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Single-cell sequencing reveals the antifibrotic effects of YAP/TAZ in systemic sclerosis

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Abstract:	Systemic sclerosis (SSc) is a heterogeneous disease with skin fibrosis. Yes-associated protein (YAP)/transcriptional coactivator with PDZ-binding motif (TAZ) is associated with fibrotic response. This work attempted to determine the precise mechanism of YAP/TAZ in SSc. Single-cell sequencing (scRNA-seq) analyzed the differential gene expression between SSc patients and healthy volunteers, showing that YAP/TAZ signaling pathway was enriched in the fibroblasts of SSc patients. Subsequently, enzyme-linked immunosorbent assay and immunohistochemical analysis examined the levels of YAP and TAZ in mild and severe SSc patients. YAP and TAZ were highly expressed in the serum and skin tissues of mild and severe SSc patients, especially severe SSc patients. Additionally, SSc mouse model was induced by bleomycin, the impact of YAP/TAZ knockdown on the pathological changes of skin and lung tissues were detected by hematoxylin and eosin staining and Masson staining. Knockdown of YAP and TAZ inhibited α-SMA mRNA and protein expressions in skin and lung tissues of SSc mice. Inhibition of YAP and TAZ reduced skin inflammation and thickness and repressed lung inflammation and fibrosis in SSc mice. Importantly, knockdown of YAP and TAZ played a synergistic effect in inhibiting inflammation and fibrosis of skin and lung tissues in SSc mice. In conclusion, this work demonstrated that knockdown of YAP and TAZ exerted a synergistic effect on alleviating SSc in mice. Thus, this work suggests that YAP/TAZ may be a potential target for SSc treatment.
Suggested Reviewers:	

Dear Editors

We would like to submit an original research article entitled “Single-cell sequencing reveals the antifibrotic effects of YAP/TAZ in systemic sclerosis” which we wish to be considered for publication in INTERNATIONAL JOURNAL OF BIOCHEMISTRY & CELL BIOLOGY.

In this paper, the aim of this study was to investigate the functional role of YAP/TAZ in systemic sclerosis. Our data indicated that knockdown of YAP/TAZ repressed inflammation and fibrosis of SSc mice, thereby alleviating SSc progression.

We have reviewed the final version of the manuscript and approve it for publication. To the best of our knowledge and belief, this manuscript has not been published in whole or in part nor is it being considered for publication elsewhere. We have no conflicts of interest to disclose.

Best Regards.

Sincerely,

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Single-cell sequencing reveals the antifibrotic effects of YAP/TAZ in systemic sclerosis

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Running title: Role of YAP and TAZ in SSc.

Abstract

Systemic sclerosis (SSc) is a heterogeneous disease with skin fibrosis. Yes-associated protein (YAP)/transcriptional coactivator with PDZ-binding motif (TAZ) is associated with fibrotic response. This work attempted to determine the precise mechanism of YAP/TAZ in SSc. Single-cell sequencing (scRNA-seq) analyzed the differential gene expression between SSc patients and healthy volunteers, showing that YAP/TAZ signaling pathway was enriched in the fibroblasts of SSc patients. Subsequently, enzyme-linked immunosorbent assay and immunohistochemical analysis examined the levels of YAP and TAZ in mild and severe SSc patients. YAP and TAZ were highly expressed in the serum and skin tissues of mild and severe SSc patients, especially severe SSc patients. Additionally, SSc mouse model was induced by bleomycin, the impact of YAP/TAZ knockdown on the pathological changes of skin and lung tissues were detected by hematoxylin and eosin staining and Masson staining. Knockdown of YAP and TAZ inhibited α-SMA mRNA and protein expressions in skin and lung tissues of SSc mice. Inhibition of YAP and TAZ reduced skin inflammation and thickness and repressed lung inflammation and fibrosis in SSc mice. Importantly, knockdown of YAP and TAZ played a synergistic effect in inhibiting inflammation and fibrosis of skin and lung tissues in SSc mice. In conclusion, this work demonstrated that knockdown of YAP and TAZ exerted a synergistic effect on alleviating SSc in mice. Thus, this work suggests that YAP/TAZ may be a potential target for SSc treatment.

Keywords: systemic sclerosis; single-cell sequencing; Yes-associated protein; transcriptional coactivator with PDZ-binding motif; fibrosis

1. Introduction

Systemic sclerosis (SSc) is a chronic, cumulative and multisystem autoimmune disease (Hughes and Herrick, 2019). Its pathological feature is fibrosis of skin and multiple organs (Denton, 2015). Fibrosis involving internal organs such as the heart, lungs, kidneys, and digestive tract can lead to serious comorbidities (Pagkopoulou et al., 2019; Perelas A et al., 2020). For instance, pulmonary fibrosis and pulmonary hypertension are the main causes of death and disability in SSc patients. The high morbidity and mortality of internal organ comorbidities bring a heavy physical and psychological burden to patients with SSc (Cutolo et al., 2019). Thus, exploring the pathogenesis of SSc is important for improving the diagnosis and treatment of this disease.

Innate immunity is the "first" barrier of organism against exogenous and endogenous damage, and plays a role in activating the immune system and in advanced tissue fibrosis (O'Reilly, 2020). A variety of cells and molecules are involved in the process of vascular injury, immune cell activation and fibrosis in the pathological process of SSc, such as macrophages, T lymphocytes, B lymphocytes and cytokines/chemokines (Stern and Denton, 2015). These immune cells and cytokines work together to ultimately regulate fibroblast differentiation and the balance of extracellular matrix (ECM) synthesis and degradation (Fuschiotti, 2018). As the most important cells in dermis, the proliferative and differential capacities of fibroblasts are crucial factors to maintain the normal structure and physiological

function of the skin (Lynch and Watt, 2018). When the skin tissue is stimulated by the exterior irritation, the activated fibroblasts enter a period of vigorous proliferation and metabolism, and synthesize ECM such as collagen and fibronectin (Sun et al., 2016). Thus, fibroblasts are key effector cells in the process of fibrosis formation in SSc, and their dysfunction is a crucial reason for SSc. Exploring abnormally expressed genes in fibroblasts could lead to the discovery of therapeutic targets for SSc.

Single-cell sequencing (scRNA-seq) is an emerging technology based on next-generation sequencing for high-throughput analysis of the internal genetic composition and function of a single cell (Nomura, 2021). ScRNA-seq has been applied in the research of tumor, developmental biology, microbiology, neuroscience, and is becoming the focus of life science research (Hedlund and Deng, 2018; Kim et al., 2018; Ximerakis et al., 2019). A previous study has employed scRNA-seq and revealed that a unique recirculating $CXCL13^{+}$ T cell cluster promotes B-cell responses in the inflamed skin of SSc patients (Gaydosik et al., 2021). ScRNA-seq analysis identify two differentially expressed genes, HSPG2 and APLNR, that affect endothelial cell injury in SSc (Apostolidis et al., 2018). Thus, applying scRNA-seq may help to find the specific genes and pathways in fibroblasts that play an important role in SSc.

In mammals, Yes-associated protein (YAP)/transcriptional coactivator with PDZ-binding motif (TAZ) is a cascade effector molecule of the Hippo signaling pathway, and its activity is regulated by the Hippo pathway (Piccolo et al., 2014). YAP/TAZ is involved in many physiological and pathological processes, including

regulation of organ volume, cell proliferation, differentiation, apoptosis, epithelial-mesenchymal transition, and inhibition of cell contact (Piccolo et al., 2014). It has been confirmed that YAP/TAZ is related to embryonic development, stem cell proliferation and differentiation, vascular remodeling in cardiovascular diseases, neurodegenerative diseases and tumorigenesis (Engel-Pizcueta and Pujades, 2021). Liu et al. have found that higher expression of YAP and TAZ are observed in fibrotic lung tissues, and the activated YAP/TAZ in fibroblasts drives the profibrotic response *in vivo* (Liu et al., 2015). The study of Toyama et al. has confirmed that dimethyl fumarate exerts antifibrotic effects in SSc dermal fibrosis, which may attribute to inhibit Akt1/GSK3β/TAZ/YAP signaling pathway (Toyama et al., 2018). Thus, targeted inhibition of YAZ/YAP may be a treatment for SSc.

In this work, we examined the levels of TAZ and YAP in the serum and skin tissues of SSc patients. SSc mouse model was constructed by administration of bleomycin, and then determined the precise mechanism TAZ and YAP in SSc.

