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Hydroxysafflor yellow A ameliorates transforming growth factor-β1-triggered fibroblast activation *via* inactivation of the NF-κB/STAT3 pathway by suppressing ADAM17 expression

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Abstract. The abnormal proliferation and activation of fibroblasts have been implicated in idiopathic pulmonary fibrosis. Herein, the present research explored the impacts of the relationship between hydroxysafflor yellow A (HSYA) and a disintegrin and metalloproteinase 17 (ADAM17) on fibroblast activation, which can provide novel insight into the treatment and management of idiopathic pulmonary fibrosis. MRC-5 fibroblasts were firstly activated with TGF-β1, followed by measurement of ADAM17 expression through qRT-PCR and Western blot. Fibrosis-related gene and protein expression levels, cell viability, proliferation, migration, and fibroblast-to-myofibroblast transdifferentiation were determined by qRT-PCR and Western blot, MTS, EdU, Transwell, and immunofluorescence assays, respectively. Moreover, the regulatory relationships among HSYA, ADAM17, and the NF-κB/STAT3 pathway in MRC-5 cells were analyzed by bioinformatics analysis, qRT-PCR, and Western blot. The results show that HSYA treatment could diminish the fibrosis-related gene and protein expression patterns, proliferation, migration, and fibroblast-to-myofibroblast transdifferentiation in TGF-β1-stimulated MRC-5 cells. Moreover, HSYA could repress the TGF-β1-triggered ADAM17 up-regulation, thereby suppressing the NF-κB/STAT3 pathway. Furthermore, over-expression of ADAM17 negated the inhibitory effect of HSYA on fibroblast activation induced by TGF-β1. The findings revealed that HSYA blocked the NF-κB/STAT3 pathway activation by down-regulating ADAM17, thereby inhibiting TGF-β1-induced fibroblast activation.

Key words: Transforming growth factor-β1 — Hydroxysafflor yellow A — ADAM17— Fibroblast activation — NF-κB/STAT3 pathway

Introduction

Idiopathic pulmonary fibrosis (IPF), as a global life-threatening, chronic, and progressive interstitial pulmonary disease, is construed as a predominant mode of idiopathic interstitial pneumonia (Zhang et al. 2018; Kishaba 2019). IPF occurs typically in the elderly population with a median age at diagnosis of roughly 65 years, and IPF is more prevalent in men and smokers (Hewlett et al. 2018). This disease is featured by the disruption of alveolar structure and the replacement of

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healthy tissues with altered extracellular matrix, which leads to disrupted gas exchange, reduced lung compliance, respiratory failure, and even death (Richeldi et al. 2017). Additionally, fibrotic diseases, including IPF, are defined by uncontrolled fibroblast activation (Epstein Shochet et al. 2020). Therefore, it is of interest for the treatment of IPF to study the molecular mechanisms behind fibroblast activation.

Hydroxysafflor yellow A (HSYA) is well-established as an active component of safflower (*Carthamus tinctorius*), which possesses various biological and pharmacological activities including pro-angiogenic, anticancer, antioxidant, and anti-inflammatory activities (Chen et al. 2020; Zhang et al. 2020). Among mice with pulmonary fibrosis, HSYA could reduce transforming growth factor (TGF)-β1 expression, collagen deposition, and lung consolidation (Jin et al. 2016). In another study, HSYA could markedly alleviate small airway thickening and collagen deposition caused by subepithelial fibrosis and lower TGF-β1 expression in rats afflicted with chronic obstructive pulmonary disorder (Wang et al. 2014). Hence, the potential mechanisms of HSYA in fibroblast activation still needs to be further studied to obtain a new IPF therapy.

In our study, we predicted a disintegrin and metalloproteinase 17 (ADAM17) as HSYA target proteins *via* PharmMapper website (http://www.lilabecust.cn/pharmmapper/submitfile.html). Numerous studies have confirmed that ADAM17 has been linked to a variety of lung diseases including lung cancer, pulmonary emphysema, and diffuse parenchymal lung diseases (Saad et al. 2019, 2021; Urban et al. 2021). Moreover, ADAM17 is involved in the TGF-β1 induction of connective tissue growth factor (CTGF) expression in the context of lung epithelial cells, which are the origin of most fibroblasts in pulmonary fibrosis (Ou et al. 2020). Notably, ADAM17 could down-regulate angiotensin-converting enzyme 2 and participate in pulmonary fibrosis (Uhal et al. 2013). Additionally, bioinformatics analysis in our assay uncovered the action of ADAM17 on the signal transducer and activator of transcription 3 (STAT3) pathway. STAT3 is an important mediator of pulmonary fibrosis, and the activation of STAT3 pathway is widely related to the progression of IPF (Prele et al. 2012; Waters et al. 2018). Based on the aforementioned findings, we hypothesized that HSYA might attenuate fibroblast activation through the ADAM17 and STAT3 signaling pathways. TGF-β1 is important in mediating IPF initiation because it promotes epithelial apoptosis, fibroblast proliferation, fibroblast to myofibroblast transformation, collagen synthesis, and epithelial-mesenchymal transformation (Tatler and Jenkins 2012). Wolters et al. (2014) have found that the pathobiology of IPF was associated with TGF-β1 activation. To elucidate the effects of HSYA, ADAM1 and STAT3 pathways, and their interactions on fibroblast activation, TGF-1 was used to stimulate human fetal lung fibroblasts.

Materials and Methods

Cell culture

The purchased MRC-5 human fetal lung fibroblasts (Chinese Academy of Medical Sciences, Beijing, China) were firstly cultured with a minimum essential medium (MEM, Thermo Fisher Scientific, Waltham, MA, USA) encompassing streptomycin (100 μg/ml), penicillin (100 μg/ml), and fetal bovine serum (10%) (all from Thermo Fisher Scientific) in an incubator (5% CO_2, 37°C). Thereafter, fibroblasts were activated with TGF-β1 by referring to literature (Pan et al. 2016). Specifically, the cells were subjected to 24 h culture in the serum-free MEM till 80–90% confluence and received 12 h starvation. Afterwards, the cells were subjected to pretreatment without or with HSYA [HSYA at low concentration (HSYA-L): 5 μmol/l; HSYA at medium concentration (HSYA-M): 15 μmol/l; HSYA at high concentration (HSYA-H): 45 μmol/l] (Pan et al. 2016) for 30 min, followed by 48 h culture in a medium without or with TGF-β1 (0, 2.5, 5, and 10 nmol/l) (Wu et al. 2020). The cells were harvested for further analysis (Chen et al. 2021).

Cell transfection

MRC-5 cells were subjected to transfection with ADAM17 over-expression plasmid (oe-ADAM17), and its corresponding negative control (oe-NC) (GenePharma, Shanghai, China) with the help of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Specifically, MRC-5 cells were evenly spread into 6-well plates and cultured to approximately 70% confluence. Thereafter, Lipofectamine 2000 and the corresponding plasmid were supplemented to 6-well plates at a ratio of 2:1. The medium was renewed after 4–6 h and cells were then collected after 48 h transfection. The transfected cells were subsequently treated with HSYA and TGF-β1 using methods as described above.

