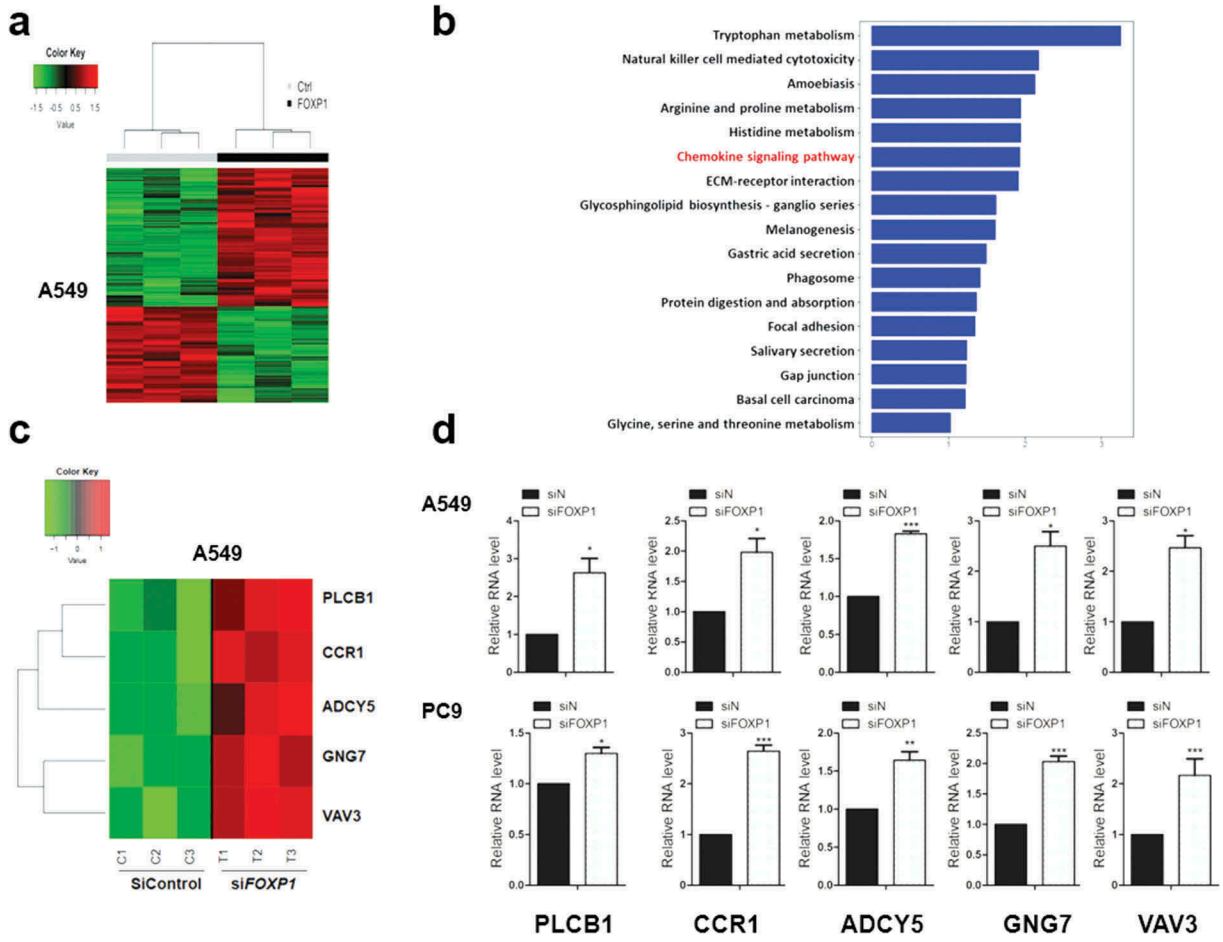

Figure 3. Chemokine signaling pathways are involved in *FOXP1*-mediated effect.
(a) Hierarchical clustering analysis reveals the alterations of transcripts in A549 cells treated with control or *FOXP1* siRNA. (b) KEGG analysis of those significantly changed genes shows their enrichment in chemokine signaling pathway. (c and d)The heatmap of representative genes (c) and the RT-PCR validations (d), showing the alterations of *CCR1, ADCY5, GNG7, VAV3*, and *PLCB1* after the knockdown of *FOXP1*. * $p < 0.05$, *** $p < 0.001$.

neoplastic transformation,[24] indicating that *FOXP1* plays important roles in cancer pathogenesis. In supporting the role of *FXOP1* in lung adenocarcinoma, we demonstrate that the expression of *FOXP1*was decreased in lung adenocarcinoma tissue using Oncomine analysis. Like other cancers, lung cancer is characterized by high growth rate and strong invasion of cancer cells. Downregulation of *FOXP1* in lung cancer cells by siRNA transfection dramatically increased the growth and invasion of lung cancer cells, PC9 and A549. Furthermore, the apoptosis of lung cancer cells was decreased when the expression of *FOXP1* was downregulated. In line with these in vitro data, we further demonstrated that downregulation of *FOXP1* enhanced the tumorigenesis of PC9 cells in cells an in vivo xenograft mouse model. These results indicate that *FOXP1* may play a protective role in lung cancer development via promoting apoptosis and inhibiting growth of cancer cells.

