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Original Article

Effect of IL-13Rα2 on TGF-β expression and collagen synthesizing induced by IL-13 in human lung fibroblasts HFL-1

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Abstract: IL-13 is a pro-fibrotic cytokine that can bind to two receptors, IL-13Rα1 and IL-13Rα2. IL-13Rα2 used to be regarded as a decoy receptor. However, recent studies indicated that it may regulate TGF-β generation. To determine whether IL-13Rα2 participate in induction of TGF-β expression in pulmonary fibroblasts, we treated human lung fibroblasts HFL-1 cells with 0, 10, 20, 50, and 100 ng/ml of IL-13 and measured the expression of IL-13Rα1, IL-13Rα2, TGF-β and collagen I with qRT-PCR and/or Western blotting. The results showed that exogenous IL-13 increased the expression of IL-13Rα2, TGF-β and collagen I in a dose-dependent manner. However, no significant change in IL-13Rα1 expression was observed. Moreover, after silencing IL-13Rα2, the expression of IL-13Rα2 mRNA and protein was significantly decreased ($P<0.01$) while TGF-β and collagen I expression was further increased ($P<0.05$). Taken together, these results indicated that IL-13Rα2 may mainly participate in inhibiting the expression of TGF-β and production of collagen I in IL-13-stiumlated human lung fibroblasts HFL-1.

Keywords: IL-13, IL-13Rα2, TGF-β, collagen I, human lung fibroblasts, HFL-1

Introduction

Pulmonary fibrosis (PF) constitutes the end stage of a broad range of heterogeneous interstitial lung diseases, characterized by the destruction of the pulmonary parenchyma, deposition of extracellular matrix and dramatic changes in the phenotype of both fibroblasts and alveolar epithelial cells [1]. The most common form of PF is idiopathic pulmonary fibrosis (IPF), which affects approximately five million people worldwide. It is a chronic, progressive and fatal interstitial lung disease with a median survival time of only two or three years after diagnosis [2, 3]. The pathologic mechanisms which account for disease progression are poorly understood and no effective treatment is available. Thus, there is an urgent need for an improved understanding of the molecular mechanisms underlying the aggressive of IPF.

Interleukin 13 (IL-13), mainly secreted by Th2 cells, is a pro-inflammatory cytokine with a molecular weight of 12 KDa [4]. It has been shown to play an important role in many inflammatory and fibrotic diseases such as asthma, idiopathic pulmonary fibrosis, systemic sclerosis, and pulmonary granulomatous disease [5-8]. IL-13 binds to two structurally and functionally distinct receptors IL-13 receptor α1 (IL-13Rα1) and IL-13 receptor α2 (IL-13Rα2). Both IL-13Rα1 and IL-13Rα2 are overexpressed in malignant glioma and other cancer cell types, including AIDS-associated Kaposi's sarcoma tumor cells (AIDS-KS), squamous cell carcinoma of head and neck (SCCHN), pancreatic ductal adenocarcinoma and ovarian cancer [9-13]. IL-13 can bind to IL-13Rα1 and the affinity between them increases significantly after the formation of an IL-4Rα/IL-13Rα1 complex that activates the STAT6 pathway to promote prolif-

Table 1. The sequence of primer

Gene	Primer	Sequence (5'-3')
IL-13Rα2	Forward	ACCTTTGCCGCCAGTCTATCTTAC
	Reverse	TGTCGGGTTTCATTTGTTGTTTTC
IL-13Rα1	Forward	GCACCAATGAGAGTGAGAAGC
	Reverse	GGAACAACCAAAGTATTGGCC
TGF-β	Forward	AAATCGTGAACTTTGTCTCCGT
	Reverse	CCCAGTGCCCTCTACTCTCAT
GAPDH	Forward	AGCCACATCGCTCAGACAC
	Reverse	GCCCAATACGACCAAATCC

eration and/or apoptotic resistance [2]. Compared with IL-13Rα1, IL-13Rα2 has a higher affinity to IL-13 [14]. Since the cytoplasmic tail of IL-13Rα2 is much shorter than that of IL-13Rα1 and lacks structures for signal transduction, IL-13Rα2 was regarded as a "decoy receptor" for a long period of time [15]. However, recent studies reported that IL-13 might bind to transmembrane monomeric IL-13Rα2 to activate downstream effectors [16-18].