2. Materials and methods

2.1. Single-cell RNA sequencing (scRNA-seq) analysis

The single-cell transcriptome data of SSc and normal cell samples (GSE138669) were downloaded from the Gene Expression Omnibus database. A total of 68249 cells from 3 SSc patients and 3 healthy volunteers were selected from GSE138669 for

scRNA-seq analysis. The low-quality cells were identified and excluded as the following thresholds: (1) unique molecular identifiers (UMIs) less than 200 or UMIs greater than 2000; (2) the number of genes expressed in each cell was less than 200; (3) 20% or more of gene expression was derived from mitochondrial genes. Subsequently, SCtransform normalization analysis was performed for each sample dataset using Seruat (version 4.0.3). PrepSCTIntegration was run to screen features for downstream integration. FindIntegrationAnchors was utilized to identify anchor genes. Finally, single-cell clustering was visualized with the aid of t-distributed stochastic neighbor embedding (t-SNE). The ligand-receptor communications of cells was analyzed applying R package CellChat.

2.2. Patients

A total of 6 mild SSc patients and 6 severe SSc patients were recruited in First Affiliated Hospital of Nanchang University. Six healthy volunteers served as control. The serum and skin tissue samples were obtained from SSc patients and healthy volunteers. These patients were diagnosed with SSc following the diagnostic criteria of SSc as previous study reported (Nadashkevich et al., 2006). These patients were classified into mild and severe SSc according the severity classification and guidelines of SSc described by Asano et al (Asano et al., 2018). The recruited patients have not systematically applied glucocorticoids, immunosuppressants, immunomodulators that may cause tissue sclerosis within 4 weeks. All participants

were signed the informed consent. All protocols were carried out according to the principle of Declaration of Helsinki with the approval of the Ethics Committee of First Affiliated Hospital of Nanchang University.

2.3. Animals

C57BL/6 male mice aged 3 months (weighting 25-30 g) were obtained from Shanghai SLAC Laboratory Animal Co., Ltd (China). Mice were raised in a temperature (21-25°C) and humidity (40-60%) controlled environment, and free access to food and water. All animal experiments were performed following the Guide for the Care and Use of Laboratory Animals (National Institutes of Health) and authorized by the Ethics Committee of First Affiliated Hospital of Nanchang University.

2.4. Animal groups

Mice were randomly divided into 6 groups (n = 6): Control, Model, shNC, sh-YAP, sh-TAZ and sh-YAP/TAZ. Mice were subcutaneously injected with 100 μL bleomycin (0.3 mg/mL; Macklin, Shanghai, China) through the back daily for 5 weeks utilizing an Alzet osmotic pump (Alzet, Durect Corporation, Cupertino, California, USA). Mice were subcutaneously injected with same volume of normal saline as control. Following 5 weeks of bleomycin administration, mice were injected

with 1×10^9 plaque-forming units (PFU) of the lentivirus particles encoding shNC, sh-YAP, sh-TAZ or sh-YAP/TAZ into caudal vein. For knockdown of YAP or TAZ, short hairpin RNA (shRNA) specifically targeting YAP (sh-YAP) or TAZ (sh-TAZ) were constructed. Scrambled shRNA (sh-NC) served as control. Lentivirus harboring shNC, sh-YAP, sh-TAZ or sh-YAP combined with sh-TAZ (sh-YAP/TAZ) were generated by GeneChem (Shanghai, China).

After modeling, the mice were euthanized by cervical dislocation. The skin and lung tissues were rapidly excised and snap-frozen at -80°C, or fixed in paraformaldehyde and embedded in paraffin for further use. Skin tissue samples were collected near the injection sites.

2.5. Immunohistochemical analysis

Skin and lung tissue sections were deparaffinized and hydrated, and then were incubated with proteinase K (Yeasen, Shanghai, China) for 20 min for antigen retrieval. Sections were treated with 3% H_2O_2 for 10 min, and then were blocked with 5% bovine serum albumin. The sections were immunostained with the primary antibodies, YAP (1:500; #ab205270) or TAZ (1:500; #ab224239) at 4°C for 12 h, and then were incubated with goat anti-rabbit IgG antibody (1:1000, #ab6720) at 37°C for 1 h. All the antibodies were obtained from Abcam (Cambridge, MA, USA). Sections were stained with diaminobenzidine (Beyotime, Shanghai, China). The YAP-positive cells and TAZ-positive cells were observed under an optical microscope (Olympus,

Tokyo, Japan), and were analyzed using Image J software.

2.6. Histological analysis

Skin and lung tissue sections were deparaffinized and hydrated. For hematoxylin and eosin (HE) staining, sections were stained with hematoxylin and eosin using HE Staining Kit (Beyotime). For Masson staining, sections were stained with Masson Stain Kit (Yeasen) as the instruction of manufacturer. The images of sections were observed under an optical microscope (Olympus). The degree of inflammation and fibrosis was evaluated applying Szapiel and Ashcroft score system as previous reported (Yavas et al., 2013). Szapiel score system contained a scale of 0-3: 0 score, normal tissues without inflammatory infiltration; 1 score, minimal inflammation with less than 20% tissue involvement; 2 score, moderate inflammation with greater than 20%, less than 50% tissue involvement; 3 score, severe inflammation with greater than 50% tissue involvement. Ashcroft score system included a scale of 0-4: 0 score, normal lung tissues; 1 score, moderate thickening of the wall without obvious damage to lung architecture; 2 score, increased fibrosis, markedly damaged lung structure, fibrous bands or small fibrous masses were observed; 3 score, severe distortion of the structure and large fibrous areas, including ‘honeycomb’; 4 score, total fibrous obliteration of the field.

2.7. Enzyme-linked immunosorbent assay

The levels of YAP and TAZ in the serum of healthy volunteers, mild and severe SSc patients were detected by performing Enzyme-linked immunosorbent assay (ELISA) utilizing Human YAP ELISA Kit (Fantai Biotech, Shanghai, China) and Human TAZ ELISA Kit (Fantai Biotech). The absorbance of YAP and TAZ standards at different concentration gradient were detected on a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA), and then established the standard curve for YAP and TAZ. The levels of YAP and TAZ were calculated according to the correspondent standard curve.

2.8. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from the skin and lung tissues applying TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and reverse transcribed to complementary DNA using the First-Strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China). PCR reactions were performed utilizing TB Green Premix ExTaq II (TaKaRa) on the 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The housekeeping gene GAPDH served as loading control. Primers used for qRT-PCR were shown in Table 1. The data were analyzed using $2^{-\Delta\Delta CT}$ method for quantification.

2.9. Western blot

The skin and lung tissues from mice were treated with RIPA Lysis Buffer (Beyotime, Shanghai, China) to obtain the total protein. Then, total protein was separated by 10% SDS-PAGE gel electrophoresis, and was transferred onto nitrocellulose membrane. The membranes were hybridized with the primary antibodies, YAP (1:500; #ab205270), TAZ (1:500; #ab224239) or α-SMA (1:1000; #ab5694) at 4°C overnight after blocked with 5% non-fat milk. Subsequently, the membranes were incubated with goat anti-rabbit HRP-IgG antibody (1:2000, #ab6721) at room temperature for 2 h. GAPDH (1:2500; #ab9485) served as housekeeping protein for normalization. All antibodies were obtained for Abcam. The WB bands were developed by ECL reagent, and were analyzed by Image J software.

2.10. Statistical analysis

Each assay was conducted in biological triplicates. All data were reported as mean ± standard deviation and analyzed by SPSS 22.0 statistical software (IBM, Armonk, NY, USA). Two-tailed Student's *t* test and one-way ANOVA were used to analyze the statistical difference. A significant difference was considered if *P* was less than 0.05.

3. Results

3.1. Profiling of scRNA-Seq and screening of marker genes

Following quality control and removal of batch effects, a total of 35063 cells derived from 3 SSc patients and 3 healthy volunteers were selected for further analysis. t-SNE algorithm was carried out to cluster the cells, showing that the cell samples were successfully classified into 25 clusters (Figure 1A). The cell type of these 25 cell clusters was identified by cell-specific marker. These cell clusters were contained secretory cells (SCGB1B2P), epidermal stem cells (KRT14), endothelial cells (VWF), pericytes (RGS5), melanocytes (PMEL), keratinocytes (KRT1), smooth muscle cells (DES), fibroblasts(COL1A1), T lymphocytes (CD3D) and macrophages/dendritic cells (AIF1) (Figure 1B-C, Supplementary figure 1).

3.2. Cell-cell communication among fibroblasts, T lymphocytes, and macrophages/dendritic cells

We carried out CellChat algorithm to analyze the ligand-receptor communications among fibroblasts, T lymphocytes, macrophages/dendritic cells in SSc patients and healthy volunteers. As shown in Figure 2A, cell communication existed between fibroblasts and T lymphocytes, fibroblasts and macrophages/dendritic cells, T lymphocytes and macrophages/dendritic cells in SSc patients. However, there were cell communication between fibroblasts and T lymphocytes, fibroblasts and macrophages/dendritic cells in healthy volunteers. The cell communication among these three types of cells in SSc patients was significantly more frequent than that in healthy volunteers. A total of 18 ligand-receptor interaction pairs were founded among

fibroblasts, T lymphocytes, and macrophages/dendritic cells, including 13 increased ligand-receptor interaction pairs and 5 decreased ligand-receptor interaction pairs (Figure 2B-C).