Chemokines have originally been recognized as critical mediators regulating the recruitment of immune cells.[25] Several lines of evidence demonstrated that cancer cells express multiple chemokines and chemokine receptors, which regulate the behaviors of cancer cells upon activation by chemokines.[26] Chemokine signaling pathways play important roles in cancer progression including tumor growth, senescence, epithelial mesenchymal transition, and immune evasion.[27] On the other hand, diverse chemokines regulate the function of T-cell, macrophage, and dendritic cells, which regulate the function of cancer cells in the microenvironment.[28] In the present study, we demonstrated that downregulation of *FOXP1* altered the expression of many genes, which are significantly enriched in chemokine signaling pathways, including *CCR1, ADCY5, GNG7, VAV3*, and *PLCB1*, indicating that chemokine signaling pathways are involved in *FOXP1*-mediated effects. *CCR1* has been shown to promote the tumor cell invasion and metastasis of colorectal cancer and liver cancer.[29,30] Consistently, we demonstrated that knockdown of *FOXP1* resulted in the increased expression of *CCR1*, which promotes the growth and invasion of lung cancer cells. Simultaneous knockdown of *CCR1* attenuated the pro-tumoral effect of *FOXP1* siRNA in lung cancer cells. These results indicate that downregulation of *FOXP1* promotes the tumorigenesis via the downstream chemokine signaling *CCR1*. In addition, we cannot rule out the possibility of that CCR1 may also act its function independent of FOXP1 in regulation of cancer development.

GNG7 is a highly specific promoter methylated gene associated with head and neck cancer. It has been shown that the expression of *GNG7* was reduced in esophageal