Transforming Growth Factor-β (TGF-β) is an important factor in regulating cell growth, migration, differentiation, immune regulation, and extracellular matrix synthesis. As one of the key fibrogenic cytokines, TGF-β generates multiple molecular pathways associated with lung fibrosis [19-22]. Mainly depending on Smad, TGF-β can signal through Smad2/3/4 complexes with co-Smad factors to translocate into nuclear thus activating a variety of fibrosis-related transcription factors (TFs). More importantly, TGF-β is thought to drive collagen deposition which is a key process to the initiation and progression of lung fibrosis [23]. Several reports have shown that IL-13Rα2 plays a signaling role that ultimately leads to oxazolone-induced colitis, bleomycin-induced mice pulmonary fibrosis and allograft fibrosis by the AP-1 (activator protein-1)/TGF-β signaling pathway [16, 24]. But the molecular signaling mechanism triggered by IL-13 binding to IL-13Rα2 is still not entirely clear.

Our study mainly investigated the expression of IL-13Rα1, IL-13Rα2 and TGF-β after stimulating with exogenous IL-13 (building IL-13-induced HFL-1 fibrosis model at the same time), then observed the expression of TGF-β and the production of collagen after siRNA silencing of *IL-13Rα2* gene. The present study aimed to elucidate the relationship between IL-13Rα2 signaling pathway and TGF-β expression in human lung fibroblasts in order to provide a theoretical basis for the pathogenesis of IPF.

Materials and methods

Reagents

Recombinant Human interleukine-13 (rhIL-13) was purchased from Peprotech (USA). The total RNA Kit II was purchased from Omega Company (USA). The Anti-IL-13Rα2 monoclonal antibody and the Anti-TGF-β ploYclonal antibody were purchased from Abcam (USA), the collagen I antibody was purchased from Affinity (USA). The lipofectamine 2000 was from Invitrogen (USA).

Cell line, cell culture and transfection

Human lung fibroblast (HFL-1) cell line was purchased from cell bank of the Chinese Academy of Sciences, Shanghai, China and it was cultured in DMEM high glucose medium (Hyclone, USA), supplemented with 10% fetal bovine serum (Biological Industries, USA). 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C in a humidified 5% CO_2 atmosphere. To investigate the effect of IL-13 on HFL-1 cells, rhIL-13 was added after serum-free culture of HFL-1 cells overnight. All transfections were carried out using lipofectamine 2000 (Invitrogen, USA) in serum-free conditions according to manufacturer's instructions. IL-13Rα2-small interfering (si) RNA and negative control-siRNA were synthesized directly (GenePharma, Shanghai, China) and transfected into cells at a final concentration of 50 nM. The sequence of IL-13Rα2-siRNA was designed by GenePharma (5'-GCCUAUCAGAUCCAGUUAUTT-3').

Ribonucleic acid (RNA) extraction and quantitative real time-polymerase chain reaction (qRT-PCR) analysis

The total RNA of HFL-1 cells or tissue specimens were extracted using the Total RNA kit II (Omega, USA) according to the user's manual. Reverse transcription was performed with a reverse transcriptase kit (Transgen, Beijing, China) using Oligo dT Primer (Transgen, Beijing, China). The mRNA levels were verified by quantitative real time-polymerase chain reaction (qRT-PCR) using Tip Green qPCR Mix (Transgen, Beijing, China) and were performed on an ABI 7500 system (Applied Biosystems, USA). Glyceraldehyde 3-phosphate dehydrogenase