3.3. YAP and TAZ serum levels were increased in SSc patients

To investigate the differences in fibroblasts between SSc samples and normal samples, C2 gene set (curated gene sets) was downloaded from the MsigDB database (http://software.broadinstitute.org/gsea/msigdb). Gene set variation analysis (GSVA) algorithm was run to analyze the signaling pathway in fibroblasts between SSc samples and healthy volunteer samples applying scRNA-seq data. YAP/TAZ signaling pathway was significantly enriched in the fibroblasts of SSc patients as compared with healthy volunteers (Figure 3A), indicating that YAP/TAZ may participate in the pathological process of SSc. Subsequently, we examined the serum levels of YAP and TAZ in mild and severe SSc patients. The results obtained from ELISA showed that YAP and TAZ serum levels were increased in mild and severe SSc patients with respect to healthy volunteers. Moreover, compared with mild SSc patients, higher serum levels of YAP and TAZ were observed in severe SSc patients (Figure 3B-C). Thus, YAP and TAZ serum levels may be positively correlated with SSc.

3.4. YAP and TAZ expressions were elevated in the skin tissues of SSc patients

Next, we measured the expression levels of YAP and TAZ in the skin tissues of SSc patients by immunohistochemical analysis. As shown in Figure 4A, the number of YAP-positive cells was significantly increased in the skin tissues of mild and severe SSc patients. Severe SSc patients displayed more YAP-positive cell number than that in mild SSc patients. Similarly, TAZ-positive cell number was elevated in the skin tissues of mild and severe SSc patients, especially severe SSc patients (Figure 4B). Thus, YAP and TAZ expressions were increased in the skin tissues of SSc patients.

3.5. The synergistic effect of YAP and TAZ knockdown reduced α-SMA expression in skin tissues of SSc mice

In the skin and internal organs of SSc patients, fibroblasts are abnormally activated with enhanced proliferative and collagen synthesis ability. Some fibroblasts transform into myofibroblasts and express α-SMA, eventually leading to skin sclerosis and internal organs fibrosis (Bhattacharyya et al., 2011). Thus, we assessed the expression of myofibroblast marker α-SMA in the skin tissues of SSc mice by qRT-PCR. Compared with normal mice, YAP, TAZ and α-SMA mRNA expression levels were significantly increased in the skin tissues of SSc mice. The increased YAP, TAZ and α-SMA mRNA expressions in SSc mice were severely decreased by knockdown of YAP or TAZ. Knockdown of TAZ combined with YAP obviously repressed the mRNA expression of YAP, TAZ and α-SMA with respect to YAP deficiency alone. Compared with YAP knockdown, knockdown of TAZ combined

with YAP further reduced α-SMA mRNA expression in the skin tissues of SSc mice (Figure 5A-C). Western blot results revealed that the protein levels of YAP, TAZ and α-SMA were increased in the skin tissues of SSc mice, which was suppressed by silencing of YAP or TAZ. Compared with TAZ deficiency, the protein expression of YAP, TAZ and α-SMA was suppressed by knockdown of TAZ combined with YAP (Figure 5D-G). Thus, YAP and TAZ knockdown may exert a synergistic effect in inhibiting α-SMA expression in skin tissues of SSc mice.

3.6. The synergistic effect of YAP and TAZ knockdown inhibited skin inflammation and thickness in SSc mice

To further investigate the influence of YAP and TAZ on the pathological changes of skin tissues in SSc mice, HE staining and Masson staining were carried out. The results of HE staining showed that normal mice exhibited normal tissue structure and cell morphology in the skin tissues. Abundant inflammatory cell infiltration and tissuse damage were observed in the skin tissues of SSc mice. Knockdown of YAP or TAZ significantly alleviated damage of skin tissues and reduced skin inflammation score in SSc mice. Compared with YAP silencing alone, YAP combined with TAZ knockdown significantly reduced the skin inflammation scores in SSc mice (Figure 6A, C). Masson staining revealed that skin was thickened in SSc mice with respect to normal mice. The skin thickness of SSc mice was repressed by deficiency of YAP or TAZ. Silencing of YAP combined with TAZ reduced skin thickness in SSc mice, but

not significantly (Figure 6B, D). Thus, YAP and TAZ knockdown may play a synergistic effect in inhibiting skin inflammation and thickness in SSc mice.

3.7. The synergistic effect of YAP and TAZ knockdown reduced α-SMA expression in lung tissues of SSc mice

SSc is a fibrosis or sclerosis of the skin and internal organs, and the most frequently affected internal organs are the lungs. Thus, we also detected the expression of α-SMA in lung tissues of SSc mice. The mRNA expression of YAP, TAZ and α-SMA was higher in the lung tissues of SSc mice than that in normal mice. The increased mRNA levels of YAP, TAZ and α-SMA in lung tissues of SSc mice were repressed by knockdown of YAP or TAZ. Knockdown of YAP combined with TAZ caused a decrease of YAP and α-SMA mRNA expression in SSc mice as compared with TAZ silencing, and inhibited TAZ mRNA expression with respect to TAP knockdown (Figure 7A-C). Western blot results uncovered that SSc mice exhibited an increase of YAP, TAZ and α-SMA protein expression in lung tissues as compared with normal mice. These protein expressions in lung tissues of SSc mice were inhibited by deficiency of YAP or TAZ. Compared with YAP deficiency, knockdown of YAP combined with TAZ led to a down-regulation of TAZ and α-SMA in SSc mice. Inhibition of YAP combined with TAZ also reduced YAP, TAZ and α-SMA protein expression in SSc mice with respect to TAZ silencing (Figure 7D-G). Taken together, deficiency of YAP and TAZ played a synergistic effect in reducing

α-SMA protein expression in lung tissues of SSc mice.

3.8. Inhibition of YAP and TAZ reduced inflammation and fibrosis of lung tissues of SSc mice

Finally, the impact of YAP and TAZ knockdown on inflammation and fibrosis of lung tissues in SSc mice was examined. As shown in Figure 8A-B, the morphology and structure of the alveoli in SSc mice were obviously destroyed, and a large number of inflammatory cells and collagen fibers were seen in the alveolar cavity. Nevertheless, the alveolar structure was not significantly damaged, and there were less inflammatory cells and collagen fibers in lung tissues of SSc mice following YAP, TAZ, YAP combined with TAZ silencing. The increased lung inflammation and fibrosis scores in SSc mice were suppressed by knockdown of YAP or TAZ. Compared with YAP or TAZ silencing alone, inhibition of YAP combined with TAZ repressed lung inflammation and fibrosis scores in SSc mice, but not significantly (Figure 8C-D). Therefore, inhibition of YAP and TAZ reduced inflammation and fibrosis of lung tissues of SSc mice.

4. Discussion

As an emerging sequencing technology, scRNA-seq analyzes the genome and transcriptome at the level of a single cell. It reflects the heterogeneity between cells,

which is conducive to reveal the mechanism of disease occurrence and development (Hedlund and Deng, 2018). For instance, Xu et al. have employed scRNA-seq and found that CXCL14 is abnormally overexpressed in a novel cell subpopulation of the positive lymph nodes in breast cancer patients, indicating that CXCL14 is closely associated with lymphatic metastasis of breast cancer (Xu et al., 2021). ScRNA-seq reveals the key divergent immune cells and their associated signaling pathways between idiopathic pulmonary fibrosis (IPF) and SSc-associated interstitial lung diseases (Valenzi et al., 2021). Apostolidis et al. have applied scRNA-seq and identified two differentially expressed genes, HSPG2 and APLNR, in endothelial cells of SSc patients (Apostolidis et al., 2018). HSPG2 and APLNR participate in injury of endothelial cells in SSs. In this work, we applied scRNA-seq and first uncovered that YAP/TAZ signaling pathway was activated in the fibroblasts of SSc patients as compared with healthy volunteers, suggesting that YAP/TAZ may be a key regulator in the progression of SSc. As an autoimmune disease, immune cells are involved in the progression of SSs (Brown and O'Reilly, 2019). We found that cell-cell communication was frequently among fibroblasts, T lymphocytes, and macrophages/dendritic cells in SSs patients. The frequent signal transduction among fibroblasts, T lymphocytes, and macrophages/dendritic cells may affect the progression of SSc.