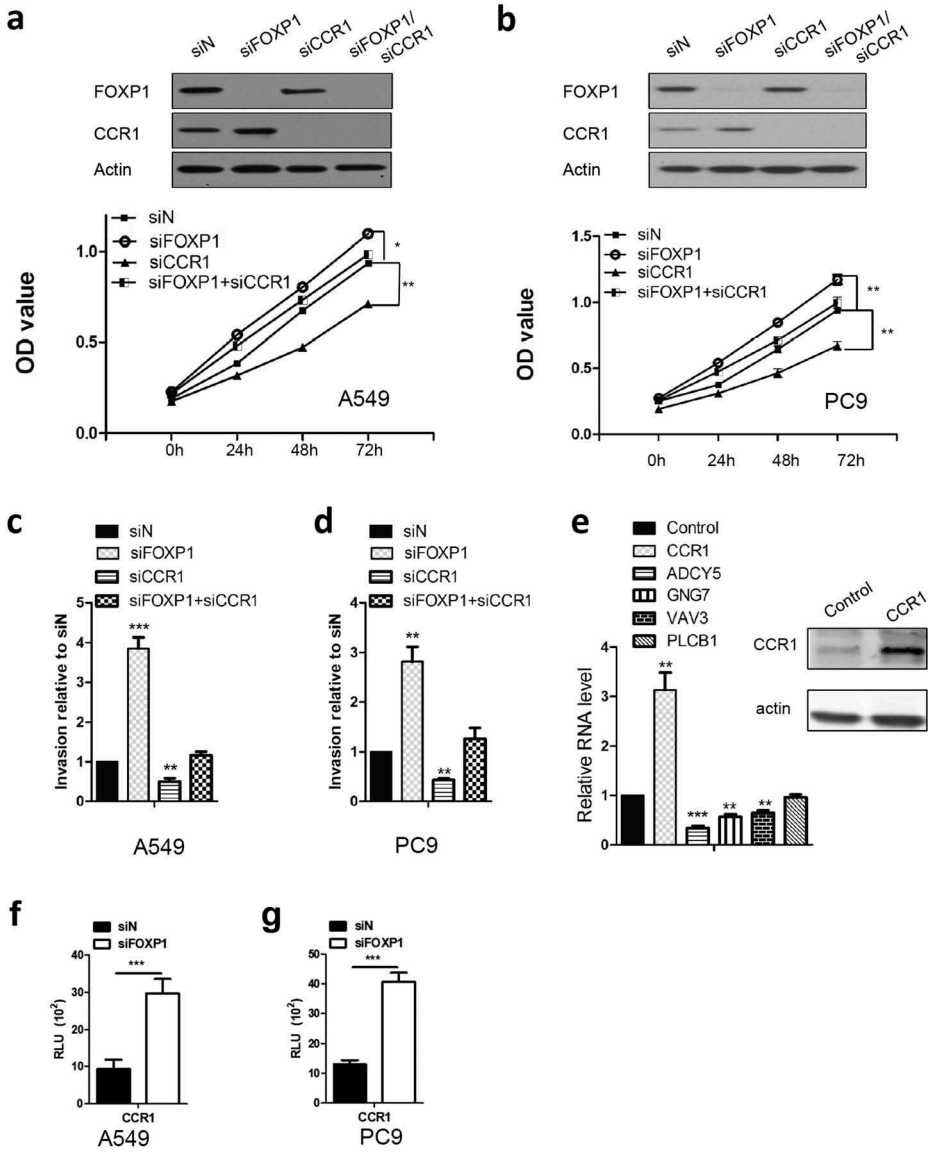

Figure 4. *CCR1* is required for *FOXP1*-mediated effect.

(a and b) OD values of A549 (a) and PC9 (b) cells at 24h, 48h, and 72h after siFOXP1, siCCR1, or siFOXP1+ siCCR1 treatment. Upper panels are Western blot images showing the FOXP1 and CCR1 expression 48 hrs after the treatment of different siRNAs. Actin serves as loading control. (c and d) Bar graphs show the relative invasion of A549 (c) and PC9 (d) cells 48 hrs after siFOXP1, siCCR1, or siFOXP1+ siCCR1 treatment. (e) RT-PCR shows the expression levels of *ADCY5, GNG7* and *VAV3* but not *PLCB1* 48 hrs after the overexpression of *CCR1*. Inset is the gel picture of Western blot examining the expression of *CCR1* in overexpressed cells. (f and g) Bar graphs show the luciferase activity in A549 (f) and PC9 (g) cells after 36 hrs of FOXP1 siRNA treatment. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

cancer and pancreatic cancer, indicating that *GNG7* functions as a tumor suppressor.[31,32] However, we demonstrated that the expression of *GNG7* was increased in lung cancer cells after the knockdown of *FOXP1*, suggesting that unlike in other cancers, GNG7 may exert oncogenic effect in lung cancer. *VAV3* is a guanine nucleotide exchange factor for Rho GTPases.[33] *VAV3* has been shown to promote breast cancer, prostate cancer, and skin tumor.[34–36] Similarly, we showed here that *VAV3* was increased after the knockdown of *FOXP1* and promoted the growth and invasion of lung cancer cells. Together with *ADCY5, GNG7* and *VAV3* are downstream targets of *CCR1* as overexpression of *CCR1* resulted in the decreased expression of *ADCY5, GNG7*, and *VAV3*. However, *PLCB1* was not affected by the overexpression of *CCR1*, suggesting that *PLCB1* might be involved in a distinct pathway following the *FOXP1* downregulation. Together, these results revealed previously unrecognized roles of *CCR1, ADCY5, GNG7, VAV3*, and *PLCB1* in *FOXP1*-mediated antitumor effect in lung cancer.

In summary, our study demonstrates that *FOXP1* is downregulated in lung cancer. Knockdown of *FOXP1* in lung cancer cells promotes the growth and invasion activities. Genes related to chemokine signaling pathways *CCR1, ADCY5, GNG7, VAV3*, and *PLCB1* are the downstream events of *FOXP1*-mediated effects. These results suggest that *FOXP1* many play a protective role in lung adenocarcinoma development and can be targeted for the development of novel diagnostic and therapeutic strategies for lung adenocarcinoma.