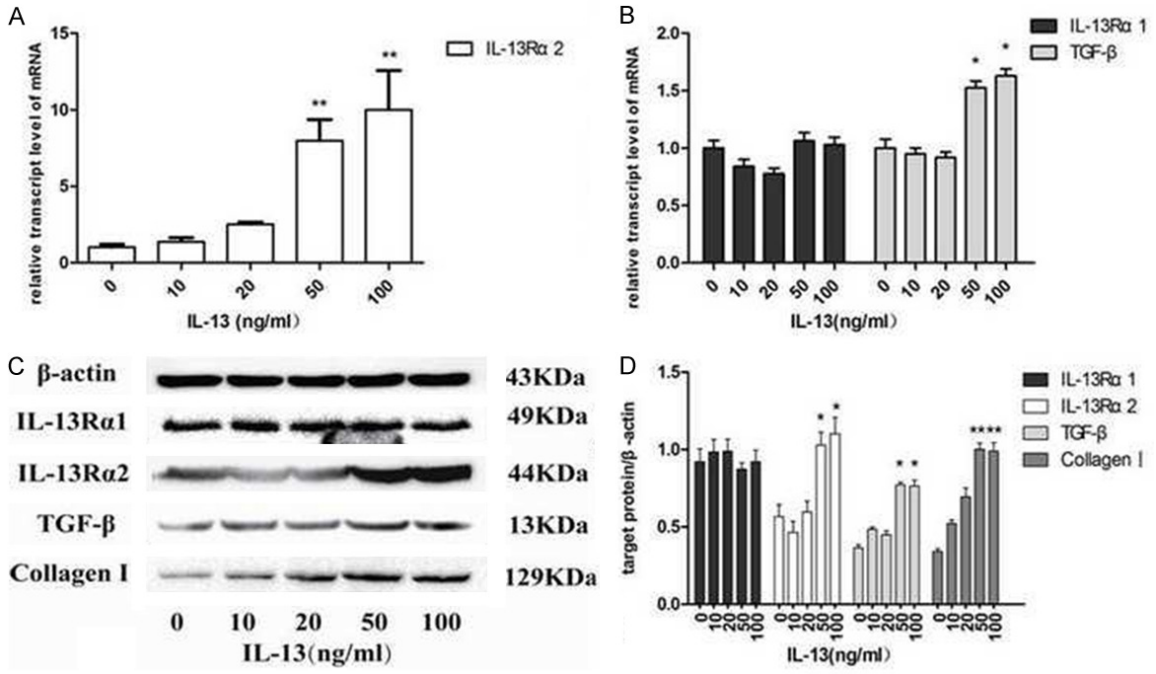

Figure 1. IL-13Rα2, TGF-β and collagen I expression increased after stimulate with high concentration (50/100 ng/ml) of IL-13 on the human lung fibroblast (HFL1). (A) q-PCR analysis of the gene IL-13Rα2 expression and (B). q-PCR analysis of the gene IL-13Rα1 and TGF-β expression and (C). Western blot analysis of the protein expression of IL-13Rα1, IL-13Rα2, TGF-β and collagen I. The IL-13Rα1 band (49 kDa), the IL-13Rα2 band (44 kDa), the TGF-β band (13 kDa), the collagen I band (129 kDa) and the β-actin band (43 kDa) are indicated. (D) Blots obtained from several experiments were analyzed using densitometry, and the densitometric values were pooled and shown as means ± SD. Protein loading was normalized based on β-actin. *P<0.05, **P<0.01 as compared with 0 ng/ml group.

(GAPDH) was used as an internal control for normalization, and the primer sequence used for qRT-PCR analysis is listed in **Table 1**. All samples were run in triplicate and the relative HFL-1 mRNA level of each sample normalized to the internal control was calculated using the $2^{-\Delta\Delta CT}$ method.