YAP/TAZ, as a transcriptional cofactor downstream of the Hippo pathway, is a key regulator of fibroblast activation. Its activity reflects the ability of cells to adhere and respond to ECM mechanical signal stimulation. Knockdown of YAP/TAZ can

weaken the function of fibroblast matrix synthesis, contraction and proliferation (Liu et al., 2015). YAP/TAZ is activated in various cancers and promotes tumor formation and metastasis, such as liver cancer, breast cancer and non-small cell lung cancer (Lo Sardo et al., 2021; Zhang and Zhou, 2019; Zhao et al., 2021). YAP/TAZ signaling pathway accelerates the production of pro-fibrotic mediators and ECM proteins in fibroblasts, which leads to tissue stiffness (Noguchi et al., 2018). Toyama et al. have confirmed that high expression TAZ is observed in the skin biopsies from diffuse SSc patients, and YAP/TAZ exerts the anti-fibrotic responses in dermal fibroblasts (Toyama et al., 2018). Consistently, we found that YAP and TAZ were highly expressed in serum and skin tissues of SSc patients. Knockdown of YAP and TAZ significantly repressed inflammation, thickness and fibrosis of skin and lung tissues of SSc mice. Nevertheless, YAP and TAZ silencing reduced α-SMA expression in the skin and lung tissues of SSc mice. Fibrosis of skin and internal organs is the important pathological feature of SSc, and the expression of α-SMA is up-regulated during the process of fibrosis (Keane, 2019; Mei et al., 2020). Additionally, inhibition of YAP combined with TAZ reduced inflammation and fibrosis, and inhibited α-SMA expression in the skin and lung tissues in SSc mice with respect to knockdown of YAP or TAZ alone. Thus, inhibition of YAP and TAZ exerted a synergistic effect in inhibiting skin and lung inflammation and fibrosis in SSc mice.

In summary, our scRNA-seq analysis uncovered that YAP/TAZ signaling pathway was activated in the fibroblasts of SSc patients. *In vivo* experiments further demonstrated that YAP/TAZ repressed inflammation and fibrosis of SSc mice, thereby

alleviating SSc progression. Thus, this work suggests that YAP/TAZ may be a potential target for SSc treatment.

In spite of our findings, several limitations can be found in this study. We initially identified the frequent intercellular communication among fibroblasts, T lymphocytes and macrophages/dendritic cells. The interaction among fibroblasts and these immune cells and its mechanism of action in the progression of SSC still need further study. Additionally, we only determined the functional role of YAP/TAZ in SSc. YAP/TAZ as a hub may affect the occurrence and development of fibrosis by regulating Hippo, Notch, Wnt/β-atenin, and TGF-β/Smad pathways (Nakamura et al., 2021; Pan et al., 2018; Piersma et al., 2015). In further work, we attempt to determine whether YAP/TAZ can affect SSc development through above pathways.

CRediT authorship contribution statement

Dongke Wu: Conceptualization, Investigation, Formal analysis, Writing-original draft. Wei Wang： Formal analysis, Writing-review and editing. Xinyue Li: Investigation, Writing-review and editing. Bo Yin: Investigation, Writing-review and editing. Yunqing Ma: Conceptualization, Funding acquisition, Formal analysis, Writing-review and editing, Supervision. All authors read and approved the final manuscript.

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Declaration of competing interest

The authors declare no conflict of interests.

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Figure legends

Figure 1. Identification of cell subpopulation. (A) Cell sample distribution via t-SNE analysis. (B) Major cell subpopulation distribution via t-SNE analysis. (C) Major cell subpopulation was identified by t-SNE analysis. Secretory cell marker (SCGB1B2P); epidermal stem cell maker (KRT14); endothelial cell maker (VWF); pericyte maker (RGS5); melanocyte maker (PMEL); keratinocyte maker (KRT1); smooth muscle cell maker (DES); fibroblast maker (COL1A1); T lymphocyte maker (CD3D); macrophage maker (AIF1).

Figure 2. Cell-cell communication among fibroblasts, T lymphocytes, and macrophages/dendritic cells. (A) Cell-cell communication among fibroblasts, T lymphocytes, and macrophages/dendritic cells. (B) Dot plot showing the ligand-receptor interactions underlying the crosstalk among fibroblasts, T lymphocytes, and macrophages/dendritic cells. (C) Dot plot showing the increased or decreased ligand-receptor interactions underlying the crosstalk among fibroblasts, T lymphocytes, and macrophages/dendritic cells.

Figure 3. YAP and TAZ levels were increased in the serum of SSc patients. (A) Signaling pathways were enriched in SSCs and healthy volunteers via GSVA analysis. The serum levels of YAP (B) and TAZ (C) in mild, severe SSc patients and healthy volunteers were analyzed by ELISA. $^{*}P < 0.05$, $^{**}P < 0.01$, vs. Normal group; $^{\#}P <$

0.05, $^{\#\#}P < 0.01$, vs. Mild SSc group.

Figure 4. YAP and TAZ expressions were elevated in the skin tissues of SSc patients. The expressions of YAP (A) and TAZ (B) in skin tissues of mild, severe SSc patients and healthy volunteers were analyzed by immunohistochemical analysis. $^{**}P < 0.01$, vs. Normal group; $^{\#}P < 0.05$, vs. Mild SSc group.

Figure 5. Inhibition of YAP and TAZ reduced α-SMA expression in skin tissues of SSc mice. SSc mouse model was induced by bleomycin, followed by injection of the lentivirus particles encoding shNC, sh-YAP, sh-TAZ or sh-YAP/TAZ. Mice were injected with normal saline as control. The mRNA expressions of YAP (A), TAZ (B) and α-SMA (C) in skin tissues of mice were examined by qRT-PCR. (D) The western blot bands of YAP, TAZ and α-SMA. The protein expressions of YAP (E), TAZ (F) and α-SMA (G) in skin tissues of mice were detected by western blot. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, vs. Control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, vs. sh-NC group; $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, vs. sh-TAZ group; $^{\&}P < 0.05$, vs. sh-YAP group.

Figure 6. Knockdown of YAP and TAZ alleviated inflammation and thickness of skin tissues in SSc mice. SSc mouse model was induced by bleomycin, followed by injection of the lentivirus particles encoding shNC, sh-YAP, sh-TAZ or sh-YAP/TAZ. Mice were injected with normal saline as control. (A) The pathological changes of skin tissues of mice were examined by HE staining. (B) The thickness of skin tissues of mice were measured by Masson staining. (C) Inflammation score in skin tissues was presented. (D) The skin thickness of skin tissues was presented. $^{***}P < 0.001$, vs.

Control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs. sh-NC group; $^{\&}P < 0.05$, vs. sh-YAP group.

Figure 7. Knockdown of YAP and TAZ reduced α-SMA expression in lung tissues of SSc mice. SSc mouse model was induced by bleomycin, followed by injection of the lentivirus particles encoding shNC, sh-YAP, sh-TAZ or sh-YAP/TAZ. Mice were injected with normal saline as control. The mRNA expressions of YAP (A), TAZ (B) and α-SMA (C) in lung tissues of mice were assessed by qRT-PCR. (D) The western blot bands of YAP, TAZ and α-SMA. The protein expressions of YAP (E), TAZ (F) and α-SMA (G) in lung tissues of mice were examined by western blot. $^{**}P < 0.01$, $^{***}P < 0.001$, vs. Control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs. sh-NC group; $^{\$}P < 0.05$, vs. sh-TAZ group; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$, vs. sh-YAP group.

Figure 8. Knockdown of YAP and TAZ alleviated the inflammation and fibrosis of lung tissues in SSc mice. SSc mouse model was induced by bleomycin, followed by injection of the lentivirus particles encoding shNC, sh-YAP, sh-TAZ or sh-YAP/TAZ. Mice were injected with normal saline as control. (A) The pathological changes of lung tissues of mice were examined by HE staining. (B) The fibrosis of lung tissues of mice was measured by Masson staining. (C) Inflammation score in lung tissues was presented. (D) The fibrosis score in lung tissues was presented. $^{**}P < 0.01$, vs. Control group; $^{\#}P < 0.05$, vs. sh-NC group.

Supplementary figure 1. Dot plot showing the proportion of cells and the scaled average gene expression of cell markers.