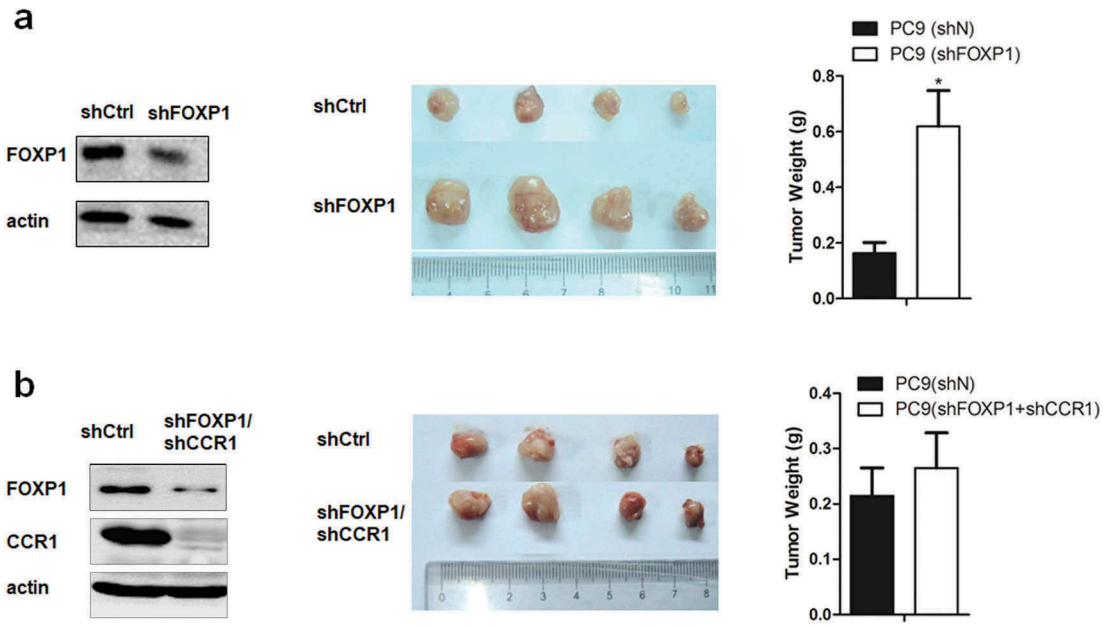

Figure 5. Downregulation of *FOXP1* promotes the tumorigenesis via *CCR1* in xenograft mouse model.
(a) Left panel shows the representative Western Blot image of PC9 cells treated with shFOXP1. Actin serves as the loading control. Middle panel shows the representative picture of tumors induced by PC9 cells treated with shCtrl or shFOXP1. Bar charts in the right panel show the weight of tumors generated by PC9 cells treated with shCtrl or shFOXP1. (b) Left panel shows the representative Western Blot image of PC9 cells treated with shFOXP1+ shCCR1. Actin serves as the loading control. Middle panel shows the picture of tumors induced by PC9 cells treated with shCtrl or shFOXP1+ shCCR1. Bar charts in the right panel show the weight of tumors generated by PC9 cells treated with shCtrl or shFOXP1+ shCCR1. * $p < 0.05$.

Materials and methods

Oncomine analysis

Oncomine, a cancer microarray database and web-based data mining platform, was used to compare mRNA level of *FOXP1* in lung cancer vs. normal patient datasets. The relative expression levels of genes were plotted using Graphpad Prism software.

Cell culture and sirna transfection

PC9 and A549 cells were cultured in RPMI and MEM, both of which were supplemented with 10% fetal bovine serum (FBS), 2 mmol/l glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin. Cells less than 30 passages were cultured in a humidified atmosphere of 95% air and 5% CO2 at 37°C. For transfection of siRNA, Lipofectamine® RNAiMAX Reagent (Invitrogen) was mixed with the siRNA construct according to the manufacturer's instructions. All the siRNAs used in this study were purchased from Sigma. The MISSION® siRNA Universal Negative Control (siN) obtained from Sigma was used as control siRNA. In detail, siFOXP1 (NM_001012505 > SASI_Hs01_00228672) and siCCR1 (NM_001295 > SASI_Hs01_00119718) were used here.

Luciferase assay

The promoter sequence of CCR1 (Sup. Table 1) was cloned into pGL4.10 reporter plasmid (Promega, USA) by PCR using forward primer (5′-3′ CGGCTAGCGAAGGAAGA ACACCAGAGACC) and reverse primer (5′-3′ ATGATAT CGGTATTTGTCATTGGCCTGG) with the digestion of the NheI/EcoRV sites. Firefly luciferase and Renilla luciferase were quantified with the Dual-Luciferase Reporter Assay system and the Stop & Glo Reagent kit according to the manufacturer's instruction (Promega, USA) after 36 hours of control siRNA or FOXP1 siRNA transfection.

MTT assay

PC9 and A549 cells were seeded in 96-well plates with 5000 cells/well. The cells were then treated with control or target siRNAs for 48 hr. MTT was added to the cells which were then cultivated for another 4 hr. Following the removal of the supernatant, DMSO (100 μl/well) was added to the cells which were agitated for 15 min. The absorbance was measured at 570 nm by an ELISA reader. Each assay was repeated three times.