Western blotting analysis

Cells was lysed in RIPA buffer (Applygen, Beijing, China) with protease inhibitors (Sangon Biotech, Shanghai, China) and centrifuged. The protein concentrations were then separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Solarbio, Beijing, China) which were activated by methanol. After blocking with 5% skimmed milk for two hours, the membranes were incubated overnight at 4°C with diluted (1:1000) primary antibodies (monoclonal mouse anti-IL-13Rα2, β-actin, polyclonal rabbit anti-TGF-β, collagen) and then subjected to incubation with horseradish peroxidase-conjugated secondary a bands were finally detected by electrochemiluminescence kit (Thermo, USA). An antibody against β-actin (Transgen, Beijing, China) confirmed equal loading of samples. The band density was quantified by Quantity One 4.6 software (Bio-Rad, Hercules, USA) and the protein level was normalized to β-actin protein.

Immunofluorescence

HFL-1 cells seeded on glass slides were fixed in 4% poly formaldehyde, permeabilized by 0.5% Triton X100 in PBS, blocked with 10% goat serum albumin, and incubated with anti-IL-13Rα2 (Abcam, USA, working concentration 1:200) and anti-TGF-β (Abcam, USA, working concentration 1:200) overnight at 4°C. Cells were washed and incubated with goat anti-mouse/goat anti-rabbit secondary antibodies for 60 min in the dark. Cells were washed three times, mounted with 4'6-diamidino-2-phenylindole (DAPI)-containing mounting media (ZSGB-BIO, Beijing, China), and visualized using a fluo-

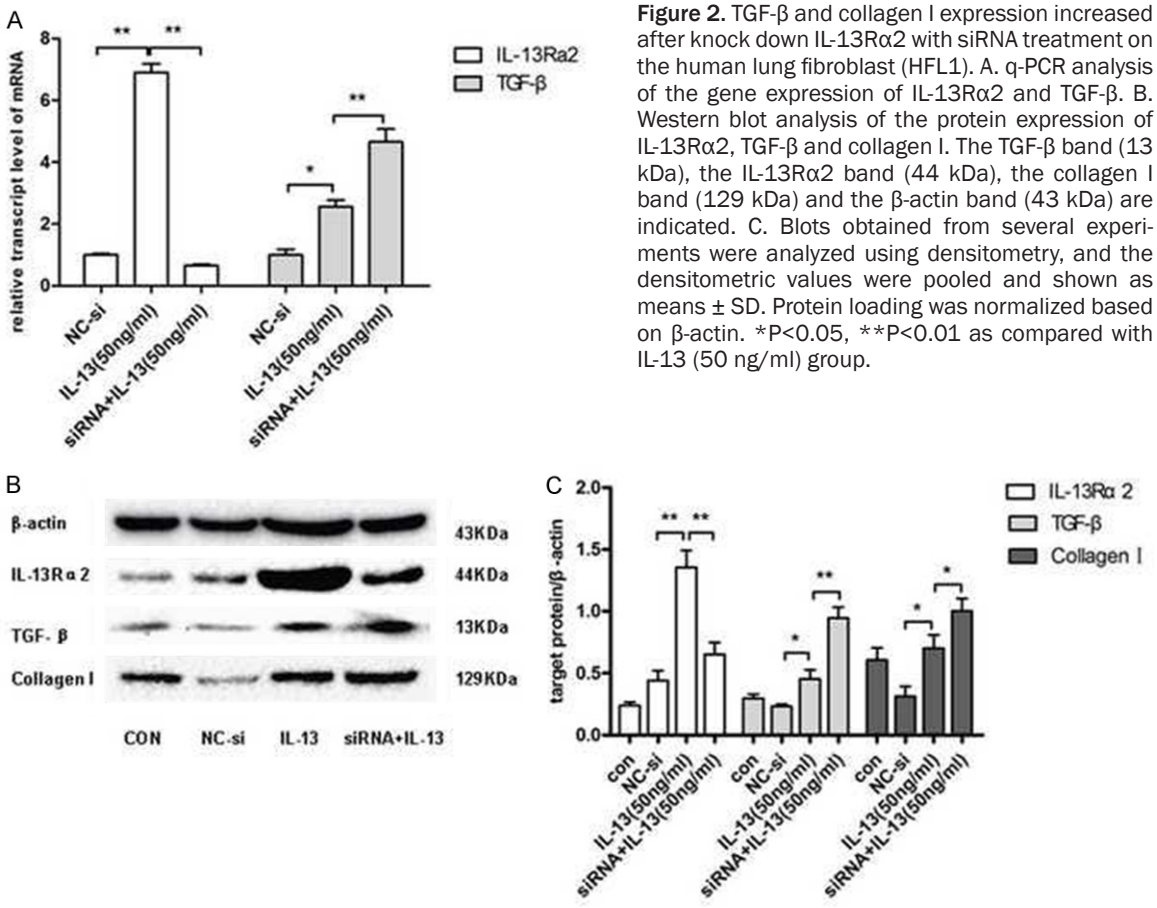

Figure 2. TGF-β and collagen I expression increased after knock down IL-13Rα2 with siRNA treatment on the human lung fibroblast (HFL1). A. q-PCR analysis of the gene expression of IL-13Rα2 and TGF-β. B. Western blot analysis of the protein expression of IL-13Rα2, TGF-β and collagen I. The TGF-β band (13 kDa), the IL-13Rα2 band (44 kDa), the collagen I band (129 kDa) and the β-actin band (43 kDa) are indicated. C. Blots obtained from several experiments were analyzed using densitometry, and the densitometric values were pooled and shown as means ± SD. Protein loading was normalized based on β-actin. *P<0.05, **P<0.01 as compared with IL-13 (50 ng/ml) group.

rescence microscope (Leica Microsystems, Germany).

Statistical analysis

All statistical analyses were performed using SPSS 20 and Graphpad prism 6.0. All data were expressed as mean ± SD and subjected to statistical analysis one-way ANOVA. Differences were taken statistically significant when $P<0.05$.

Results

IL-13Rα2, TGF-β and collagen I expression increased after IL-13 stimulation in a dose-dependent manner

After HFL-1 lung fibroblasts were stimulated by IL-13 at high concentrations (50, 100 ng/ml), and exogenous IL-13 stimulation had no detectable effect on IL-13Rα1 expression, q-PCR and western blot assays showed that the levels of mRNA and protein expression of IL-13Rα2, TGF-β and collagen I were increased proportionally to the concentration of the stimulating IL-13. The 100 ng/ml group showed the biggest increase in IL-13Rα2 and TGF-β expression as well as the 50 ng/ml group had the biggest change rate among treatment groups and this change was significant by comparison with 0 ng/ml ($P<0.01$) (**Figure 1**).

TGF-β and collagen I expression increased after IL-13Rα2 siRNA treatment

We added 50 ng/ml IL-13 (the biggest change rate) to stimulate HFL-1 lung fibroblasts and observed a significant increase in the expression of IL-13Rα2 ($P<0.01$). After IL-13Rα2 was silenced by siRNA transfection, the mRNA and protein levels of IL-13Rα2 were significantly reduced ($P<0.01$). In addition, the expression of TGF-β and collagen I were low before siRNA silencing of IL-13Rα2 and significant increase in TGF-β expression occurred after IL-13 stimulation ($P<0.05$). After IL-13Rα2 silencing, the expression of TGF-β and collagen I significantly increased again ($P<0.05$) (**Figure 2**).

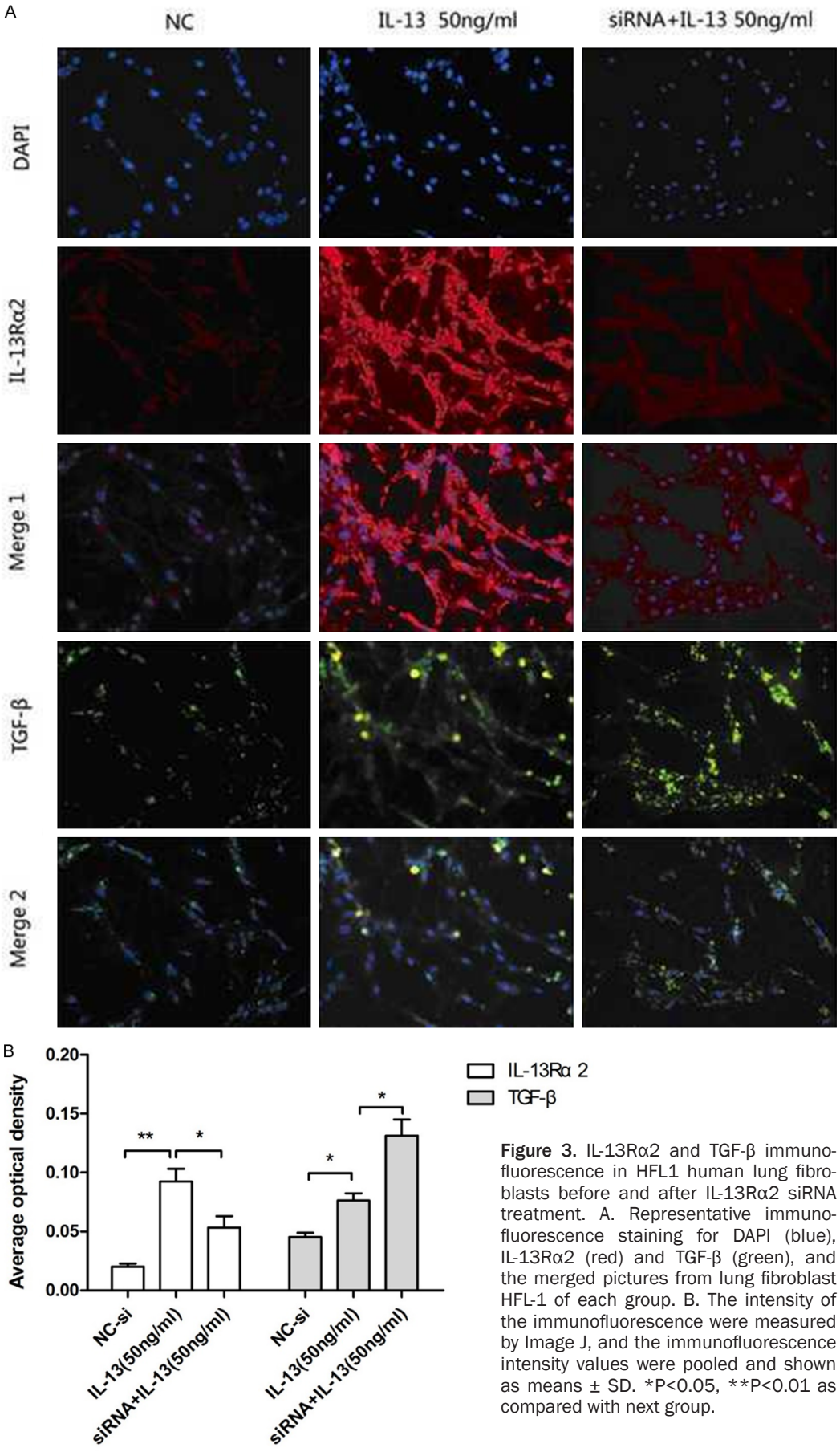

Figure 3. IL-13Rα2 and TGF-β immunofluorescence in HFL1 human lung fibroblasts before and after IL-13Rα2 siRNA treatment. A. Representative immunofluorescence staining for DAPI (blue), IL-13Rα2 (red) and TGF-β (green), and the merged pictures from lung fibroblast HFL-1 of each group. B. The intensity of the immunofluorescence were measured by Image J, and the immunofluorescence intensity values were pooled and shown as means ± SD. *P<0.05, **P<0.01 as compared with next group.

Immunofluorescence double labeling revealed a decrease in IL-13Rα2 and an increase in the expression of TGF-β after IL-13Rα2 silencing

We measured the expression of IL-13Rα2 and TGF-β in HFL-1 human lung fibroblasts after IL-13 and IL-13Rα2 siRNA treatments using immunofluorescence labeling. Cellular localization was determined by staining nuclei with DAPI. The results showed that the expression of both IL-13Rα2 and TGF-β increased in the IL-13 (50 ng/ml) group. After IL-13 stimulation was applied, the expression of IL-13Rα2 and TGF-β significantly increased ($P<0.05$), after siRNA treatment, IL-13Rα2 expression significantly decreased in the siRNA + IL-13 (50 ng/ml) group ($P<0.05$) and TGF-β expression further increased in the siRNA + IL-13 group ($P<0.05$) by comparison with the IL-13 (50 ng/ml) only group (**Figure 3**). This result was consistent with the q-PCR results.

Discussion

This is the first study to supply the relationship between IL-13Rα2 and TGF-β in IL-13-induced HFL-1 fibrosis model *in vitro*. We investigated the role of IL-13Rα2 in HFL-1 lung fibrosis model with exogenous IL-13 stimulation to determine whether it may induce TGF-β expression or only as a decoy receptor. Exogenous high concentrations (50, 100 ng/ml) of IL-13 stimulation increased the expression of IL-13Rα2, TGF-β and collagen I but did not affect IL-13Rα1 expression (**Figure 1**). Following siRNA silencing of IL-13Rα2, the mRNA and protein expression of IL-13Rα2 decreased, while the mRNA and protein expression of TGF-β further increased. Besides, the production of collagen I increased too (**Figure 2**). This finding was further verified by immunofluorescence labeling experiments (**Figure 3**). Based on these results, we believe that IL-13Rα2 is not capable of inducing TGF-β generation and may not be associated with intracellular signaling transduction, in other word, cannot lead to lung fibrosis.

TGF-β, especially TGF-β1, is the strongest known inducing factor for extracellular matrix accumulation. TGF-β activates TβRII, sequentially phosphorylates TβRI, Smad2, and Smad3, finally Smad4. Smad2/3/4 tripolymer translocates into nuclear to promote TFs through interaction with specific binding motifs in gene regulatory regions and those abnormal expressing TFs are thought to cause a series of fibrosis-related processes [25]. The argument of IL-13Rα2 mainly focuses on this fibrogenic cytokines. The pioneers to raise the IL-13Rα2/TGF-β signaling transduction pattern were Fichtner-Feigl S *et al* [16] in rodent models of oxazolone-induced colitis, bleomycin-induced pulmonary fibrosis, they observed that IL-13Rα2 could induce TGF-β1 expression through the binding of AP-1 to *TGF-β* promoter, thereby enhancing fibrosis. Similarly, Brunner SM *et al* [24] found that in allograft models of fibrosis, IL-13Rα2 could induce TGF-β1 generation, resulting in graft fibrosis. In addition, Fujisawa T *et al* [10] reported that IL-13Rα2 was a key receptor binding chain for its ligand IL-13 that favored the production of MMPs, through MAPK/AP-1 pathway after binding, which in turn, initiates invasion and metastasis in ovarian cancer. However, our data does not agree with those who think IL-13Rα2 has its own signaling transduction (**Figures 2**, **3**). After silencing IL-13Rα2, we observed that the expression of TGF-β and collagen I increased, whatever in qPCR, Western blot or immunofluorescence, indicating that IL-13Rα2 may inhibit fibrosis. It is not a unique instance, but has its counterpart. Recent reports of Lumsden R V [26] and Chandriani S [27] were also quite correspondent to our results. Lumsden R V found that overexpression of IL-13Rα2 inhibited the IL-13 induction of fibrotic markers (including soluble collagen, TGF-β, and CCL17) as well as Chandriani S showed that endogenously expressed IL-13Rα2 does not activate any unique IL-13-mediated gene expression patterns.

IL-13Rα1 and IL-13Rα2 are two receptors for IL-13 which is also a fibrogenic cytokine. As a transmembrane receptor, IL-13Rα1 can combine with IL-4Rα to form stable complexes [28]. These complexes then activate the JAK/STAT signaling pathway to induce TGF-β generation, eventually leading to fibrosis [29]. So increasing the concentration of IL-13 resulted in increased TGF-β expression (**Figure 1**). Turning to IL-13Rα2, STAT6 which activated by IL-4 or IL-13 acting through IL-13Rα1 and NF-κB which activated by TNF-α acting through its receptor, transactivate the *IL-13Rα2* promoter [16]. However the short cytoplasmic tail and lacking of signaling transducing structures limit IL-13Rα2 to have its own signaling pathway [26]. Therefore, "decoy receptor" becomes its longstanding

label. Different from constitutively expression receptor IL-13Ra1, IL-13Rα2 has three subtypes listing as soluble IL-13Rα2 (sIL-13Rα2), intracellular IL-13Rα2 (iIL-13Rα2) and membrane IL-13Rα2 (memIL-13Rα2). Three subtypes are able to circulate between each other and this mechanism might have a relationship with our finding. Compared with those using drug stimulation to build the tissue fibrosis model, we used the IL-13-induced HFL-1 fibrosis model in this study. Generally, the content of IL-13Rα2 is quite low in normal human tissues and sIL-13Rα2 occupies a very small fraction of all IL-13Rα2 [30]. Previous reports about IL-13Rα2 signaling transduction in a large scale showed the function of memIL-13Rα2 which has the possibility to lead signaling pathway. However, the IL-13Rα2 which was stimulated by high concentration IL-13 might exist in sIL-13Rα2 format in our model. SIL-13Rα2 and memIL-13Rα2 are actually different splice variants of the *IL-I3Rα2* gene, with the sIL-13Rα2 lacking the transmembrane domain of the molecule and therefore lacks signaling ability [31]. The study of Chen W *et al* [32] showed that unlike mice, human lacked IL-13Rα2 in serum. They also revealed that sIL-13Rα2 was derived exclusively from the memIL-13Rα2 transcript in humans through MMPs/MMP-8 cleavage of memIL-13Rα2 and those derived-sIL-13Rα2 further bind IL-13 with high affinity hence sequestered IL-13 away from IL-13Ra1. Notably, TGF-β dominates in fibrosis when concentration of IL-13 is limited because the induction of TGF-β through the memIL-13Rα2 has an amplifying effect on IL-13-mediated induction of fibrosis as well as IL-13 is probably able to promote TGF-β by more direct situations. On the contrary, when HFL-1 is stimulated with large amounts of IL-13, sIL-13Rα2 becomes paramount to compete with IL-13Rα1 but cannot fully reverse IL-13Rα1-induced fibrosis. What's more, the increasing sIL-13Rα2 can also recombine with TβRII to reduce the expression of the signal transduction proteins Smad3 and STAT6 [33].

Taken together, our study provides the primary evidence for the function of IL-13Rα2 in IL-13-stimulate HFL-1 fibrosis model *in vitro*. We found that under high concentrations of IL-13 in HFL-1 cell line, IL-13Rα2 did not exhibit its TGF-β-related signaling transduction pattern which might attribute to the stimulation of sIL-13Rα2. However, this study mainly worked on the total expression level of IL-13Rα2. Further experiments on this subject are required to clarify the expression of sIL-13Rα2 and memIL-13Rα2 and provide more precise mechanisms on IL-13-meditated IPF.

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Disclosure of conflict of interest

None.

Authors' contribution

Xiang-Tong Lu is responsible for making experiment. Yu Zhang is in charge for thesis writing and modification. Ran Wei is in charge of making chart. These authors contributed equally to this work.

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