Figure 1

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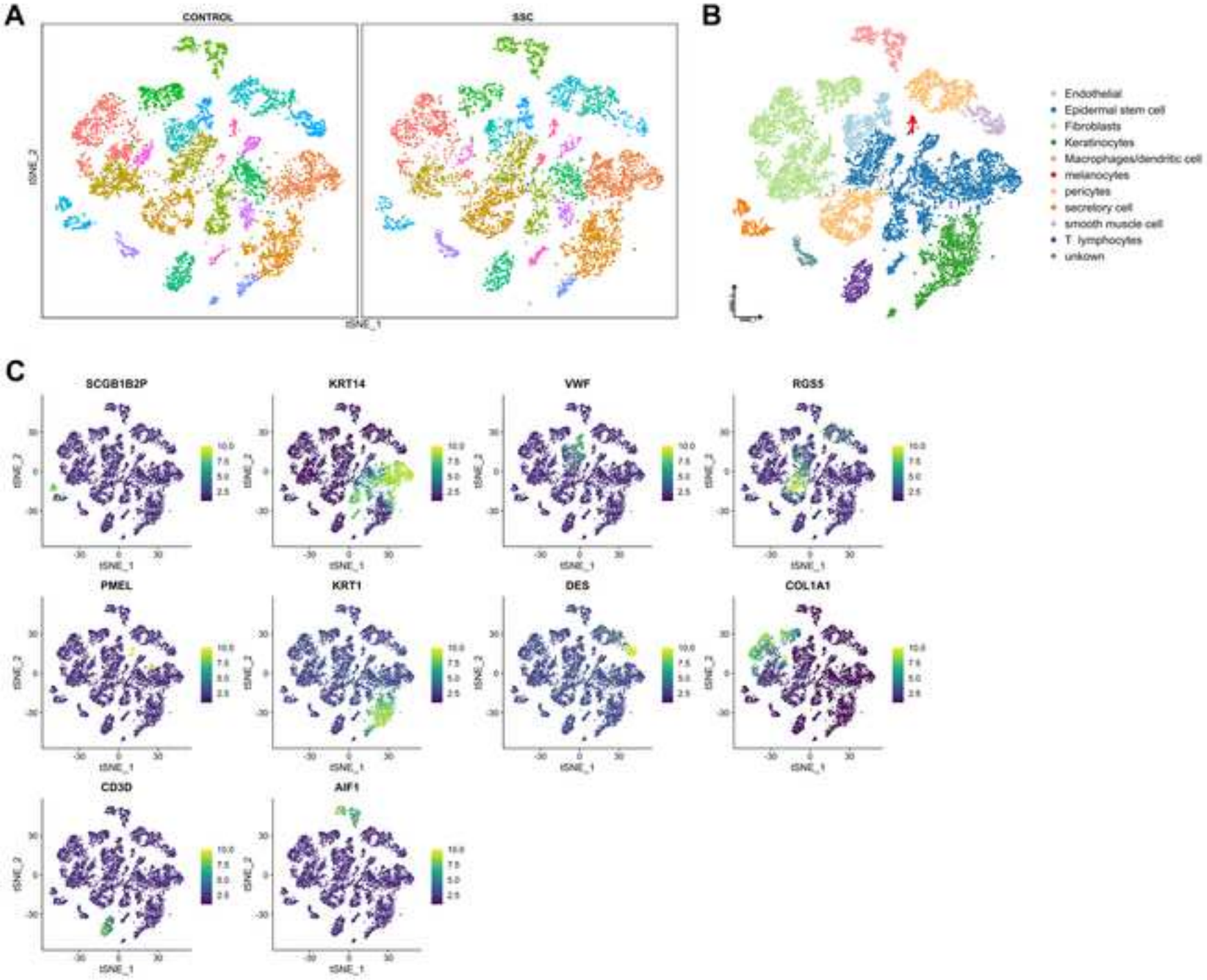

Figure 2

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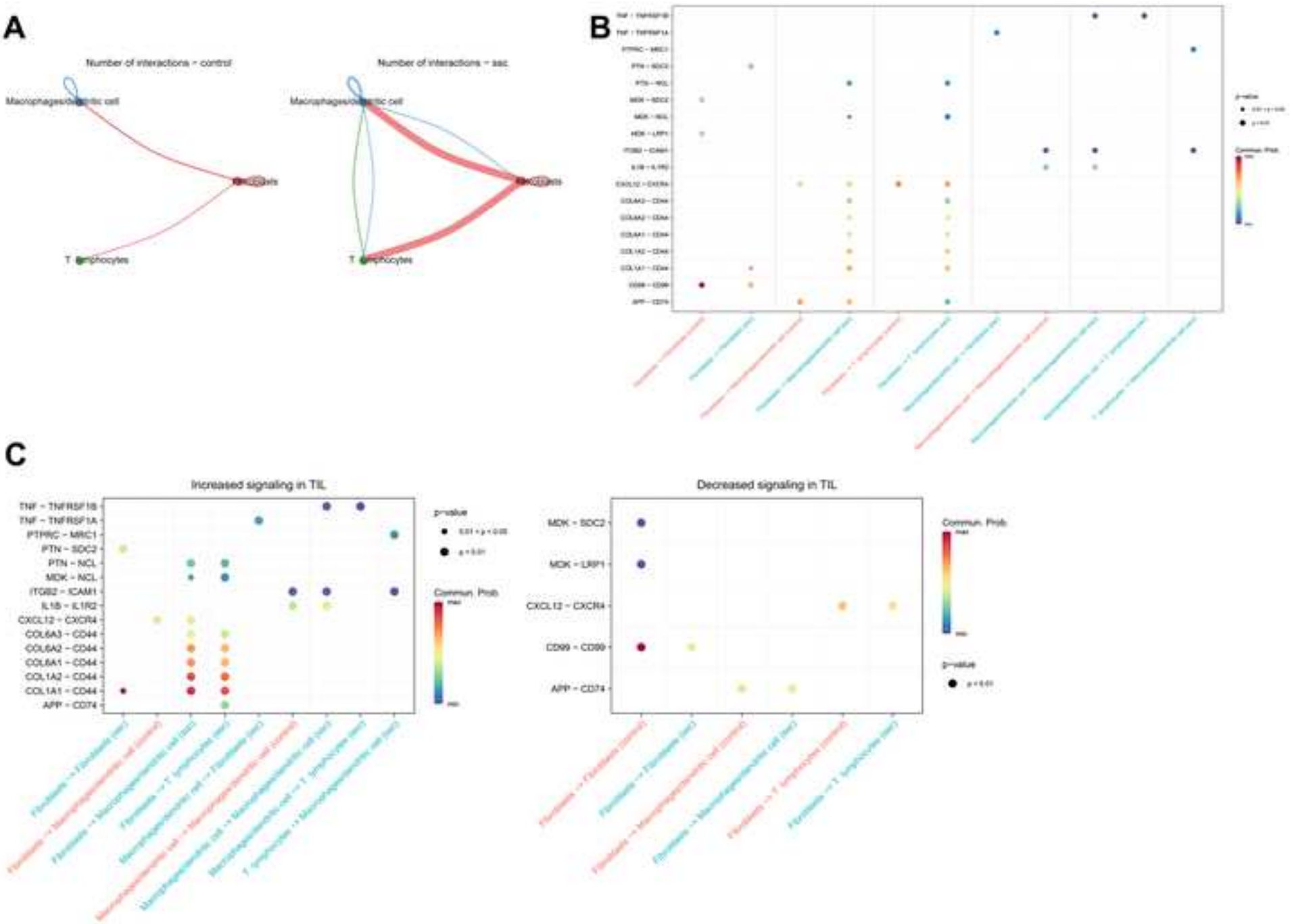