Detection of apoptosis

Activation of apoptosis signaling in A549 and PC9 cells was assessed by Annexin V/PI double staining assay using FITC Annexin V Apoptosis Detection kits (Beyotime Institute of Biotechnology, Haimen, China). After treatments, cells were trypsinized, washed, and resuspended in Annexin V/PI binding buffer subjected to staining according to the manufacturer's instructions. The stained cells were analyzed by flow cytometry (BD Biosciences).

Western blot

Whole-cell lysates from PC9 and A549 cells were prepared and 20 μg of protein was separated using 10% SDS-PAGE gel.

After blocking with Tris-buffered saline/0.05%Tween 20 containing 5% skim milk or 3% bovine serum albumin (Sigma, St. Louis, MO), blots were incubated at 4 °C overnight with the primary antibodies against human FOXP1 protein (Abcam, ab16645), CCR1 (Abcam, ab13240), and actin (Abcam, ab8227), respectively. A horseradish peroxidase conjugated IgG was used as secondary antibody according to manufacturer's instruction.

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

The total RNA from PC9 or A549 cells was prepared using the Trizol reagent (Invitrogen, Carlsbad, CA). 1 μg of total RNA was treated with DNase I (Invitrogen) and the cDNA was synthesized in vitro from the mRNA template using SuperScript® III First-Strand Synthesis Kit (Invitrogen). cDNA was amplified by PCR using specific primer pairs. Taqman probes were taken to examine the gene expression, including CCR1 (Hs00928897_s1, ADCY5 (Hs00766287_m1), GNG7 (Hs00192999_m1), VAV3 (Hs00916818_m1), PLCB1 (Hs01001930_m1). Actin served as the internal control. The relative gene expression was calculated using the equation: $2^{-\Delta\Delta Ct}$.

Invasion assay

Lung cancer cell invasion was measured by using the Matrigel-coated transwell culture chambers as described previously.[37] Briefly, PC9 or A549 cells transfected with control or FOXP1 siRNA were maintained in serum-free-F12 medium in the upper chamber. The lower chamber was filled with 10% FBS-containing culture medium. Cells were incubated for 18 h at 37°C in a humidified atmosphere with 95% air and 5% O_2. The invaded cells penetrated through the Matrigel in the lower chamber were fixed and counted under a light microscope.

RNA sequencing and differentially expressed genes (DEGs) analysis

Three control and three *FOXP1* knockdown A549 cell lines were subjected to RNA sequencing. Total RNA was extracted 48 h after the FOXP1 siRNA or control siRNA transfected. With the RNA sequencing data prepared, the expression abundance (FPKM) value of each gene was estimated by running cufflinks,[38] and the differential expressed genes were assessed by cuffdiff. Only those genes with |fold change|> 2 and adjusted p value < 0.01 were recognized as statistically differentially expressed between two groups. The adjusted p value was obtained through applying Benjamini and Hochberg's (BH) false discovery rate correction on the original p value, and fold change threshold was selected based on our purpose of focusing on significantly differentially expressed genes.

Hierarchical clustering and GO analysis

We performed hierarchical clustering to classify analyzed samples based on gene expression profiles.[39] Hierarchical clustering was carried out using differentially expressed genes to observe the global gene expression patterns. We utilized R packages – GO.db to detect Gene Ontology categories with significant enrichment in DEGs comparing to which across all measured genes. The significantly enriched biological processes were identified as p value less than threshold value 0.01.

Xenograft mouse model

Following the methods described in the reference to implant PC9 cells subcutaneously into both flanks of nude mice at 2×10^6 cells in 100 μl per spot, the tumors were collected after the implantation about 28 days.[40] Control cells or those with either shFOXP1 or sFOXP1/shCCR1 were injected into opposite flanks. All animal experiments were approved by the Animal Care and Use Committee. ShRNA-FOXP1 (Sigma, SHCLNV-NM_032682> TRCN0000015664) and ShRNA-CCR1 (Sigma, SHCLNV-NM_009912> TRCN00 00027778) were introduced to cell using Lentiviral system

Statistical analysis

Data are presented as mean ± s.e.m. of three independent experiments. Significance of means between two groups is determined by student's t-test. Difference in cell growth between control and PRAME siRNA-treated groups was evaluated by repeated measures analysis of variance (ANOVA). A P-value of < 0.05 was considered significantly different.

Acknowledgments

Not applicable.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Funding

This work is supported by Project of Shanghai Municipal Commission of Health and Family Planning (No. 20134084).

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