3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay

MRC-5 cells (3×10^3 cells/well) were put and cultured for 24 h in complete media of 96-well plates. Following 12 h serum starvation, MRC-5 cells were classified to the following two groups: (1) exposed for 48 h to HSYA (0, 5, 15, and 45 μmol/l) to delve into the impact of HSYA on MRC-5 cell viability; (2) stimulated for 48 h with TGF-β1 (0, 2.5, 5, and 10 nmol/l) to assess the role of TGF-β1 in cell viability. Thereafter, each well was cultured at 37°C for 1 h with 20 μl CellTiter 96® AQueous One Solution Reagent (Promega, Madison, WI, USA). Ultimately, the optical density (A) values at 490 nm were determined by means of a microplate reader (Bio-Tek, Winooski, VT, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

The extraction of RNA was conducted with RNeasy Mini Kits (Qiagen, Germantown, MD, USA), followed by reverse-transcription into cDNA utilizing Reverse Transcription Kits (RR047A, Takara, Tokyo, Japan). Thereafter, qRT-PCR was implemented utilizing SYBR® Premix Ex TaqTM II (Perfect Real Time) Kits (DRR081, Takara) with the help of a RT fluorescence quantitative PCR apparatus (ABI 7500, ABI, New York, NY, USA). Reaction conditions were detailed as below: 30 s of pre-denaturation at 95°C; next 5 s at 95°C and 34 s at 60°C (×40 cycles). Each sample was set up with 3 replicate wells. Primers (Sangon, Shanghai, China) were detailed in Table 1. The Ct value was recorded, and with β-actin acted as an internal reference, the $2^{-\Delta\Delta Ct}$ method is employed to compute the relative expression patterns of genes.

Western blot

Following lysing MRC-5 cells in enhanced radio-immunoprecipitation assay buffer comprising protease inhibitors (BOSTER, Wuhan, China), protein concentration was determined utilizing bicinchoninic acid kits (BOSTER). Thereafter, proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then electro-transferred to polyvinylidene fluoride membranes. Next, the membranes were subjected to 2 h treatment with 5% bovine serum albumin for blocking nonspecific binding before overnight incubation with diluted primary rabbit antibodies (Abcam, Cambridge, UK) against ADAM17 (ab39162, 1:1000), β-actin (ab8227, 1:5000), Collagen I (ab138492, 1:1000), Collagen III (ab7778, 1:5000), fibronectin 1 (Fn1, ab2413, 1:1000), alpha-smooth muscle actin (α-SMA, ab5694, 1:1000), phosphorylated p65 (p-p65, ab76302, 1:1000), p65 (ab32536, 1:2000), STAT3 (ab109085, 1:2000), and phosphorylated STAT3 (p-STAT3, ab76315, 1:2000) at 4°C. Following rinsing, the membranes were probed for 1 h with horseradish peroxidase-labeled goat anti-rabbit secondary immunoglobulin G (IgG) antibodies (ab205718, 1:2000, Abcam). The color development was performed with the help of enhanced chemiluminescence working solution (EMD Millipore, St. Louis, MO, USA). For subsequent gray-scale quantification of bands in Western blot images, Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA) was utilized with β-actin as an internal control.

5-ethynyl-2'-deoxyuridine (EdU) assay

MRC-5 cells (5×10^3 cells/well) were cultured for 2 h with 50 μM EdU solution (200 μl) utilizing Cell-Light EdU DNA cell proliferation kits (RiboBio, Guangdong, China) and next fixed in 4% paraformaldehyde (PFA), followed by incubation in a shaker comprising glycine (2 mg/ml). Subsequently, proliferating cells were subjected to staining with Apollo solution. After nuclear staining with 4',6-diamidino-2-phenylindole (DAPI), cells were photographed under an inverted fluorescence microscope (TE2000, Nikon, Shanghai, China).

Transwell assay

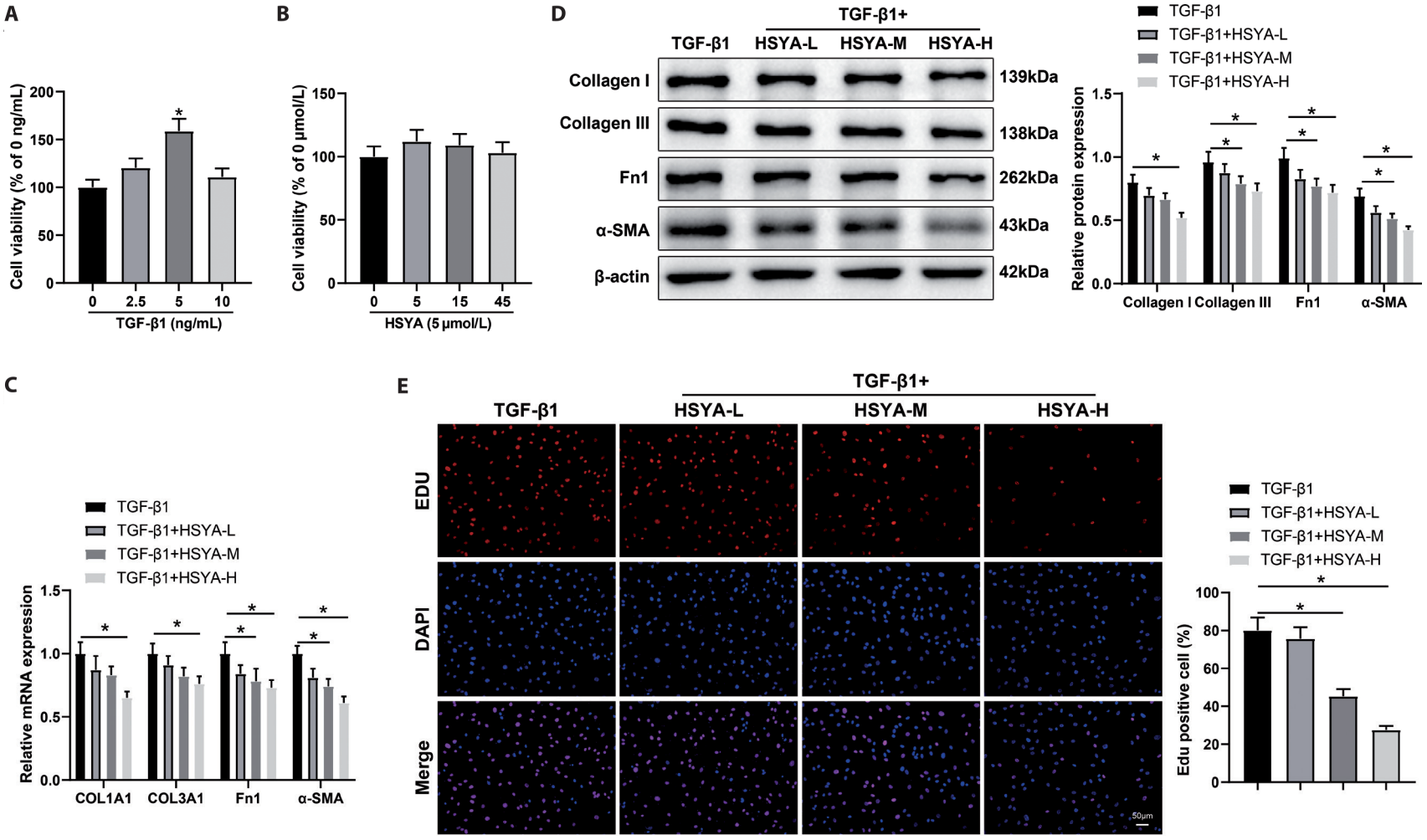
Following resuspension of MRC-5 cells (5×10^5 cells/ml) in a MEM, the apical chamber was supplemented with 200 μl cell suspension and the basolateral chamber was supplemented with 1 ml complete MEM. Following 24 h culture, the cells were subjected to fixing in 4% PFA, followed by 0.1% crystal violet staining. Migratory cells were ultimately observed and counted with the help of a microscope (Leica, Wetzlar, Germany).