Figure 3

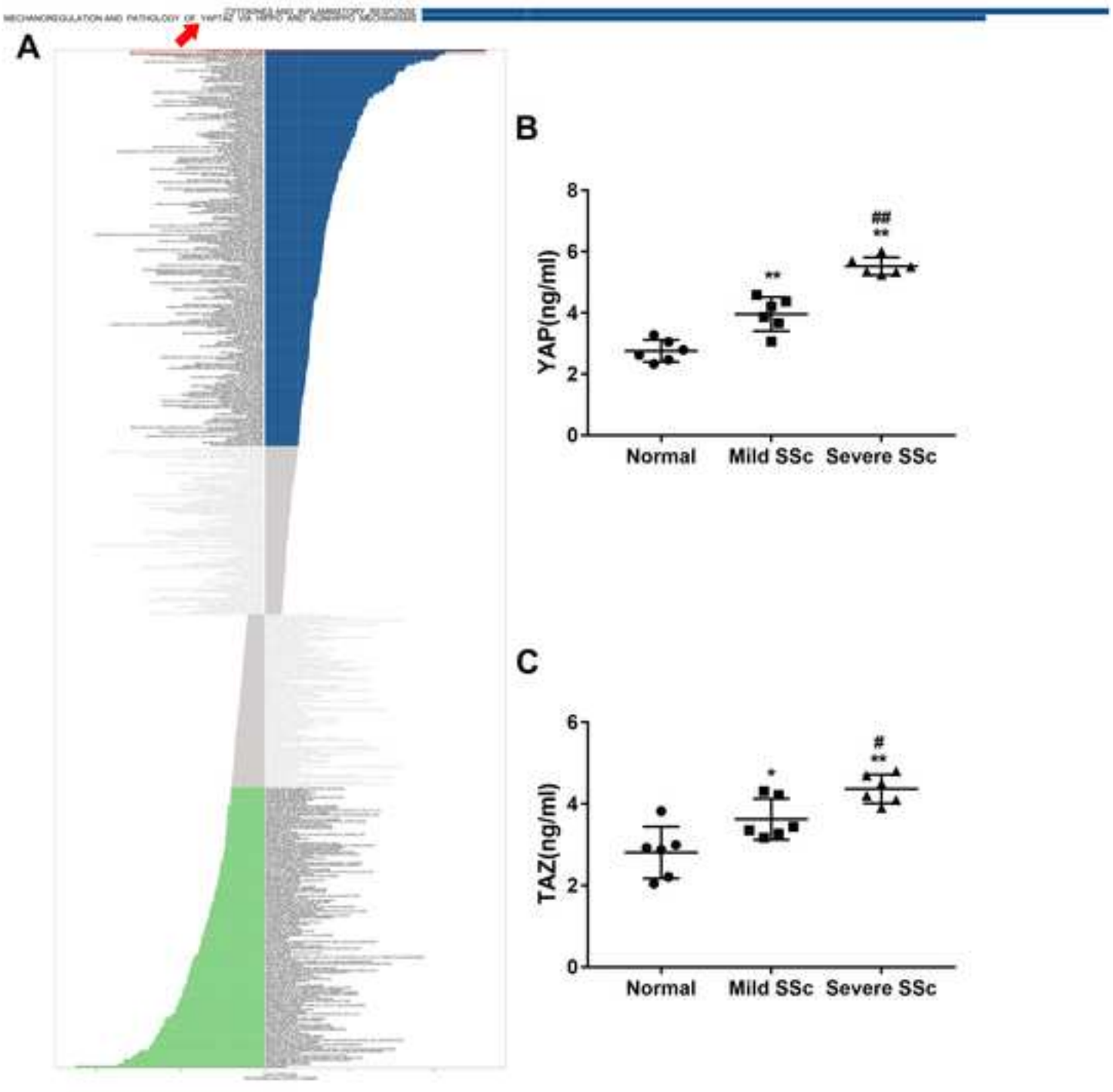
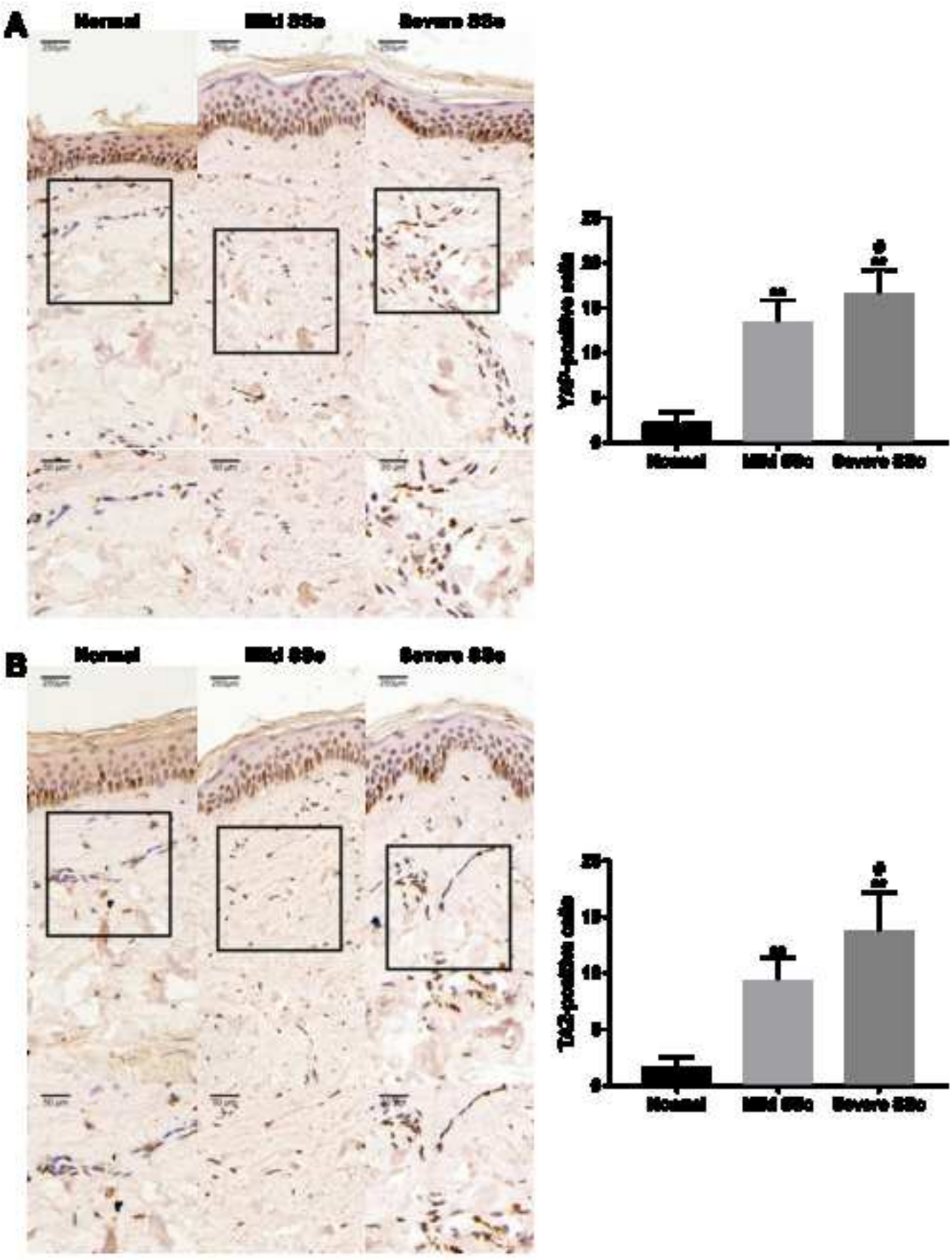


Figure 4



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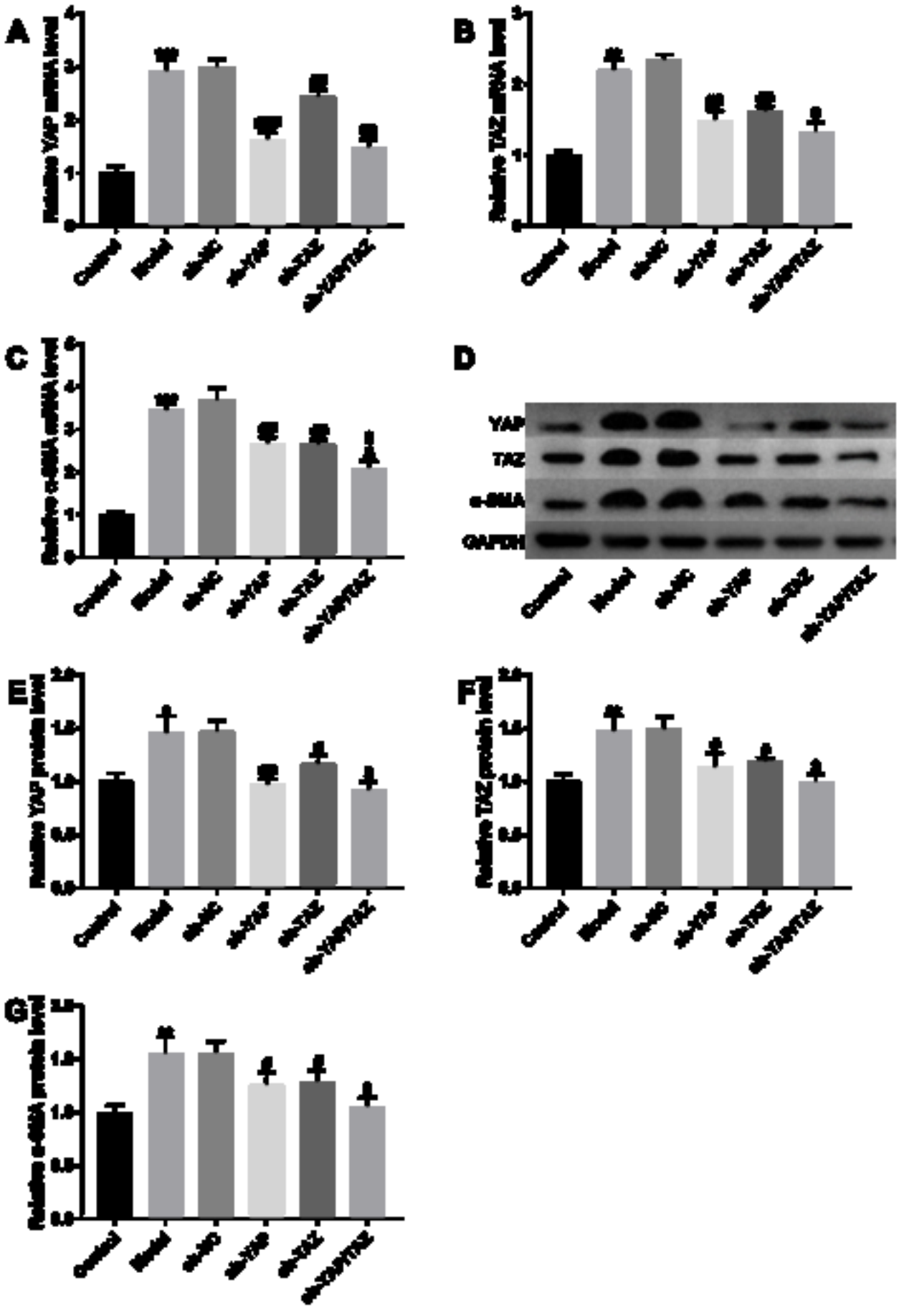