Immunofluorescence assay

MRC-5 cells were firstly fixed for 30 min with 4% PFA and subsequently permeabilized with 0.1% Triton X-100. Following blocking with 50% goat serum, cells were probed overnight at 4°C with anti-rabbit α-SMA antibody (1:100, ab7817, Abcam), following by 1 h incubation with anti-rabbit IgG (H+L) (Alexa Fluor 488 conjugate) (1:500, 4412, CST, Beverly, MA, USA) under conditions devoid of light at room temperature. Thereafter, DAPI was utilized for the staining of nuclei. Finally, immunofluorescence images

Table 1. Primers used for qRT-PCR

Genes	Forward primer (5'-3')	Reverse primer (5'-3')
ADAM17	GTGCAGGGTCCGAGGT	GCGAGCACAGAATTAATACGA
COL1A1	GGATCGACCCTAACCAAGGC	GATCGGAACCTTCGCTTCCA
COL3A1	AGTGGCCATAATGGGGAACG	CACCTTTGTCACCTCGTGGA
Fn1	GGATCCCCTCCCAGAGAAGT	GGGTGTGGAAGGGTAACCAG
α-SMA	GTCCCAGACATCAGGGAGTAA	TCGGATACTTCAGCGTCAGGA
β-actin	AAGGACTCCTATAGTGGGTGACGA	ATCTTCTCCATGTCGTCCCAGTTG


A
Cell viability (% of 0 ng/mL)
200
150
100
50
0
*
0
2.5
5
10
TGF-β1 (ng/mL)
B
Cell viability (% of 0 μmol/L)
150
100
50
0
0
5
15
45
HSYA (5 μmol/L)
D
TGF-β1+
TGF-β1
HSYA-L
HSYA-M
HSYA-H
Collagen I
Collagen III
Fn1
α-SMA
β-actin
139kDa
138kDa
262kDa
43kDa
42kDa
TGF-β1
TGF-β1+HSYA-L
TGF-β1+HSYA-M
TGF-β1+HSYA-H
Relative protein expression
1.5
1.0
0.5
0.0
Collagen I
Collagen III
Fn1
α-SMA
C
TGF-β1
TGF-β1+HSYA-L
TGF-β1+HSYA-M
TGF-β1+HSYA-H
Relative mRNA expression
1.5
1.0
0.5
0.0
COL1A1
COL3A1
Fn1
α-SMA
E
TGF-β1+
TGF-β1
HSYA-L
HSYA-M
HSYA-H
EDU
DAPI
Merge
50μm
TGF-β1
TGF-β1+HSYA-L
TGF-β1+HSYA-M
TGF-β1+HSYA-H
Edu positive cell (%)
100
80
60
40
20
0

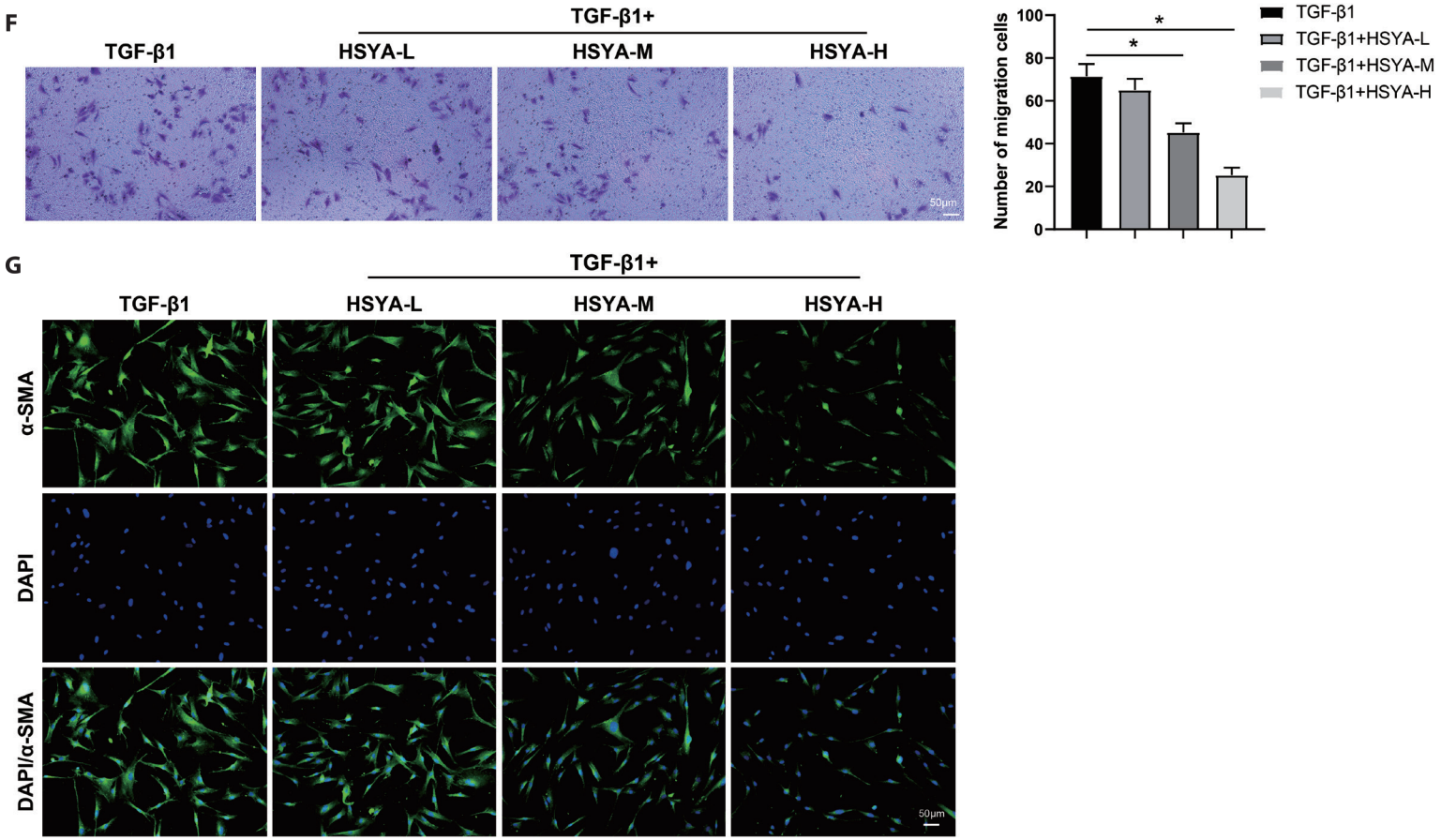


Figure 1. Effect of HSYA on TGF-β1-stimulated MRC-5 cell proliferation. The viability of MRC-5 cells with 0, 2.5, 5 and 10 nmol/l TGF-β1 (**A**) or 0, 5, 15, and 45 μmol/l HSYA (**B**) was measured by MTS assay. **C.** The expression patterns of fibrosis-related genes COL1A1, COL3A1, Fn1 and α-SMA were measured by qRT-PCR. **D.** Expression levels and quantitative analysis of Collagen I, Collagen III, Fn1 and α-SMA proteins (obtained from different gels when the loading amount and internal reference were consistent) were measured by Western blot. **E.** EdU incorporation assay was performed to directly measure active DNA synthesis. **F.** Cell migration was determined by Transwell assay. **G.** Fibroblast-to-myofibroblast transdifferentiation was assessed by immunofluorescence assay. Cell experiments were repeated three times. Data are presented as mean ± SD and analyzed with one-way analysis of variance, followed by Tukey's *post-hoc* test. * $p < 0.05$ *vs.* TGF-β1 group.

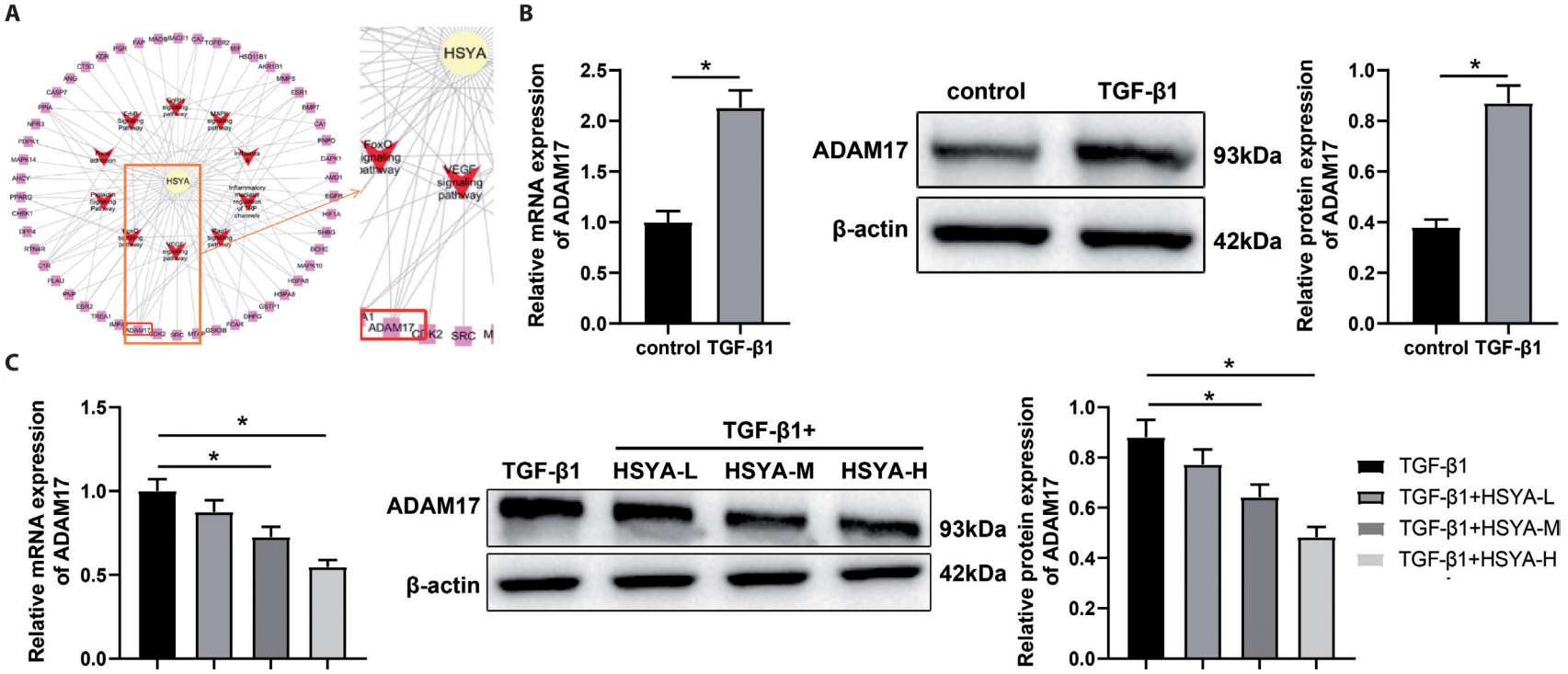


Figure 2. The impact of HSYA on the expression levels of ADAM17 gene and protein obtained from different gels when the loading amount and internal reference were consistent. **A.** PharmMapper website predicted that HSYA could target ADAM17 protein obtained from different gels when the loading amount and internal reference were consistent. **B, C.** ADAM17 protein expression levels in fibroblasts were assessed by qRT-PCR and Western blot. Cell experiments were repeated three times. Data were expressed as mean ± SD and analyzed with one-way analysis of variance, followed by Tukey's *post-hoc* test. * $p < 0.05$ *vs.* control or TGF-β1 group.

were acquired by means of a fluorescence microscope (Olympus Life-Sciences, Tokyo, Japan) for assessing fibroblast-to-myofibroblast transdifferentiation (Chen et al. 2021).

Statistical analysis

Statistical analysis and graphing were processed by means of SPSS 21.0 (IBM, Armonk, NY, USA) as well as GraphPad Prism 9.0 (GraphPad Software Inc., San Diego, CA, USA). Measurement data were shown as mean ± standard deviation. The examination for normal distribution and homogeneity of variance was conducted. For data conforming to normal distribution and homogeneity of variance, two-group comparisons were implemented with the *t*-test, and multiple-group comparisons were carried out utilizing one-way or two-way analysis of variance, followed by Tukey's test. A p value was evaluated by a two-sided test; $p < 0.05$ indicated statistical significance.

Results

HSYA alleviated TGF-β1-triggered fibroblast activation

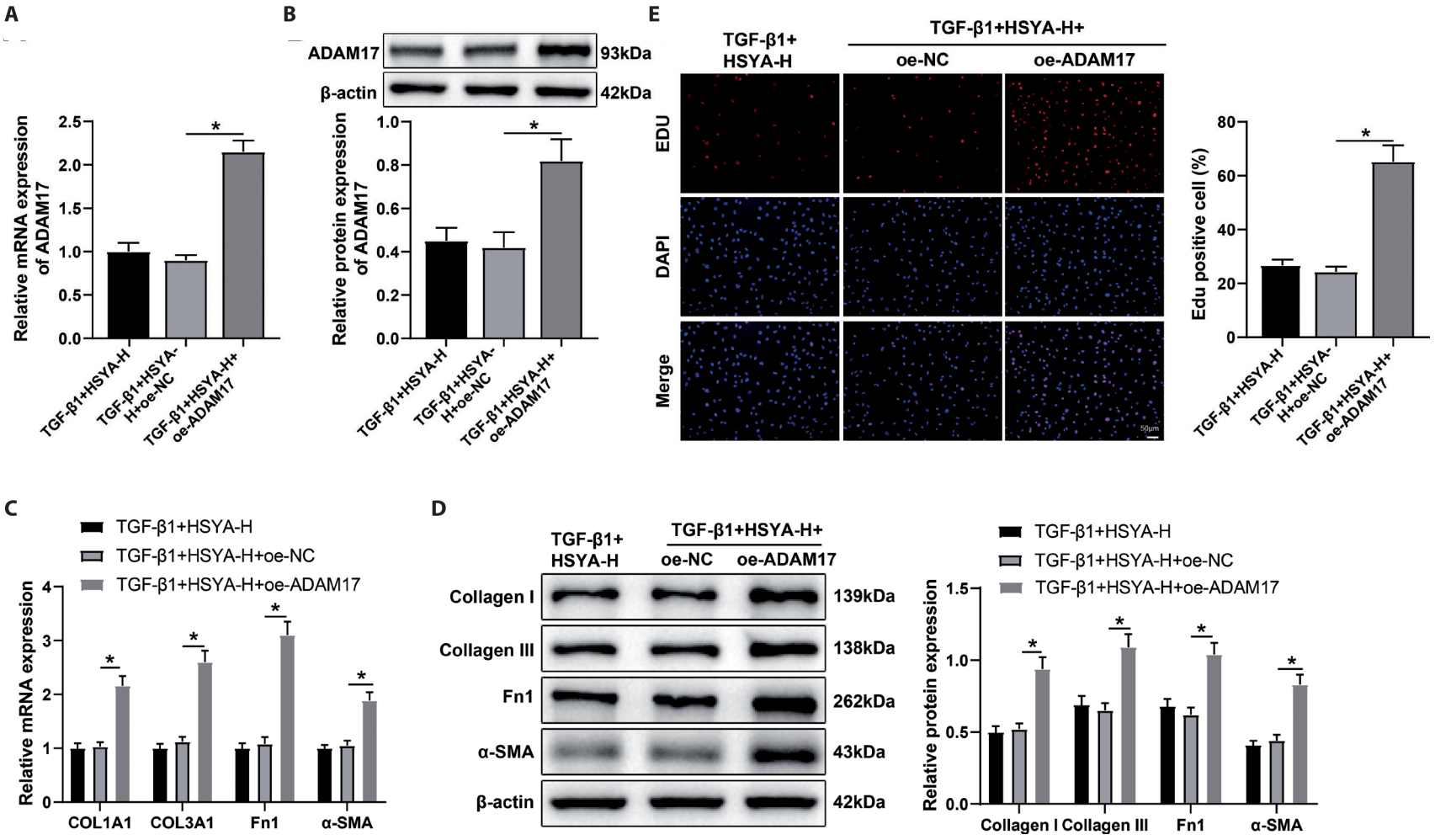
Accumulating studies have confirmed that HSYA could alleviate the function of multiple organ fibrosis (Li et al. 2017; Cong et al. 2022). However, the regulatory mechanisms of HSYA in fibroblast activation need further investigation. In this study, fibroblast activation was firstly induced by TGF-β1 treatment. The impact of different concentrations of TGF-β1 treatment on fibroblast viability was evaluated by using MTS assay. Figure 1A showed that the most significant increase in cell viability was observed in cells treated with 5 ng/ml TGF-β1. Hence, this study used 5 ng/ml TGF-β1 to evaluate its effect on fibroblast activation, implying that treatment with 5 ng/ml TGF-β1 induced activation of fibroblasts (Fig. S1 in Supplementary material). Moreover, the MTS assay was used to evaluate cytotoxicity of HSYA in fibroblasts. Figure 1B showed no statistically significant difference in cell viability in the 0, 5, 15, and 45 μmol/l HSYA groups, indicating that HSYA is not cytotoxic to fibroblasts. Subsequently, the effect of various concentrations of HSYA on fibroblast activation was estimated. The expression patterns of fibrosis-related genes and proteins (Collagen I, Collagen III, Fn1, and α-SMA) were examined by qRT-PCR and Western blot. Figure 1C and D showed that the TGF-β1+HSYA group exhibited lower mRNA and protein expression patterns of fibrosis-related genes than the TGF-β1 group. Additionally, EdU, Transwell, and immunofluorescence assays demonstrated decreases in cell proliferation, migration, and fibroblast-to-myofibroblast transdifferentiation in the TGF-β1+HSYA group (*vs.* the TGF-β1 group) (Fig. 1E–G). Additionally, the impact of HSYA on fibroblast activation showed a dose-dependent mode. Specifically, the inhibitory effect of HSYA on fibroblast activation increased significantly with the increase of HSYA concentration. HSYA (45 μmol/l) exhibited the most apparent effect, which was utilized for subsequent analyses. In summary, HSYA mitigated TGF-β1-induced fibroblast activation.

HSYA negatively targeted ADAM17

The above experiments revealed that HSYA was capable of attenuating TGF-β1-triggered fibroblast activation. However, the related molecular mechanism was still unclear. The PharmMapper website predicted that HSYA could target ADAM17 (Fig. 2A). A prior study has documented the association between ADAM17 and IPF progression (Liu et al. 2018). Therefore, we hypothesized that HSYA might affect fibroblast activation by targeting ADAM17. ADAM17 expression in TGF-β1-activated fibroblasts was determined to confirm our conjecture. The obtained results elucidated that ADAM17 was more prominently up-regulated in the TGF-β1 group compared with the control group (Fig. 2B). Then, TGF-β1-activated fibroblasts were treated with multiple concentrations of HSYA. In contrast to the TGF-β1 group, ADAM17 was notably down-regulated in the TGF-β1+HSYA group, and the reduction in ADAM17 expression was more pronounced as the concentration increased (Fig. 2C). Taken together, HSYA reduced ADAM17 expression in TGF-β1-activated fibroblasts.

HSYA relieved TGF-β1-stimulated fibroblast activation by down-regulating ADAM17

The rescue experiments were conducted to verify whether HSYA orchestrated TGF-β1-triggered fibroblast activation through ADAM17. Firstly, fibroblasts were introduced with oe-ADAM17 or oe-NC prior to HSYA and TGF-β1 treatment, and then ADAM17 expression was measured. Relative to the TGF-β1+HSYA-H+oe-NC group, the TGF-β1+HSYA-H+oe-ADAM17 group showed substantially elevated ADAM17 expression (Fig. 3A,B). Next, fibroblast activation was assessed, which revealed that oe-ADAM17 negated the inhibition action of HSYA in TGF-β1-stimulated fibroblast activation. Specifically, fibrosis-related gene and protein expression patterns, cell proliferation, cell migration, and fibroblast-to-myofibroblast transdifferentiation were prominently enhanced in the TGF-β1+HSYA-H+oe-ADAM17 group (*vs.* the TGF-β1+HSYA-H+oe-NC group) (Fig. 3C–G). In conclusion, HSYA repressed TGF-β1-provoked fibroblast activation *via* ADAM17 down-regulation.


A
Relative mRNA expression of ADAM17
2.5
2.0
1.5
1.0
0.5
0.0
*
TGF-β1+HSYA-H
TGF-β1+HSYA-H+oe-NC
TGF-β1+HSYA-H+oe-ADAM17
B
ADAM17
93kDa
β-actin
42kDa
Relative protein expression of ADAM17
1.0
0.8
0.6
0.4
0.2
0.0
*
TGF-β1+HSYA-H
TGF-β1+HSYA-H+oe-NC
TGF-β1+HSYA-H+oe-ADAM17
E
TGF-β1+HSYA-H
TGF-β1+HSYA-H+
oe-NC
oe-ADAM17
EDU
DAPI
Merge
50μm
Edu positive cell (%)
80
60
40
20
0
*
TGF-β1+HSYA-H
TGF-β1+HSYA-H+oe-NC
TGF-β1+HSYA-H+oe-ADAM17
C
TGF-β1+HSYA-H
TGF-β1+HSYA-H+oe-NC
TGF-β1+HSYA-H+oe-ADAM17
Relative mRNA expression
4
3
2
1
0
*
COL1A1
COL3A1
Fn1
α-SMA
D
TGF-β1+HSYA-H
TGF-β1+HSYA-H+
oe-NC
oe-ADAM17
Collagen I
Collagen III
Fn1
α-SMA
β-actin
139kDa
138kDa
262kDa
43kDa
42kDa
TGF-β1+HSYA-H
TGF-β1+HSYA-H+oe-NC
TGF-β1+HSYA-H+oe-ADAM17
Relative protein expression
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1.0
0.5
0.0
*
Collagen I
Collagen III
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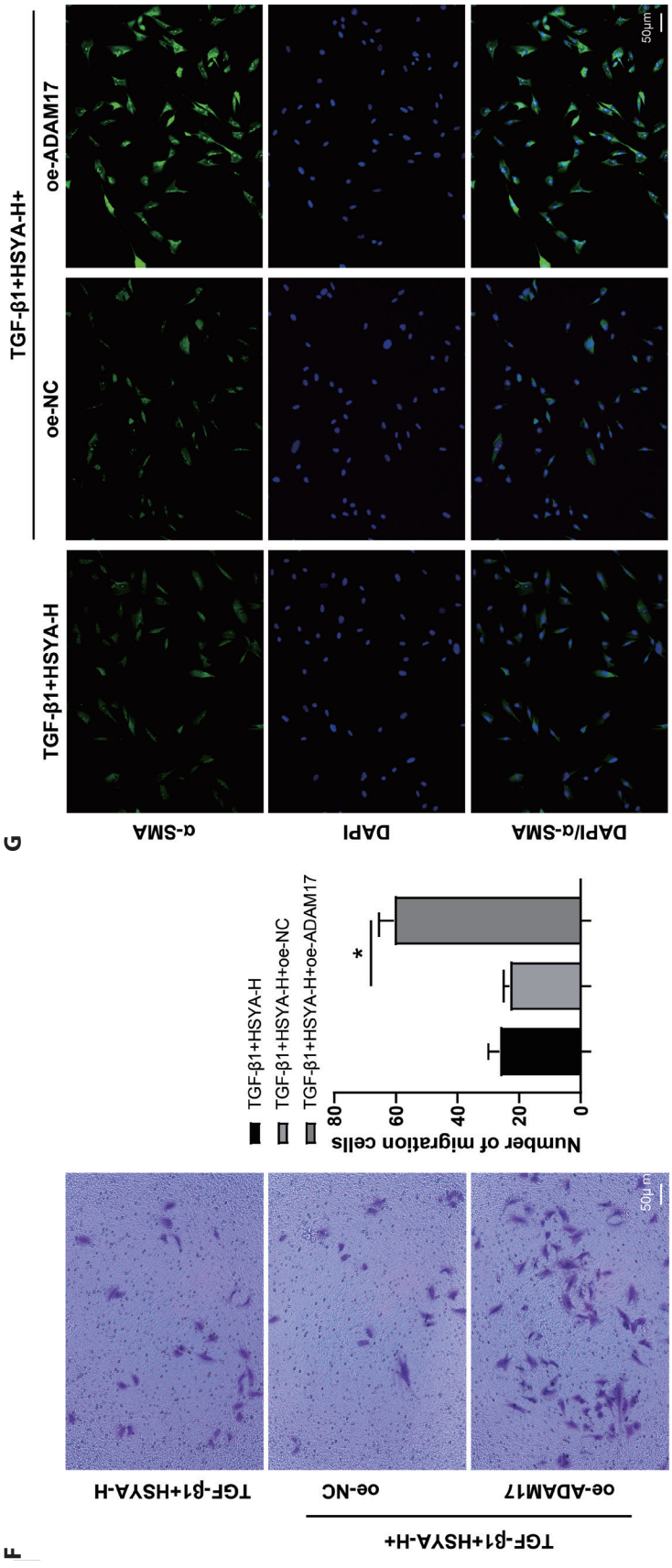

Figure 3. The effect of HSYA on TGF-β1 induced fibroblast activation. **A, B.** qRT-PCR and Western blot were performed to determine ADAM17 expression in fibroblasts, respectively. **C, D.** qRT-PCR and Western blot were carried out to measure the expression levels of fibrosis-related genes (COL1A1, COL3A1, Fn1, α-SMA) and proteins (Collagen I, Collagen III, Fn1, and α-SMA obtained from different gels when the loading amount and internal reference were consistent), respectively. **E.** Cell proliferation was examined by EdU assay. **F.** Cell migration was detected by Transwell assay. **G.** Fibroblast-to-myofibroblast transdifferentiation was analyzed by immunofluorescence assay. Cell experiments were repeated three times. Data were exhibited as mean ± SD and analyzed with one-way analysis of variance, followed by Tukey's *post-hoc* test. * $p < 0.05$ *vs.* TGF-β1+HYSA-H+oe-NC group.

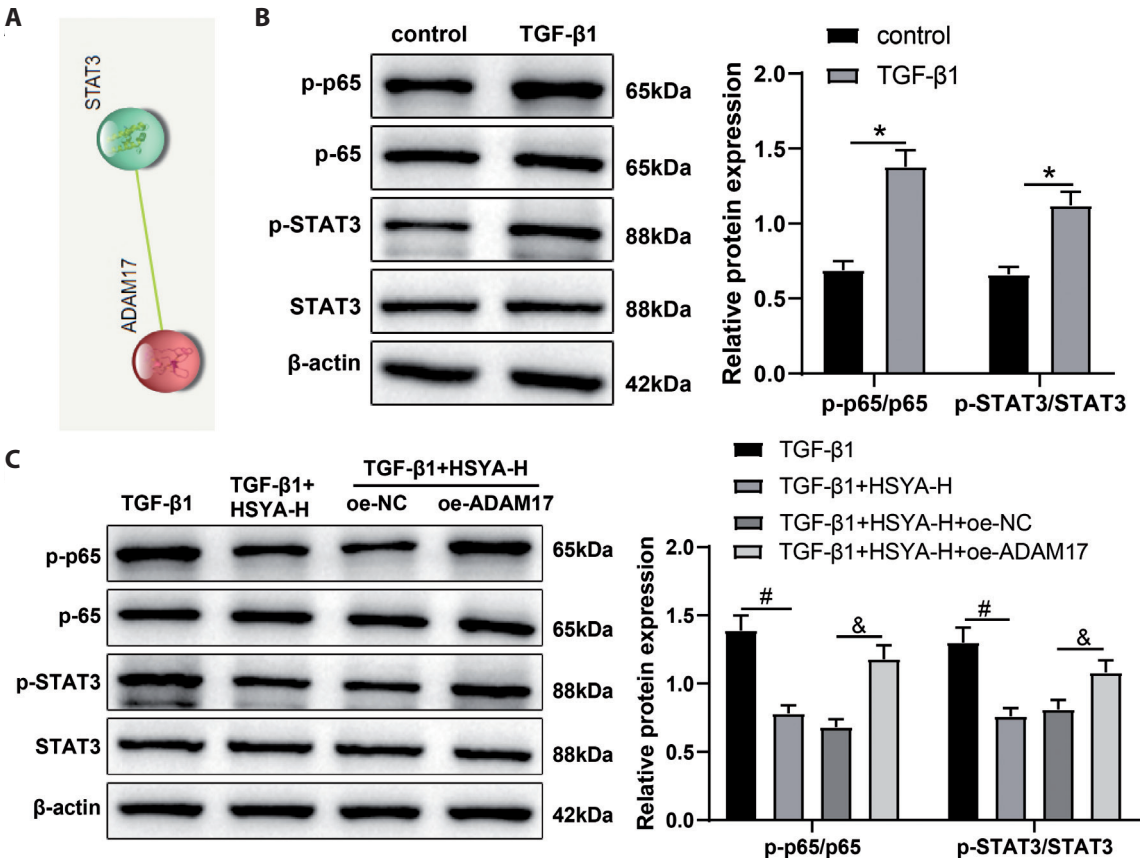

Figure 4. HSYA blocked the NF-κB/STAT3 pathway by inhibiting ADAM17. **A.** STRING website predicted that ADAM17 could act on the STAT3 pathway. **B, C.** p-p65, p65, p-STAT3, and STAT3 proteins (the above proteins obtained from different gels when the loading amount and internal reference were consistent) expression levels were measured by Western blot. Cell experiments were repeated three times. Data were expressed as mean ± SD and analyzed with one-way analysis of variance, followed by Tukey's *post-hoc* test. * $p < 0.05$ *vs.* control group; # $p < 0.05$ *vs.* TGF-β1 group; & $p < 0.05$ vs. TGF-β1+HYSA-H+oe-NC group.

HSYA mitigated fibroblast activation by reducing ADAM17 expression and impeding the NF-κB/STAT3 pathway activation

Subsequently, the downstream related molecular mechanism implicated in HSYA-repressed fibroblast activation *via* ADAM17 was investigated. STRING website showed that ADAM17 could act on the STAT3 pathway (Fig. 4A). Therefore, the expression levels of STAT3 signaling-related proteins were determined, which unveiled that p-p65/p65 and p-STAT3/STAT3 levels were evidently augmented in the TGF-β1 group (*vs.* the control group) (Fig. 4B). Additionally, fibroblasts were delivered with oe-ADAM17 or oe-NC, followed by HSYA and TGF-β1 treatment. The obtained results unraveled that p-p65/p65 and p-STAT3/STAT3 were noticeably diminished in the TGF-β1+HSYA-H group (*vs.* the TGF-β1 group) (Fig. 4C) but considerably elevated in the TGF-β1+HSYA-H+oe-ADAM17 group (*vs.* the TGF-β1+HSYA-H+oe-NC group) (Fig. 4C). These results suggested that HSYA inactivated the NF-κB/STAT3 pathway *via* ADAM17 down-regulation, which in turn extenuated TGF-β1-provoked fibroblast activation.

Discussion

IPF is a progressive and fatal lung disease (Wilson and Wynn 2009). Lung fibroblasts, derived from mesenchymal cells, are the main effector cells in the process of pulmonary fibrosis. TGF-β1 is a key cytokine in inducing pulmonary fibroblast activation, which can promote fibroblast proliferation and differentiation into α-SMA-positive myofibroblasts and modulate ECM production. In this study, our results indicated that HSYA (5, 15, and 45 μmol/l) was non-toxic to cells (Fig. 1B,C). Moreover, HSYA could inhibit the mRNA and protein expression levels of α-SMA, Collagen I, Collagen III, and Fn1 fibronectin in MRC-5 cells (Fig. 1D).

TGF-β1/Smad signal transduction is activated by TGF-β1 binding to type II serine/threonine kinase receptors of TGF-b1 (TβRII) on to the cell membrane. Upon ligand binding, TbβII oligomerizes with transmembrane type I serine/threonine kinase receptors of TGF-b1 (TβRI), leading to the phosphorylation of TβRI, which further stimulates transcription factors Smad2/Smad3 phosphorylation causing them to bind to Smad4. This heteromeric complex translocates into the nucleus and promotes the expression of profibrotic genes (Savary et al. 2019; Yao et al. 2019). Smad7 can bind to the tetramer formed by TGF-β1, TGFRβ II, and TGFR I to inhibit the expression of extracellular matrix. These mediating processes play a key role in the pathogenesis of IPF and AE-IPF and are widely recognized by the academic community. In this study, we found that HSYA exerted inhibitory effects on TGF-β1-induced expression of fibrotic-related biomarkers (α-SMA, COL1A1, COL3A1, and Fn1) (Fig. 3C). Interestingly, we found that the inhibitory effect of ADAM17 cotreatment with 45 μmol/l HSYA were nearly to the same level (Fig. 3C). This suggested that HSYA may act on the same pathway and that the inhibitory effect of HSYA on TGF-β1-induced activation of MRC-5 cells may depend on TβRII signalling.

Increasing study has confirmed that HSYA was associated with organ fibrosis. Luan et al. (2022) have shown that HSYA could alleviate bleomycin-induced pulmonary fibrosis in mice, and its mechanism of action might be related to the regulation of the TGF-β1/Smad signaling pathway. Li et al. (2022) found that HSYA inhibited the expression levels of Smad3 protein in the TGF-β1/Smad signaling pathway, thereby reducing the secretion of related inflammatory factors, the deposition of collagen in the mouse lung, and the destruction of alveolar structures. However, the mechanism by which HSYA alleviates IPF is not fully understood. This study aims to investigate the protective role of HSYA in TGF-β1-induced fibroblast activation by lowering the expression of ADAM17 and inactivating the NF-κB/STAT3 pathway (Figs. 3, 4). The fibroblasts closely related to IPF were selected as the research object (Rajasekaran et al. 2015), and the IPF cell model was established by inducing MRC-5 cells with TGF-β1.

The fibroblast-to-myofibroblast transdifferentiation caused the accumulation of myofibroblasts, which were critical for collagen-producing processes in pulmonary fibrosis and participate in the formation of fibrotic lesions (Zhang et al. 2019). HSYA slowed down renal fibrosis progression by depressing TGF-β1-triggered EMT (Hu et al. 2016). Herein, this research attempted to address the specific protective mechanism of HSYA against fibroblast activation for deepening the knowledge of IPF pathogenesis. Our data elaborated that HSYA alleviated fibroblast activation by blocking the NF-κB/STAT3 pathway through ADAM17 down-regulation (Fig. 2).

TGF-β1 superfamily consists of many proteins that modulate various biological processes including proliferation, differentiation, EMT, and fibrosis (Zhang et al. 2021). Wei et al. (2019) have observed that TGF-β1 was an essential fibrogenic cytokine in IPF progression, which led to the transdifferentiation of lung fibroblasts to myofibroblasts. Numerous study have confirmed that IPF could be attenuated by inhibiting the action of TGF (Bellaye et al. 2018; Liu et al. 2020; Ruan et al. 2020). Hence, TGF-β1 was employed in our research to induce fibroblast activation. As a dominant biological active and hydrosoluble compound obtained from safflower, HSYA is not only effective in the treatment of both angina and cerebral infarction, but also exhibits anticancer properties, mediates metabolism, and protects liver, lung, endothelial cells, and neurons (Ao et al. 2018). Additionally, HSYA confers protection against carbon tetrachloride-induced liver fibrosis (Zhang et al. 2012; Liu et al. 2014). Jin et al. have found that HSYA exerted alleviatory effects on chronic pulmonary fibrosis with the increase of HSYA concentration and lowers TGF-β1 expression (Jin et al. 2018). Similar to the above studies, we also found that HSYA could suppress fibrosis-related gene and protein expression levels. Besides, HSYA could attenuated fibroblast activation in TGF-β1-activated fibroblasts in a dose-dependent mode (Fig. 3). Consistently, Pan et al. (2016) found that HSYA has inhibitory effects on TGF-induced proliferation and activation of MRC-5 cells, while down-regulating the levels of fibrosis-related genes.

To further ascertain the underlying impacts of HSYA on fibroblast activation, ADAM17 was predicted as a target protein of HSYA through bioinformatics analysis, and the targeted inhibition of ADAM17 by HSYA was verified experimentally in TGF-β1-activated fibroblasts. ADAM17 controls the protease-driven detachment of over 70 growth factors, cell surface receptors, and membrane-tethered cytokines (Saad et al. 2019). In human lung fibroblasts, ADAM17 expression was elevated in response to hypoxia which mediates lung fibrosis in individuals suffering with chronic obstructive asthma (Chen et al. 2017). ADAM17 up-regulated CTGF in human lung fibroblasts, and both ADAM17 and CTGF are involved in lung fibrosis formation (Chen et al. 2018). ADAM17 down-regulation caused by miR-708-3p could repress IPF (Liu et al. 2018). Concordant with the results of Ge et al. (2020), ADAM17 was more expressed in TGF-β1-induced fibroblasts in this study (Fig. 3). Moreover, ADAM17 over-expression annulled the suppressive action of HSYA on TGF-β1-provoked fibroblast activation (Fig. 3). Conjointly, HSYA down-regulated ADAM17 to palliate TGF-β1-provoked fibroblast activation.

TGF-β, NF-κB, and STAT3 are essential fibrotic pathway proteins that have been shown to act together in unilateral ureter obstruction-induced renal fibrosis and inflammation (Lu et al. 2018; Meng et al. 2019). Targeting NF-κB and

STAT3 signals effectively mitigated pulmonary fibrosis (Saber et al. 2022; Zhu et al. 2022). HSYA was documented to depress JAK2/STAT3 activation in rats with middle cerebral artery occlusion (Yu et al. 2020). In the context of human fetal lung fibroblasts, HSYA repressed tumor necrosis factor-α-triggered inflammation by blocking the NF-κB pathway (Liu et al. 2019). ADAM17 induced NF-κB transcriptional activity in nasopharyngeal carcinoma (Fei et al. 2019). ADAM17 was associated with the upregulation of STAT3, which contributed to IPF (Liu et al. 2018). Our article also discovered that in TGF-β1-induced fibroblasts, HSYA disrupted the NF-κB/STAT3 pathway activation by suppressing ADAM17 (Fig. 4). However, this work also has limitations. Firstly, our study lacks a more in-depth morphological analysis of the phenotype of myofibroblasts. Secondly, the experimental results have not been validated *in vivo*. Therefore, it is necessary to conduct further research to explore its molecular mechanisms in the future.

In conclusion, this research exhibited that HSYA could confer protection against TGF-β1-induced fibroblast activation *via* NF-κB/STAT3 pathway inactivation by reducing ADAM17 expression, indicating that HSYA is expected to be a potential treatment option for IPF. This work proposes some novel ideas for IPF therapy through the study of fibroblast activation.

Conflict of interest. The authors declare that they have no competing interests.

Author contributions. Yu W., Yan W. conceived the ideas; designed the experiments. Yan W., W.Z., S.W., Y.H. performed the experiments. Yan W., W.Z., S.W., X.S., X.W. analyzed the data. W.Z., S.W. provided critical materials. X.S., Y.H., X.W. wrote the manuscript. Yu W. supervised the study. All the authors have read and approved the final version for publication.

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