Figure 6

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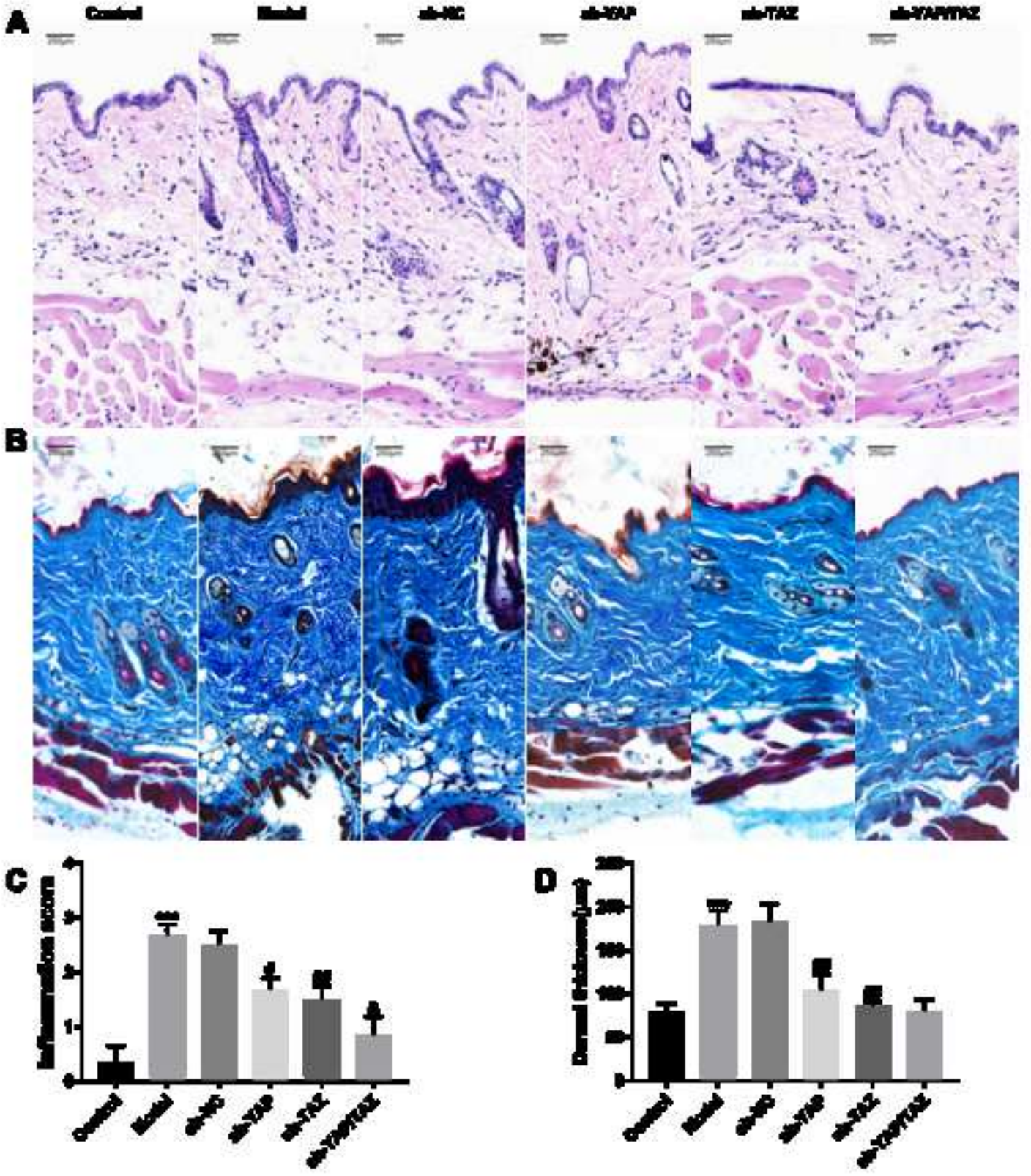


Figure 7

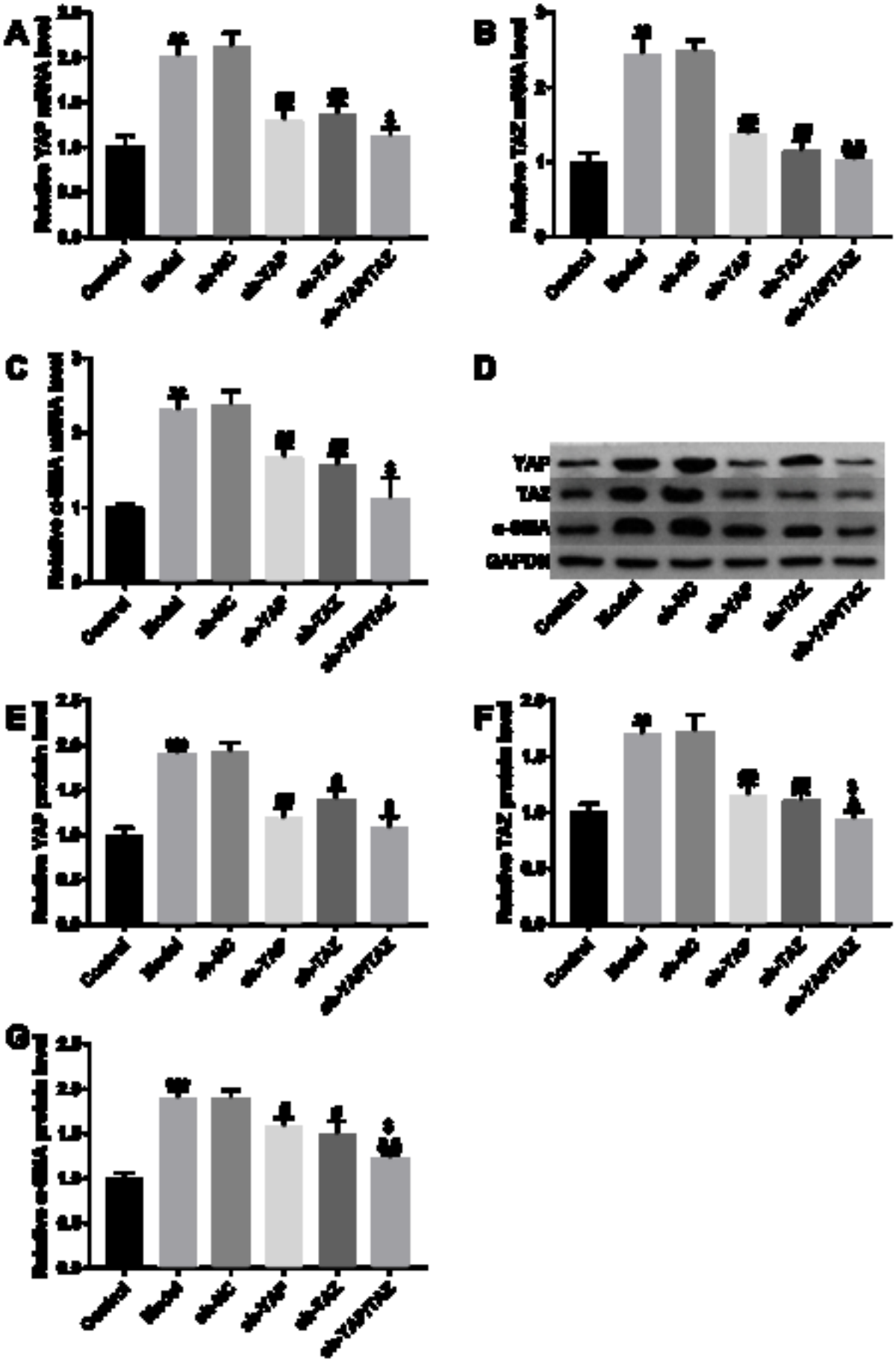
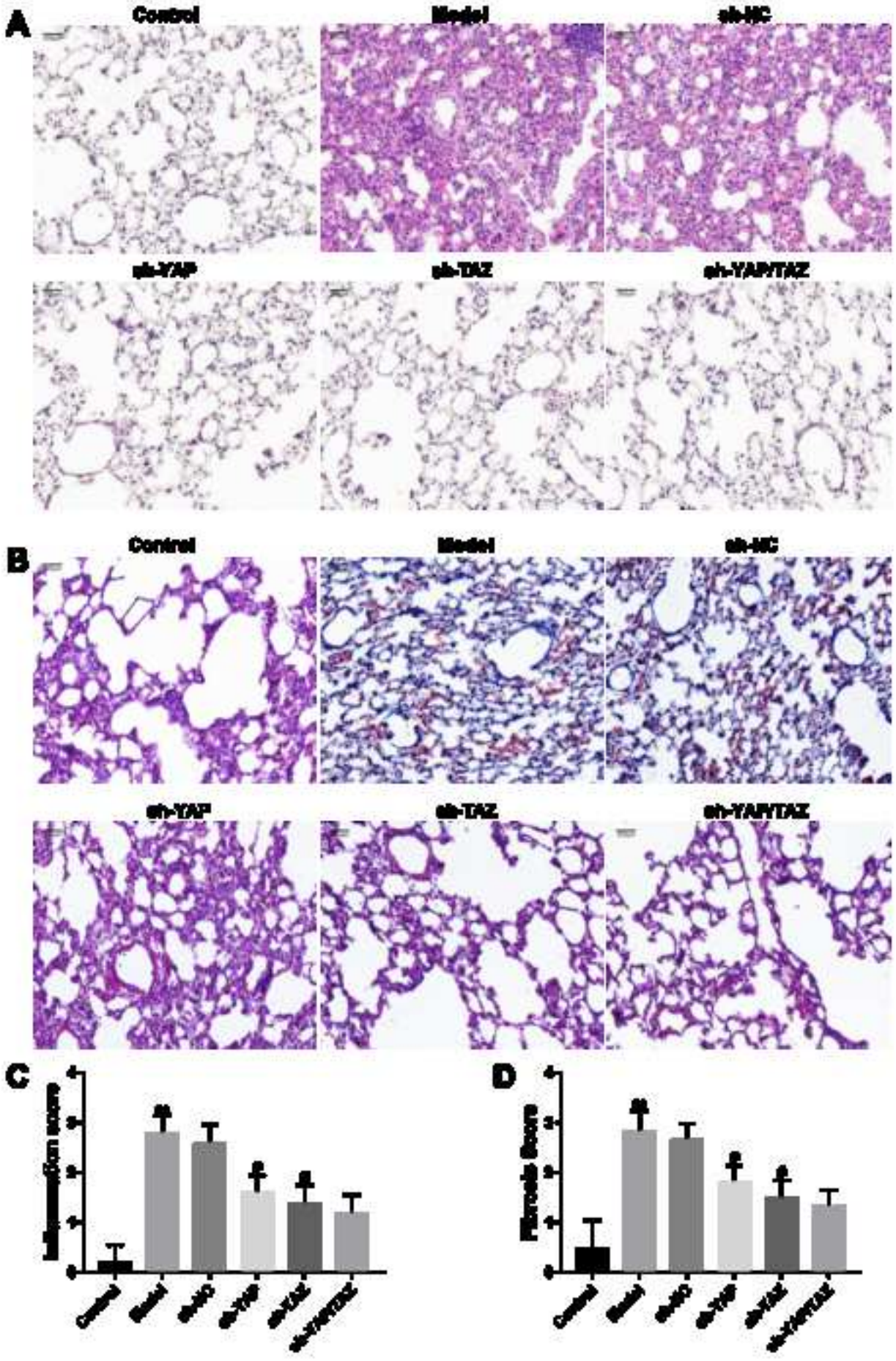


Figure 8



Supplementary figure 1

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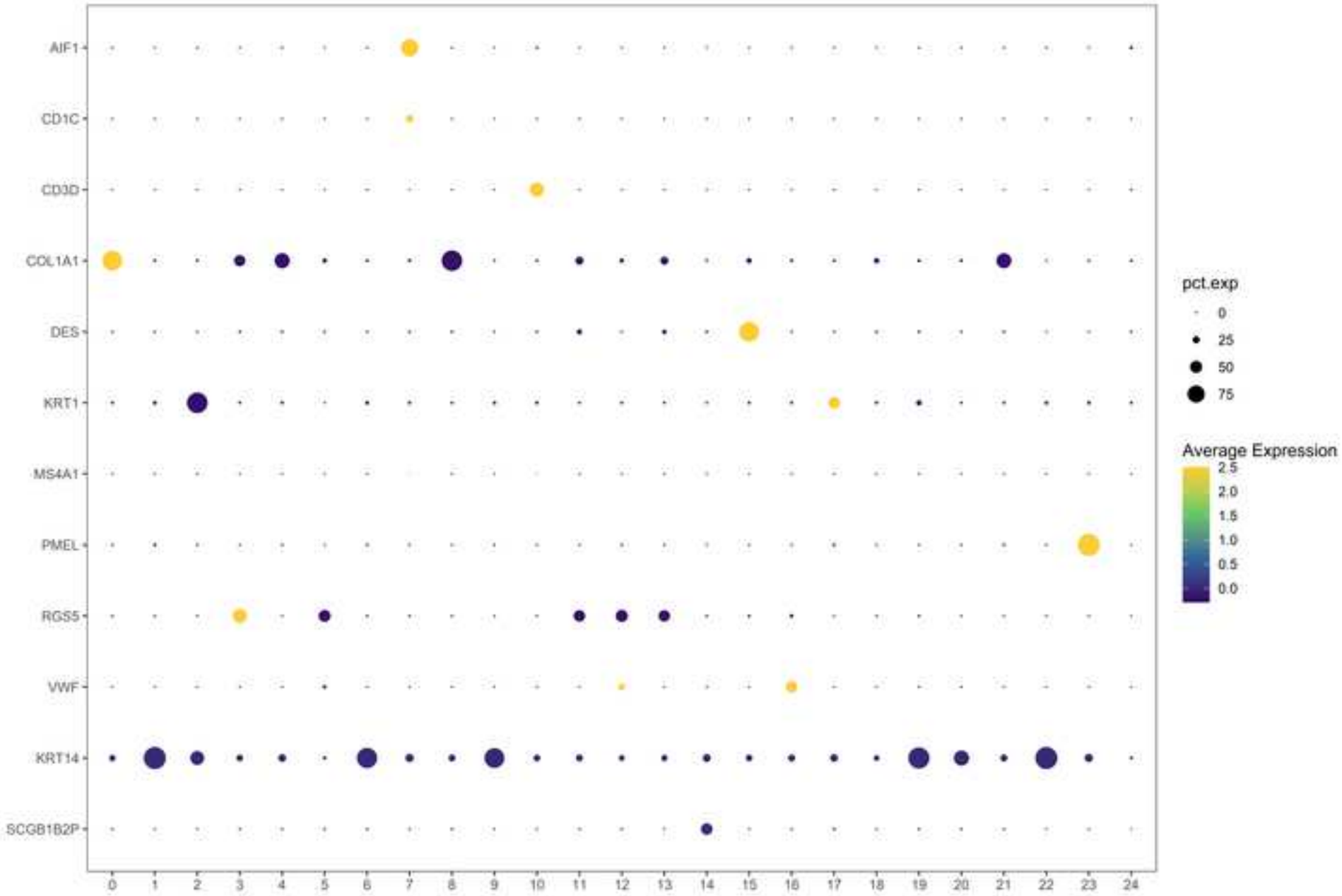

Table 1 Primers used for qRT-PCR.

Gene	Forward Primer	Reverse Primer
TAZ	F-CCCCGACTCACCTGGACCCT	R-GGGAATGCAGCTCCTTGGTGA
YAP	F-TCGGTGTCTCCGGCCGGGAC	R-GAGACAACACCACTGGCCGT
α-SMA	F-TCCCATCCATCGTGGGACGT	R-AGAGGGACAGCACAGCCTGA
GAPDH	F-GTCAACGGATTTGGCCGTATT	R-CTTCTCCATGGTGGTGAAGAC

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:
