Glucocorticoid Counteracts Reactive Oxygen Species-Drived Fibroblast Activation through LINC00605-Dependent mRNA Decay of NOXA1

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Abstract

Background: Aberrant activation of fibroblasts is the main cause of the occurrence and development of fibrotic diseases and poses a serious threat to human health. Excessive reactive oxygen species (ROS), traditionally induces oxidative stress damage, are recently found in some fibroblasts' pathological transformation. However, the roles of the increased ROS in fibroblasts activation and the potential interventions have not been reported.

Methods: Single-cell RNA sequencing was performed to demonstrated that the elevated ROS drives fibroblasts activation and promotes hypertrophic scar fibroblasts' (HSFBs) features, such as excessive proliferation and collagen synthesis. Flow cytometry and immunofluorescence was conducted to evaluate Glucocorticoids effected ROS level. Two ROS-activated models were performed to evaluate intracellular ROS generation specifically increase collagen synthesis and proliferation. Differentially expressed proteins and lncRNA were analyzed by high-resolution spatial proteomics analysis and lncRNA sequencing. The role and mechanism by which lncRNA

LINC00605-dependent mRNA decay pathway, leading to ROS reduction and normalization of HSFBs were investigated in ROS-activated models.

Results: ROS drives fibroblasts activation and inhibiting ROS reduced proliferation and fibrosis phenotypes of HSFBs. Notably, glucocorticoids suppress ROS generation in HSFBs. This effect was mediated by downregulating NOXA1, a key gene responsible for promoting ROS production. Mechanistically, the GR bound to NOXA1 mRNA, while glucocorticoid-upregulated LINC00605 directly bound to the glucocorticoid receptor-mediated mRNA degradation (GMD) factor YBX1. This LINC00605-YBX1 complex was then specifically recruited to NOXA1 mRNA through lncRNA-mRNA interactions, facilitating the assembly of the GMD complex and ultimately triggering NOXA1 mRNA decay.

Conclusion: These results indicated that elevated ROS-drived fibrotic phenotypes and cellular proliferation but glucocorticoids could suppress ROS and subsequently reverse pathological transformation of fibroblasts, highlighting ROS-targeted strategies as promising avenues for early interventions in fibrotic diseases.

Keywords: Reactive oxygen species, fibroblasts activation, hypertrophic scar fibroblast, glucocorticoid, LINC00605, glucocorticoid receptor-mediated mRNA decay

Highlights

- Increased intracellular ROS drived fibroblasts activation and led to typical fibrosis phenotypes
- Glucocorticoids reduce cellular ROS in activated fibroblasts through degrading NOXA1.
- In fibroblast cell model, elevated cellular ROS did not induce cell damage but promote proliferation and function of fibroblasts.
- We investigated the role of glucocorticoid receptor-mediated mRNA degradation (GMD) in cellular ROS and identified a novel LINC00605-dependent GMD machinery for the NOXA1.

Introduction

Fibroblasts, which are widely distributed in connective tissue, play important roles in numerous physiological processes including tissue integrity, tissue repair and homeostasis by synthesizing extracellular matrix (ECM) such as collagen, elastin, and glycosaminoglycans[1]. Whereas, fibroblasts can also be activated and undergo phenotypic transformation (eg. transformation into myofibroblasts) in pathological conditions, leading to excessive cell proliferation and ECM deposition, thereby causing fibrotic diseases or pathological scars. Traditional theory holds that normal physiological concentrations of reactive oxygen species (ROS) act as messenger molecules to transduce cellular signals, while high concentrations of ROS induce cellular oxidative stress and DNA damage[2]. However, in recent years more and more evidences have shown that the increase of cellular ROS, caused by chronic inflammatory stimulation during the development of pulmonary fibrosis and pathological scars, does not seem to lead to damage and apoptosis of fibroblasts, but may be related to excessive activation of fibroblasts[3,4]. This makes ROS become a hot topic in activation of fibroblasts and developing the potential anti-fibrotic strategies.

Glucocorticoids (GC), the most important regulatory hormones for stress response, play various roles in human growth, metabolism, and immune processes[1]. Increasing evidences indicate that the generation and elimination

of ROS are also potentially related to GC, but the roles and mechanisms of glucocorticoids on intracellular ROS have not been fully elucidated to date[5,6]. For example, GC produce ROS, causing oxidative damage in the hippocampus and impairing cognitive function in rats[7]. Glucocorticoids dexamethasone exposure leads to osteoblast apoptosis and autophagy in osteoblasts, affecting their viability through elevated intracellular ROS generation[8]. However, recent studies have shown that glucocorticoids may also lower intracellular ROS levels. Exposure to GC markedly reduced lipopolysaccharide (LPS)-induced oxidative stress in macrophages[9]. Low-dose GC dexamethasone demonstrated cardioprotective effects in myocardial ischemia-reperfusion (MIR) mouse models and MI cell cultures by reducing oxidative stress through ROS scavenging, suppressing inflammatory activation, and upregulating antioxidant defense mechanisms[10]. These seemingly opposite conclusions suggest that glucocorticoids may have different effects, promoting or inhibiting cellular ROS, among different special cell types or under different pathological conditions, and their mechanisms may also be more complex and diverse. However, so far there have been no reports on the specific roles and mechanisms of glucocorticoids on ROS in fibroblasts.

The ways in which glucocorticoids exert physiological and pharmacological effects have long been believed to activate glucocorticoid signaling pathway and initiate gene transcription by binding to GRE sites[11]. Until recent years, glucocorticoid receptors-mediated mRNA degradation, which is different from the GRE pathway, was discovered as a special way for glucocorticoids to regulate gene expression[12,13]. Further, our previous research found that glucocorticoids can also target and regulate the expression of specific genes through lncRNA mediated GMD, revealing the new role of glucocorticoids in post transcriptional regulation of genes, making it possible to further study more underlying functions and mechanisms of glucocorticoids[1].

In this study, we found that the elevated ROS active fibroblasts including proliferation and collagen synthesis in the hypertrophic scar pathological model. Conversely, suppression of ROS transformed the HSFBs to normal-like fibroblasts, which suggested ROS inhibition was an effective strategy for reversing fibroblasts' activation. Further, we uncovered the novel function of glucocorticoids that it could decrease intracellular ROS of fibroblasts. Mechanistically, we demonstrated that glucocorticoids specifically declined NOXA1, a key regulator to promote ROS generation, through a LINC00605-dependent GMD pathway, leading to ROS reduction and normalization of HSFBs. These results not only offer new therapeutic strategies based on ROS inhibition in fibroblasts for fibrosis and pathological scar diseases, but also provide a novel perspective for understanding the hidden biological roles and mechanisms of glucocorticoids.

Materials and Methods

Materials

The glucocorticoid TA was obtained from Abcam (ab142469, MA, USA). Western blot analysis was conducted using NOXA1 (1:500, ab68523, Abcam), α-SMA (1:400, ab7817, Abcam), GR (NR3C1, 1:500, ab305050, Abcam), and reference gene β-actin (1:1000, ab8227, Abcam) antibodies. Actinomycin D was used to inhibit RNA synthesis, which was a product of VWR International, LLC (ab141058, Abcam). Cellular ROS assay kit was purchased from Abcam (ab113851). LPS and TGF-β was purchased from Sigma-Aldrich (82857-67-8, 446859-

33-2, MO, USA). ROS inducer Tert-butyl hydroperoxide (TBHP, 75-91-2) and ROS scavenger N-acetyl-l-cysteine (NAC; 616-91-1) was purchased from Merck (Darmstadt, Germany).

HS samples

HS tissues were collected from patients (range: 18 to 65 years) with a hypertrophic scar present for less than 2 years. Patients were given GC lidocaine or lidocaine only for more than three months, resulting in softening and shrinking. The collected HS specimens were performed for histopathological examination and subject to ISH and IHC assay.

Cell culture and drug treatment

HSFB/NSFB collected from minced HS/NS tissue were cultured with Dulbecco's modified eagle's medium (DMEM, Invitrogen, CA, USA) containing 10% fetal calf serum. Cells were incubated in a 37°C incubator with 5% (v/v) CO_2. Primary (P0) HSFbs were used for cell biology and molecular mechanism experiments. In the experimental groups, HSFBs were treated with 200 nM of GC for 72 hours at density of 1.5×10^6. The primary human immortalized fibroblast cell line WS1 (CRL-1502, ATCC) was maintained in T-75 flasks and used between passages 3–5. For coculture experiments, THP-1 macrophages (TIB-202, ATCC) were initially treated with LPS for 72 h in RPMI-1640 culture medium within 24-well BioCoat cell culture inserts (BD Biosciences, USA). Following LPS stimulation, the THP-1 medium was exchanged for RPMI medium without serum, and the inserts were moved to wells that contained WS1 fibroblasts in DMEM lacking serum. To further induce pro-inflammatory signaling, THP-1 macrophages were exposed to 10 μM phorbol 12,13-dibutyrate (PDBu, Invitrogen) for a duration of 24 hours. Subsequently, cell lysates were collected for intracellular ROS detection and proliferation assays[14,15]. To investigate fibrosis-induced intracellular ROS production, fibroblast cell line WS1 were exposure to TGF-β to establish an ROS-induced cell model[16].

Tissue section preparation and laser microdissection technology (LCM)

This fully integrated spatial cell-type annotation and proteomics quantification technology, named LCM-SISPROT, is therefore precise for spatial-resolution proteome profiling of HSFB from GC-treated HS tissues. Tissue sections were prepared with a thickness of 12 μm, and then transferred to a PEN-membrane glass slides. For cell type-fibroblast proteomic analysis of HS, α-SMA stained HSFBs were collected by LCM with an accumulated capturing area of 5 mm^2, following with H&E staining. An initial whole stained tissue sections scan at 20× magnification (NanoZoomer S60 system, Hamamatsu, Japan) was performed prior to LMD. The pathological images and cell type examination results of the acquired whole slide images were initially assessed by utilizing a LMD7000 microscope (Leica, Weizler, Germany). An optimized cutting parameters were adjusted to minimize possible laser-induced to the neighboring cells. Subsequently, ten pathological regions exhibiting HSFB morphological appearances were dissected under the supervision of seasoned pathologists. All the collected cumulative area of 1 mm^2 of HSFB samples were gathered into PCR tube for next step[17].

Simple and integrated spintip-based proteomics technology (SISPROT)

The microdissected HSFBs were disrupted, de-crosslinked with brief centrifugation and and fragmented through sonication. The HSFB lysates were handled following a modified SISPROT protocol[18]. Briefly, the samples were

acidified to pH 3 and then placed onto an activated SISPROT tip filled with mixed POROS beads of SAX and SCX fractionation and one plug of Empore C18 disk (GL Science, Japan). Proteins were rinsed with Acetonitrile, reduced with 10 mM TCEP, followed by alkylation with 40 mM chloroacetamide, digested 1 hour at 37°C by adding 10 ng of trypsin. The digested peptides were loaded onto the C18 layer, and dried under vacuum. After that, all peptide samples were resuspended in 0.1% FA with mixed iRT peptides (Biognosys, Switzerland). Every sample was measured two times for ddaPASEF and diaPASEF method analysis, individually.

Liquid chromatography/mass spectrometry (LC/MS)

LC/MS analysis was conducted on a timsTOF Pro mass spectrometer combined with a nanoElute UPLC system via a captive spray ion source (Bruker Daltonik Captive Spray, Germany). Digested peptides were first transported to a trapping column and then separated inline on a 50 mm capillary column packed with C18 beads (Dr. Maisch GmbH, Ammerbuch, Germany) at 100 nL/min. Peptides for protein analysis were extracted and utilized in a linear gradient ramped from 2% to 22% phase B over 50 minutes, linear gradient to 35% in 10 minutes, and the gradient was then washed at 80% phase B for 20 minutes. The LC/MS-MS platforms were operated in ddaPASEF or diaPASEF mode based on prior descriptions to identification differential expression protein in spatial-resolution proteome profiling and GC-treated HSFBs proteome profiling.

MS data analysis

The original MS file data were submitted to spectronaut software v16.2 for data analysis (Biognosys). MS raw files obtained from peptide fractionation samples and LMD injection samples were utilized for the generation of the spectral library. The raw files were compared with Homo sapiens protein database from UniProt (Uniprot Proteome ID: UP000005640). The final library used for this experimental work comprised 2379 protein groups. Subsequently, a standardized analytical pipeline was employed for the comprehensive identification and measurement of proteins across Data-Independent Acquisition (DIA) raw files[19]. Supplementary table 1 includes all the differential expression proteins for MS data analysis.

RNA sequencing

For RNA sequencing analysis, we harvested HSFBs that were treated with GC or control group. Total lncRNA or mRNA extracted by TRIZOL method (Invitrogen), and ribosomal RNA and fragmentation was removed, followed by reverse transcription to generate complementary DNA (cDNA) for examining the differentially expressed lncRNAs. RNA sequencing experiment was carried out by Gene Denovo Biotechnology Co. (Guangzhou, China). Various steps were performed, including purification, 3' terminal addition, terminal repair, library quality inspection, ligation, primer addition, and PCR amplification. Finally, sequencing of the resulting libraries was performed on an Illumina HiSeq 3000 instrument (Illumina Inc, USA) with PE150. Probes that changed by < 1.5-fold and had a $P \geq 0.05$ were excluded from subsequent analysis.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from NSFB/HSFB/FB with TRIzol reagent (Invitrogen, USA). cDNA was converted from cellular RNA via reverse transcription and used as template in qRT-PCR. qRT-PCR was carried out using the ABI7500 quantitative PCR quantitative PCR instrument (Applied Biosystems, USA) for evaluating their

expression levels, including human lncRNA, NOXA1 and GR, also using GAPDH (housekeeping gene) for normalization. The gene expression levels were determined by the method of $2^{-\Delta\Delta Ct}$ calculation[20]. Supplementary table 3 includes all the primers for qRT-PCR.

Western blotting

The lysate of GC-treated HSFBs (50 µg protein) was loaded on 10% polyacrylamide gels, followed by SDS-PAGE electrophoresis. The separated protein bands were subsequently moved onto PVDF membranes and incubated with diluted primary antibodies (GR, YBX1, NOXA1 and β-actin). Bound labeled secondary antibody was detected using enhanced chemiluminescence (ECL) reagent[21].

Bioinformatic analysis

Single-cell RNA sequencing (scRNA-seq) data from NS/HS samples was retrieved from the Gene Expression Omnibus (GEO) database (accession: GSE156326). The analysis was performed on HS samples to compare gene expression profiles across major cell types between HS and NS. To assess fibroblast activity, the AUCell algorithm from the SCENIC R package was applied to compute AUCell scores for fibroblast signature genes in individual cells within each cluster[22]. The Gene Ontology (GO) annotations from the Gene Ontology site (http://www.geneontology.org/) were used for analyzing DEGs. A 1537-base pair (bp) sequence of LINC00605 was retrieved from NCBI (Gene ID: 100131366). To identify the tissues where LINC00605 is differentially expressed, the gene read counts of LINC00605 were observed using https://www.gtexportal.org/. Genome browser depiction of LINC00605 and its conserved analogs sequences in multiple vertebrate species were then queried from http://genome.ucsc.edu/. Evolutionary conservation was evaluated based on data from the UCSC Genome Browser Database (GBD, http://genome.ucsc.edu/). Predicted three-dimensional structure of protein GR (UniProt ID: P04150) and YBX1 (UniProt ID: P67809) were from UniProt, and structure of protein YBX1 dimer prediction was based on the sequence by using the AlphaFold software. Three-dimensional structure files of protein monomers, protein dimers, and LINC00605 docking was performed by using AutoDock Vina software[23]. The GR and YBX1 proteins were represented as slate cartoon models, nucleic acid as a green cartoon model, and endcaps and internal binding sites as corresponding-colored stick structures. The hydrogen bonding interaction participating numerous groups of residues used to involved hydrogen bonds between GR (or YBX1) and nucleic acids[24]. To predict the hairpin formation in mRNA sequences of NOXA1 RNA, their secondary structure was examined by online tools RNAfold (http://rna.tbi.univie.ac.at)[25]. Furthermore, the GR motif binding sites of the NOXA1 promoter region were also examined in silico[26]. To amplify these regions for PCR in CHIP assay, the relevant primer pairs were designed by premier 5.0 software. RBPMAP, a computational tool for mapping of RNA binding proteins (RBPs) binding site (http://rbpmap.technion.ac.il/) was employed to predict the LINC00605 binding sites of RBPs[27].

Size-exclusion chromatography (SEC)

The samples were subjected to molecular weight measurements upon their preparation using a SEC Hiload 16/60 Superdex 75 column (GE healthcare, USA). The column was calibrated using SEC protein standards (1.3-670 kDa/mol in molecular weight) (Agilent, USA). Sample solutions had a concentration of 20 mg/1000 µl, an

injection volume of 150 µl, and a flow rate of 1 ml/min. The calculations for multidetector GPC were performed using Agilent software[28].

Fluorescence in situ hybridization and immunofluorescence (FISH/IF) co-analyses

The LINC00605 probes and YBX1 antibody (ab76149, 1:300, Abcam) were applied for RNA fluorescence and immunofluorescence co-localization analysis. Cy3-labeled LINC00605 probes were designed using Stellaris RNA FISH designer. GC-treated HSFBs climbing was fixed in 4% paraformaldehyde. The LINC00605 probe hybridization solution with a concentration of 500 nM was added. After incubated the probe and anti-YBX1 antibody overnight, HSFBs were incubated with secondary antibodies and stained with DAPI. HSFBs were imaged using FSX100 fluorescence microscope (Olympus, Japan). Primer sets used in FISH are listed in Supplementary table 3.

Photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP)

PAR-CLIP assay was demonstrating GR in recognition of the motif sequences in NOXA1 mRNA and its directions of transcription[1]. For in vivo GC-RNA interaction study, HSFBs were labeled in media containing 100 µM 4-thiouridine for 16 hours, followed by irradiate cell plates with UV light at 365 nm. GC-treated cells were incubated for another 12 hours in 200 µM 4-thiouridine. To further explore cross-link NOXA1 RNA to endogenous GR, the Spectrolinker XL-1500 UV cross-linker was used to irradiate the HSFBs. Treated HSFBs were collected, and then lysed with ribonuclease T1. Specific anti-GR antibody linked to protein A/G dynabeads was utilized to immunoprecipitate the endogenous GR functional protein. The RNA residing in the immunoprecipitate was further trimmed with RNase T1. Then, lysis buffer was used to wash the beads, deoxyribonuclease I and proteinase K were consecutively employed to remove the DNA and proteins contamination. Finally, the pull-downed RNA was recovered and identification by PAR-CLIP qPCR. The sequences of the PAR-CLIP qPCR primers are provided in Supplementary table 3.

RNA immunoprecipitation (RIP)

Cross-linking of GC-treated HSFBs was performed under condition of 1500 µJ cm^2. The collected HSFBs were subject to nuclear/cytoplasmic fractionation according to manufacturer's procedures (Nuclei EZ Lysis Buffer, Sigma, USA). Collected cytoplasmic fraction was pre-clearned by yeast RNA and recombinant protein G agarose performed at 4°C for 30 minutes. An aliquot of HSFB lysates was saved as input (control), and the remaining HSFB lysates were used for immunoprecipitation by an incubation wit GR or YBX1 antibody or IgG at 4°C overnight. LINC00605-YBX1 antibody complexes were then captured by protein G beads (Invitrogen). Subsequently, RNA that was immunoprecipitated was extracted and analyzed using qRT-PCR. Primer sets used in RIP are listed in Supplementary table 3.

Vector construction

The overexpression plasmid of LINC00605 were constructed using cDNA obtained from HSFBs for subcloning with pcDNA-3.1 plasmid (Invitrogen). The mutant form of LINC00605 expression plasmid was also made by subcloning the insert which contains mutation sequence of the putative binding site into pcDNA-3.1 vector. Meanwhile, the cDNA containing the coding sequence of YBX1 gene was obtained from HSFBs and amplified

by PCR as an insert which was then verified by DNA sequencing and subcloned into pcDNA-3.1 vector. The cDNAs of NOXA1 were also amplified for construction of their ectopic expression plasmid with pcDNA-3.1 vector. The names of those constructs were given, according to the location of the promoter region for transcription of their mRNAs. To evaluate the effect of CDS or CDS mutation on NOXA1 expression, we generated constructed vectors according to our previous report[1]. The primers used for developing those constructs are listed in Supplementary table 3.

Vector construction and lentivirus packaging

The the full-length open reading frame (ORF) of human NOXA1 was PCR-amplified from HSFBs cDNA. The NOXA1 ORF was ligated into the corresponding sites of the pcDNA3.1vector (Invitrogen). Full-length of human LINC00605 was synthesized as an insert to be cloned into the vector pMD-18T (Takara), as verified by DNA sequencing. The obtained DNA was further subcloned into pLenti-6.3 destination vector (Invitrogen) for next lentiviral packaging step. Packaging of lentiviral plasmids were performed in human embryonic kidney 293T (HEK293T) cells by co-transfection of pMD.2G and pPAX2 plasmids for 48 hours, using lipofectamine 2000 (Invitrogen). Following this, the medium was centrifuged, and the precipitate was harvested, followed by collection of supernatants containing viral particles, the title of which could be assessed by multiplicity of infection (MOI). Subsequently, lentivirus transfection HSFBs was performed following instructions. The primers for plasmid constructs are listed in Supplementary table 3.

siRNAs and transfection

In this study, we designed siRNAs targeting LINC00605 RNA, NOXA1 or GR mRNA, which were synthesized by Ribobio company (Guangzhou, China). The transfection of both siRNAs and pcDNA3 plasmid was performed as previously reported[29]. Briefly, HSFBs (2.5×10^5 cells per well in 6-well plate obtained from Falcon) were transfected with siRNAs and plasmid for a duration of 48 hours using lipofectamine 2000 (Invitrogen). The primers used for validation of transfection efficiency are listed in Supplementary table 3.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was carried out with ChIP-IT Express Enzymatic kit (Millipore, USA) following the guidelines from the manufacturer. To summarize, HSFBs were cross-linked with 1% formaldehyde, followed by the preparation of cell lysates in a ChIP lysis buffer. Chromatin was fragmented through either sonication or enzymatic digestion, with the resulting sheared chromatin (ranging from 200 to 1500 base pairs) verified via agarose gel electrophoresis. A mixture containing 1.5 μg of sheared chromatin DNA and either anti-YBX1 antibody or control (IgG) was incubated with magnetic protein A-agarose beads and incubated overnight at 4°C on a rotating device[30]. The DNA isolated from the antibody-protein a agarose-beads complex was subsequently used as a template in PCR amplification with specific designed primers, which are listed in Supplementary table 3.

Analysis of intracellular ROS by flow cytometry

The impact of ROS production and proliferation on HSFBs was determined by flow cytometry. Briefly, HSFBs were treated in triplicate with either a control or GC at 37°C for 24 hours. Following a washing step, a total of 1×10^6 cells were stained with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Invitrogen) at 37°C for 15

minutes. The stained cells were collected on a FACSCalibur (Life Technologies, USA), and the fluorescence intensity data was quantified utilizing CELLQuest software version 5.1 (BD Biosciences, USA).

ROS immunofluorescence

HSFBs with different treatment were then incubated with DCFH-DA (Invitrogen), and the HSFBs nuclei was stained with diamidino-phenyl-indole (DAPI). An excitation wavelength of 488 nm and an emission wavelength of 535 nm were used to monitor fluorescence intensity. Images showing fluorescence were taken using a confocal microscope (Olympus, Japan).

Immunohistochemistry (IHC) and In Situ Hybridization (ISH)

For IHC, deparaffinized and hydrated paraffin-embedded GC-treated HS and control tissues were immunostained with anti-NOXA1 antibodies (1:200, ab222852, Abcam). Signals were amplified using an HRP-DAB kit (DakoCytomation, Denmark). For ISH, tissue sections were deparaffinized, rehydrated, and permeabilized prior to hybridization. Oligonucleotide probes targeting LINC00605 and NOXA1 were designed and synthesized with Alexa647 labeling (Thermo Fisher, USA). Hybridization was performed in a buffer containing 2× SSC, 25% formamide, 10% dextran sulfate, and yeast tRNA at 42°C for 3-4 h in a humidified chamber. Post-hybridization washes were conducted in graded SSC buffers to remove nonspecific binding. The imaging process utilized a Nikon 90i microscope with Volocity software, and signals were manually scored[31].

Enzyme-linked immunosorbent assay (ELISA)

The Col1 and Col3 of GC-treated HSFBs secretion levels into the supernatant were quantified utilizing an ELISA kit (Invitrogen). All standards, samples and blanks were measured by SpectraMax M5 microplate reader (Molecular Devices, USA) to measure absorbance at 450 nm. A standard curve was generated by regression fitting with computer software, and the protein concentrations were subsequently calculated based on the absorbance values obtained.

MTT assay

Cell proliferation and viability of human skin fibroblasts (HSFBs) treated with GC were assessed using the MTT assay. Briefly, HSFBs were incubated with MTT reagent (Invitrogen) for 1 h, and the formazan crystals were subsequently dissolved in 200 μL of DMSO. The measurement of optical density was taken at 570 nm (OD 570).

Statistical analysis

All numerical values are shown as mean ± SD of 3 independent replicates and analyzed using two-sided student's *t*-tests. The statistical analysis software is GraphPad PRISM (version 9.5, GraphPad, USA). Correlations between flow cytometry examine ROS level, and between the flow cytometry examine proliferation level and collagen ELISA level were analyzed using Pearson correlation[32]. Stoichiometry curve plot and three-dimensional surface plots were plotted using Origin (version 2025, Origin Labs, USA). $P < 0.05$ was considered statistically significant.

Results

scRNA-seq analysis revealed that hypertrophic scar fibroblasts exhibit elevated ROS levels, excessive collagen synthesis, and hyperproliferative characteristics

To characterize disease-associated cellular states in hypertrophic scars (HS), we analyzed single-cell RNA sequencing (scRNA-seq) data from HS and normal skin (NS) samples in the GEO database (Figure 1A). Unsupervised clustering identified eight major cell populations based on canonical marker expression (Figure 1B). Fibroblasts (FBs) were the predominant cell type in both HS and NS, accounting for over 40% of all cells (Figure 1C). GO enrichment analysis of highly differentially expressed genes in HSFB and NSFB found that they were mainly enriched in ROS, collagen synthesis and proliferation (Figure 1D). Using AUCell to evaluate pathway activity, we found that HSFBs exhibited significantly higher enrichment scores for the three signaling pathways compared to NSFBs (Figure 1E and F). Furthermore, ROS AUCell score positively correlation with collagen synthesis and proliferation, indicating specific cell type enrichment (Figure 1G). Overall, scRNA-seq uncovers that fibroblasts in hypertrophic scars exhibit heightened ROS generation, aberrant collagen synthesis, and enhanced proliferative capacity.

High intracellular ROS levels correlate with aberrant collagen production and hyperproliferation in HSFBs

Hypertrophic scar is a common skin condition resulting from an excessive healing response to injury, marked by abnormal fibroblast growth and excessive collagen production and deposition[1]. To investigate the role of intracellular ROS generation in HS development, we isolated HSFBs and NSFBs from HS and NS tissues, respectively (Figure 2A). Intracellular ROS levels were assessed using fluorescent microscopy and flow cytometry (Figure 2B-E). Compared to NSFBs, HSFBs exhibited significantly higher intracellular ROS generation (Figure 2F and G). Additionally, HSFBs showed elevated expression of Col1, Col3 and proliferation rate (Figure 2H-J). Previously studies reported that constitutive intracellular production of ROS enhances fibroblast proliferation and collagen synthesis[33,34], therefore, we hypothesized that the critical role of high-level intracellular ROS may related to hypertrophic scarring in abnormal wound healing process, characterized as enhanced collagen synthesis and proliferation in HSFBs. To test this hypothesis, we analyzed their correlation. The results revealed a strong positive association between intracellular ROS levels and the Col1/Col3 expression and proliferation rate (Figure 2L-M). Meanwhile, consistent with the previously results[35,36], primary HSFBs (P0), exhibiting activated fibroblasts property *in vitro*, displayed a much higher α-SMA mRNA and protein content compare to NSFBs (FigureS1. A and B). In addition, consistent with the result ROS contribute to α-SMA induction[37], intracellular ROS levels and α-SMA protein level indicated a significant positive correlation (Figure S1. C). These findings collectively demonstrate that intracellular ROS generation positive collation with collagen synthesis and proliferation processes in HSFBs under pathophysiological conditions.

Elevated ROS levels drive collagen formation and proliferation processes

Previous studies have reported that the inflammatory microenvironment and pro-fibrotic factors can activate fibroblasts, promoting collagen synthesis and excessive fibroblast proliferation during hypertrophic scarring[38-40]. Concurrently, the inflammatory microenvironment and pro-fibrotic factors have also been demonstrated to induce ROS production[14-16,35]. Therefore, we hypothesized that ROS may serve as a key driver in fibroblast activation, leading to excessive collagen deposition and fibroblast hyperproliferation. To test this hypothesis, we firstly mimics the elevated ROS microenvironment of HSFB in HS tissue, and we utilized both macrophage fibroblast

co-culture system and TGF-β induce intracellular ROS generation to activate FBs (Figure 3A)[14-16,35,36]. Figure 3B and C indicates experimental design for the timing, concentration and ROS level in the of LPS and TGF-β treated FBs. The ROS concentration was at peak at 24 h with 100 ng/mL LPS and 10 ng/mL TGF-β, respectively. We considered the concentration and timepoint to be the critical point for analyses between ROS concentration of mimics inflammation pathophysiological environment. Thus, 100 ng/mL LPS and 10 ng/mL TGF-β at 24 hours pretreatment was selected as the optimum concentration and time point in the subsequent experiments. Meanwhile, activated fibroblasts marker α-SMA, Col1/Col3 and proliferation rate are significant increase in two ROS-activated model (Figure 3D-F, Figure S2). To explore whether intracellular ROS generation specifically increase collagen synthesis and proliferation, ROS scavenger N-acetyl-l-cysteine (NAC) was added in two models. Interestingly, NAC almost completely abolished intracellular ROS levels measured by flow cytometry (Figure 3G-J). Meanwhile, scavenge ROS decreased levels of α-SMA expression, collagen synthesis and proliferation (Figure 3K-M, Figure S3). These findings indicated that elevated ROS levels participate in promoting collagen synthesis and proliferation process.

GC inhibit collagen production and fibroblast proliferation through ROS-*dependent mechanisms*

Glucocorticoid triamcinolone acetonide is the first-line medications for patients with hypertrophic scar. Glucocorticoids selectively decrease collagen synthesis and proliferation of HSFBs, inhibiting fibrosis and scarring[41]. However, the regulation role of GC on ROS is still unknown. To test the effects of GC on ROS-inhibition in HSFBs, ROS concentration and cell viability were used to screening optimum concentration and time interactive point, respectively[42,43]. The ROS concentration was inhibited by GC in two cell model. The ROS concentration was most significantly reduced at 48 h with 200 nM GC in two cell model, respectively (Figure 4A-D). We considered 200nM at 48 hours to be the critical point for analyses between ROS concentration under pathophysiological conditions. Thus 200nM at 48 hours were selected as the optimum concentration and time point in the subsequent experiments. Flow cytometry confirmed that GC scavenging intracellular ROS ability (Figure 4A-D). Meanwhile, GC downregulated collagen expression and cell proliferation rate (Figure 4E-G). To explore intracellular ROS downregulation specifically decrease collagen expression and cell proliferation, ROS inducer tert-butyl peroxide (TBHP) was added in GC treated cell model. Interestingly, TBHP almost evaluated intracellular ROS levels measured by flow cytometry (Figure 4H-K). In contrast, increased ROS rescued collagen expression and cell proliferation in GC treated cell model (Figure 4L-M). These findings indicated that reduction of ROS underlies GC-mediated inhibition of collagen production and FB growth.

Deep spatial proteomics reveals NADPH oxidase activator 1 NOXA1 was downregulated by GC

Glucocorticoid (GC) triamcinolone is typically the initial choice in the treatment of hypertrophic scar. After administering GC through an intralesional injection, there was a marked and a decrease in HSFBs proliferation and collagen deposition[1]. Fibroblasts are generally recognized as the major effector cells responsible for hypertrophic scars, in essence, we want to know what its intracellular protein expression in GC-treated HS[1]. Based on accurately defining single-cell boundaries and HSFBs typing results, a laser microdissection (LMD) technique was used to automated microdissection, and subject to an integrated proteomic technology called SISPROT analysis HSFBs proteomics in GC-treated HS tissues (Figure 5A). HSFB proteins of HS tissues are quantified in 6 samples, on average 13,220 proteins were identified per sample (Figure 5B). 2379 proteins were

identified with a high degree of reliability, demonstrating robust quantitative reproducibility and low coefficient of variation (CV) values (Figure S4A and B). Principal component analysis (PCA) indicated distinct distribution patterns of the proteome across the two lineages (Figure S4C). The identified proteins spanned six orders of magnitude and exhibited a moderately high intra-region correlation (Figure S4D). The total number of 2,379 protein groups across six samples were quantified (Supplementary table 1). Differential expression analysis and volcano plots revealed a total of 741 different expression proteins between control and GC groups, of these 344 genes were up-regulated and 397 proteins were down-regulated in response to GC treatment (Supplementary table 1), like downregulated NOXA1 (Figure 5C and D). The NADPH oxidase activator 1 NOXA1 is vital for generation of ROS which are involved in pathophysiological states cell functions[44]. Further GO analysis of the differentially dysregulated proteins were associated with biological processes, such as oxidative stress, cell proliferation and collagen synthesis, while GSEA results showed that ROS pathway was closely correlated with down regulated proteins expression by GSEA analysis (Figure 5E, Figure S4E and F). The results showed the ROS pathway enrichment in in-depth spatial proteomic profiling and recapitulating the biological features in HSFBs in GC treated HS tissue. Meanwhile, the SISPROT data (Figure 5F), GC-treated cell model (Figure 5G and H), ISH and IHC (Figure 5I and J) indicated that NOXA1 is downregulated in GC treated HS and HSFBs. The data indicate that deep spatial proteomic analysis demonstrated that NOXA1 expression was significantly downregulated in response to GC treatment

Depleting NOXA1 attenuated collagen synthesis and proliferation by blocking ROS generation

The NADPH oxidase activator 1 NOXA1 is vital for generation of ROS which are involved in pathophysiological states cell functions[44,45]. We first determined NOXA1 expression is upregulated in two cell models (Figure 6A and B). Next, the downregulated of NOXA1 was investigated using siRNA and conformed by qRT-PCR and western blotting in FBs (Figure 6C and D). Therefore, we hypothesized that NOXA1 resulted ROS production may explain the ability of collagen expression and proliferation to promote hypertrophic scarring. To test this hypothesis, our data showed that downregulated of NOXA1 protein decreased ROS production in two cell models (Figure 6E-L). Meanwhile, it decreased the collagen synthesis and cell proliferation rate in two models (Figure6M-P). These finding indicated that silencing NOXA1 reduced intracellular ROS levels, thereby mitigating ROS-triggered collagen synthesis and cell proliferation.

NOXA1 overexpression reverses GC's anti-collagen synthesis and proliferation effects by restoring ROS production

To confirm specificity of intracellular ROS generation inhibition by GC-treatment in inflammation and fibrosis cell model, we used a lentivirus-mediated delivery system to stably overexpression NOXA1 (Figure S5). Overexpression of NOXA1 significant rescued intracellular ROS generation in GC-treatment inflammation and fibrosis cell model (Figure 7A-H). Meanwhile, overexpression of NOXA1 relief repression of GC suppress collagen synthesis and proliferation (Figure 7I-K). These findings, together with the data of previous studies, indicated that NOXA1 overexpression increased intracellular ROS production and reversed GC-mediated suppression of collagen synthesis and proliferation.

GC repress NOXA1 expression in a glucocorticoid receptor-mediated mRNA decay-dependent manner

Recently, our previous study reported that the glucocorticoid-GR system regulates the degradation of multiple target mRNAs independently of a translation event in the clinical glucocorticoid HS treatment, which was named as glucocorticoid receptor-mediated mRNA decay (GMD)[1,12,13]. To explore its potential RNA stability influence on the decay of NOXA1 mRNAs, HSFBs underwent treatment with actinomycin D, a transcriptional inhibitor, and the levels of NOXA1 mRNA were evaluated at different time points. Notably, in presence of GR, HSFBs exhibited a markedly accelerated degradation of NOXA1 mRNAs compared to control HSFBs (Figure 8A). In contrast, knocking down GR, not RNA binding protein YBX1, significantly impeded the decay of these target mRNA, suggesting the pivotal role of GR in maintaining NOXA1 mRNA stability (Figure 8B, Figure S6, Figure S7). Given that glucocorticoids function by binding to the GR[46,47], we questioned whether the mediation of mRNA expression downregulation by LINC00605 operates independently or is contingent on GR. After specifically knocking down GR in GC-treated HSFBs, we observed an increase in the NOXA1 mRNAs (Figure 8B).

For GMD to function, it requires the binding of degraded mRNA to activate the mRNA decay process. To discern whether GR interacts with NOXA1 mRNAs, we analyzed and identified the functional residues, categorizing them based on their interactions with GR. During the hydrogen-bonding interaction, several residue groups were identified hydrogen bonds between GR and NOXA1 mRNA nucleic acids were identified (Figure 8C). Additionally, it was discerned that GR binds target mRNAs in a position-independent fashion[47]. To confirm the specific binding of GR to NOXA1 mRNAs in HSFBs, we found that 636-663bp region was the potential GR bound region (Figure 8D, Supplementary table 2). Beyond theoretical calculations, we extracted data from a previous study's immunoprecipitation of GR-mRNA complexes followed by microarray analysis[46]. This analysis highlighted a pronounced enrichment of GR protein binding to NOXA1 mRNAs (Figure 8E), suggesting potential role of GR in neurogenesis *via* the degradation of these mRNAs. The existence of binding regions of NOXA1 mRNA motif was validated by analyzing their predicted secondary structures (Figure 8F). This evidence suggests that GR modulates NOXA1 mRNA decay rely through the GMD mechanism.

Knockdown LINC00605 decrease NOXA1 in GMD-dependent manner

LncRNAs are a diverse and functionally rich subset of non-coding RNAs. With the continuous discovery of new lncRNA classes, their categorization is primarily based on their functional roles and conservation. To better understand the molecular mechanism, we employed lncRNA sequencing analysis to study gene expression changes in HSFB treated with GC (Figure 9A). Notably, LINC00605 was upregulated in GC-treated HSFBs (Figure 9B). This lncRNA (gene ID 100131366) drew our attention due to its validation in the NCBI database, its confirmed upregulation in GC-treated HSFBs *via* ISH and qRT-PCR (Figure 9C and D). Further examination of genome-batch conversion revealed a degree of conservation in the genomic region surrounding the LINC00605 between species (Figure S8). To further identified that LINC00605 positive regulated NOXA1 in GMD-depend manner, we examine NOXA1 expression in LINC00605 knockdown HSFBs with or without GC treatment. qRT-PCR and western blotting revealed that knockdown LINC00605 downregulated NOXA1 mRNA and protein expression (Figure 9E and F, Figure S9). These findings indicated that knockdown LINC00605 decrease NOXA1 was dependent on GC.

Interaction of LINC00605 with YBX1 influences GMD of NOXA1

Given the occurrence of GMD in GC-treated HSFBs, the mechanisms by which LINC00605 regulates lipid accumulation and impacts mRNA decay remain elusive. We hypothesized that LINC00605 might interact with specific protein factors. Utilizing the online tool RBPmap, we identified the RNA-binding protein motifs in LINC00605 and predicted the potential functional residues of YBX1 that can bind to LINC00605 with AACAUCA sequence (Figure 10A, and Figure S10). An RNA pull-down assay confirmed presence of RNA bind protein YBX1 within the pull-down samples by the LINC00605-sense RNA probe (Figure 10B). To validate this RNA-protein interaction in a cellular context, we conducted a cross-linked RNA immunoprecipitation assay, revealing a significant association between YBX1 and LINC00605 in GC-treated HSFBs (Figure 10C). Notably, LINC00605 was more enriched in samples immunoprecipitated with anti-YBX1 antibodies than in those with IgG. These findings confirm the binding of YBX1 to LINC00605 RNA, rather than other factors. Next, we assessed the stoichiometry of YBX1 and YBX1-LINC00605 complexes by analytical size-exclusion chromatography (SEC). YBX1 eluted as a single peak at 9.91 mL, corresponding to an apparent molecular weight (MW) of 71 kDa. Given the predicted molecular weights of YBX1 (~35.6 kDa), it is plausible to infer that the observed elution profile corresponds to YBX1 homodimers (Figure 10D). Subsequently, we conducted an incubation of LINC00605 with YBX1, resulting in a shift of approximately 0.3 mL in elution volume to ~9.62 mL, accompanied by a calculated MW of 81 kDa. These findings are in agreement with the anticipated MW of two YBX1 molecules forming a complex with two LINC00605 (Figure 10D). Furthermore, immunofluorence analyses demonstrated co-localization of YBX1 and LINC00605 in the nucleus of GC-treated HSFBs (Figure 10E). Finally, the validation was conducted through molecular docking of YBX1 homodimer and two LINC00605 (Figure 10F). The visualization outcomes underscore the reliability of the docking program in elucidating compound binding mechanisms and propose a 2:2 YBX1-LINC00605 stoichiometry. This finding aligns with previous studies indicating that YBX1 protein exists as a dimer in solution[48-50].

Given this specificity, we ruled out LINC00605's role as a scaffold in connecting GMD complex factors. While the LINC00605/YBX1 interaction was established both in vivo and in vitro, the mechanism by which LINC00605 facilitates the decay of NOXA1 via YBX1 remains unclear. To this end, we considered pivotal role of YBX1 in determining mRNA fate and investigated its impact on NOXA1 mRNA stability. Using actinomycin D to inhibit de novo transcription, we studied GC-treated HSFBs transfected with either LINC00605, YBX1, or YBX1 mutation plasmid (Figure 10G, Figure S7, Figure S9). We then measured the endogenous NOXA1 mRNA levels at specified intervals, normalizing them to GADPH mRNA. The resulting data indicated that overexpressing either YBX1 or LINC00605 accelerated the degradation of NOXA1 mRNAs, an effect amplified by their combined expression. This also suggests that the binding between LINC00605 and YBX1 does not inhibit functions of YBX1, negating the possibility of LINC00605 acting as a decoy molecule. In summary, our findings demonstrate that LINC00605 interacts with YBX1, promoting mRNA degradation through YBX1.

LINC00605 recruits YBX1 to target NOXA1 mRNA through specific sequence binding

From our prior experiments, we established that LINC00605 facilitates the degradation of NOXA1 mRNA through YBX1. However, the precise mechanisms underlying the binding of LINC00605 to target RNAs remain unclear. YBX1, a GMD-specific factor, binds to the GMD complex I containing target mRNA/GR/UPF1-PNRC2, which then evolves into the GMD complex II, ultimately activating mRNA decay. The exact process by which

YBX1 is recruited to target mRNAs and its sequential recruitment machinery is still under investigation[12,13]. LncRNAs are known to guide proteins to target DNAs, mRNAs, or proteins, thereby modulating gene expression. Our earlier research suggested that the pivotal role of lncRNAs in recruiting YBX1 to bind target mRNA might elucidate mRNA degradation in GC-treated HSFBs.

Considering the induction of GMD by glucocorticoids, we explored whether the sequence of LINC00605 specificity played a role in the degradation of NOXA1 mRNAs. Our earlier findings showed that GC treatment reduced the NOXA1 mRNA levels (Figure 8A). We hypothesized that specific binding sites on LINC00605 might be involved. An exploration of the interaction partners of LINC00605 highlighted sequences associated with it at various sites (Figure 11A). Using sequence blast online tools, we designed an overexpression vector containing the binding sites of LINC00605, encompassing both wild-type and deletion binding sites in NOXA1 mRNA (Figure 11B). To pinpoint the regions within LINC00605 that influence NOXA1 mRNA decay, we overexpressed a series of LINC00605 deletions. Notably, the LINC00605 deletion at positions 1047-1052 bp did not enhance the stability of NOXA1 mRNA (Figure 11B). This finding supports the idea that LINC00605 interacts with NOXA1 genes through specific elements in LINC00605. Mutations at this specific binding sites nullified the mRNA decay, as confirmed by qRT-PCR. Other binding sites within LINC00605 did not impact the NOXA1 mRNA levels, leading us to focus on the two identified binding sites for subsequent experiments. We then examined whether mutations in key regions of LINC00605 affected the recruitment YBX1 to the NOXA1 coding sequence region (CDS), leading us to mutate this region (Figure 11C). As anticipated, the qRT-PCR results showed that this mutation curtailed the enrichment of YBX1 in the NOXA1 CDS region (Figure 11D). To validate the impact of binding specificity on mRNA decay, we assessed effect of LINC00605 on mRNA stability. Using actinomycin D to halt de novo transcription in GC-treated HSFBs, we transfected cells with either LINC00605 or its mutant (Figure 11E). Subsequent measurements of the endogenous mRNA levels, normalized to GADPH mRNA (known to remain stable during actinomycin D treatment), revealed that LINC00605 overexpression expedited mRNA degradation. This accelerated decay was significantly reversed by binding site mutation (Figure 11E and F). In conclusion, our findings underscore the role of LINC00605 in recruiting YBX1 to mRNA, facilitating mRNA degradation through a sequence-specific binding mechanism.

To further substantiate the direct interaction between LINC00605 and the mRNA of NOXA1, we constructed luciferase reporter vectors, including those containing mutations at the predicted LINC00605-binding sites (Figure 11G). In comparison to the control reporter construct, the overexpression of LINC00605 led to a decrease in luciferase mRNA levels from the NOXA1 reporter constructs, while no such reduction was observed in the mutant plasmid within GC-treated HSFBs. Cotransfecting cells with plasmids expressing NOXA1 restored luciferase mRNA expression from wild-type plasmid, likely by competing with the luciferase transcript containing NOXA1 in LINC00605 binding. This indicates that LINC00605 recruits YBX1 to NOXA1 mRNAs, enhancing their GMD through sequence-specific binding (Figure 11H and I). The findings showed that LINC00605 attracted YBX1 to NOXA1 mRNAs, enhancing their GMD through specific lncRNA-mRNA binding. Ultimately, LINC00605 directed YBX1 to NOXA1 mRNAs, facilitating their GMD process and suppresses ROS during glucocorticoid treatment.

Discussion

The aberrant activation and transformation of fibroblasts often interact with inflammatory stimulation, forming an inflammation-fibrosis cascade reaction, which is a primary cause of fibrotic diseases in the lungs, liver, and kidneys, as well as scar hyperplasia-related disorders and tumor progression[51]. Previous studies have widely suggested that inflammatory stimulation is the main driver of abnormal fibroblast activation, making anti-inflammatory strategies a key therapeutic approach for fibrosis[52]. Intriguingly, recent studies have uncovered novel links between fibroblast dysregulation and ROS. For example, Amara reported that pulmonary fibroblasts in animal models of pulmonary fibrosis exhibited significantly higher ROS levels compared to those in normal models, therefore promoting a profibrogenic environment [53]. De Felice et al. demonstrated a significant increase in ROS levels in HSFBs during the development and progression of hypertrophic scar tissue[54]. These findings suggest that elevated ROS may enhance fibroblast proliferation and secretory function, which appears inconsistent with the traditional view that excessive ROS induces DNA damage and oxidative stress, leading to cellular impairment. This discrepancy highlights the need to explore the complex roles of ROS in pathophysiological processes, particularly its mechanisms in fibroblast dysregulation-related diseases.

In this study, using hypertrophic scar as a pathological model, we found that the intracellular ROS levels in scar-derived fibroblasts were significantly higher than those in normal skin fibroblasts and were closely associated with the degree of inflammatory response[54,55]. Although these studies have confirmed elevated ROS in fibroblasts under pathological conditions, the impact of increased ROS on fibroblast biological behavior remains unexplored. Our single-cell RNA sequencing and clinical specimen analyses provide the first evidence that elevated ROS in HSFBs positively correlates with intracellular ROS levels and aberrant fibroblast proliferation coupled with excessive collagen production. Subsequent *in vitro* experiments demonstrated that inflammatory stimulation induces ROS elevation and fibroblast activation, whereas ROS reduction reverses the hyperproliferation, excessive collagen synthesis, and myofibroblast transdifferentiation triggered by inflammation. These results experimentally confirm that elevated ROS serves as a driving force behind aberrant fibroblast activation and offer novel ROS-based therapeutic strategies for fibrosis-related diseases.

Glucocorticoids, a classic class of anti-inflammatory agents, have seen their biological roles continuously expanded with ongoing research, exhibiting strikingly divergent effects across different cell types. For instance, they induce apoptosis in T cells[56], pancreatic β-cells[57], and osteoblasts[58] while suppressing tumor cell growth[59], yet they promote neutrophil and T-cell polarization[60]. Regarding ROS, traditional theory holds that glucocorticoids may induce inflammation and accumulate intracellular ROS production, thereby induce oxidative stress damage and subsequent apoptosis. For example, Khanka reported GC induced osteoblast apoptosis and ROS generation at cellular and mitochondrial levels[5]. However, recent studies have revealed that GC can also suppress inflammation-induced ROS generation in certain specialized cells. For instance, Liu reported GC Dexamethasone upregulates PIEZO1 expression in macrophages via SGK1, leading to suppressed inflammation, increased ROS generation, and enhanced apoptosis[61]. Furthermore, GC conferred cardioprotection in MIR mice and MI cell models by improving cardiac function, attenuating ROS levels, inhibiting inflammation, and enhancing antioxidative responses[10,62]. These seemingly contradictory findings highlight a key point: the regulatory effect of glucocorticoids on ROS exhibits complex, context-dependent variations across different

pathological conditions and cell types. Yet, the role of glucocorticoids in modulating ROS in fibroblasts remains unclear. Notably, the role of GC in modulating ROS in fibroblasts remains unclear. Using hypertrophic scar as a model, we uncovered for the first time that glucocorticoids inhibit ROS-actived in fibroblasts. Furthermore, ROS reduction reversed fibroblast activation and phenotypic transformation, including hyperproliferation, collagen synthesis, and myofibroblast marker expression. Interestingly, glucocorticoids did not reduce ROS levels in fibroblasts under non-inflammatory conditions, a phenomenon consistent with Huo's findings on glucocorticoid regulation of ROS in microglia[63]. This suggests that glucocorticoid-mediated ROS modulation depends not only on cell type specificity but may also be influenced by distinct pathological microenvironments.

ROS regulation is a dynamic balance between ROS production and clearance, and its dysregulation often involves abnormal expression of multiple genes, including NADPH oxidases (NOX), a major source of ROS that regulate the intracellular redox state. One of these, NADPH oxidase activator 1, NOXA1 gene encodes a enzyme that is a key component of the activation of NOX1, enzymes which catalyze a reaction generating ROS[44]. Vendrov et al. demonstrated that systemic and vascular smooth muscle cell-specific NOXA1 deletion reduced vascular ROS levels, proliferation, and migration in a murine model of atherogenesis[45]. Our study demonstrates that glucocorticoids suppress inflammation-activated ROS production in fibroblasts by downregulating NOXA1 mRNA stability. While most previous studies suggest glucocorticoids regulate gene expression primarily through glucocorticoid receptor (GR) binding to glucocorticoid response elements (GREs) to activate or repress transcription, our in-depth research reveals a novel regulatory mechanism-GMD (Glucocorticoid receptor-mediated mRNA decay). GMD represents a newly identified pathway for glucocorticoid-mediated gene regulation, the biological functions of which remain largely unexplored. The GMD pathway shares protein factors, including UPF1 and PNRC2, with other pathways, such as nonsense-mediated decay (NMD)[1]. However, unlike NMD, which requires an elongating ribosome to recognize a premature mRNA termination codon, GMD is translation-independent and inducible by glucocorticoids. In GMD, GR is preloaded onto the target mRNA, and glucocorticoid binding initiates the recruitment of PNRC2 and UPF1[12,13,64,65]. Cho reported that GMD functions in the chemotaxis of human monocytes by targeting chemokine CCL2 mRNA[4]. Our earlier work also revealed that GMD is involved in glucocorticoid-resistant cellular mechanotransduction[1]. This study is the first to uncover GMD's role in modulating ROS generation under inflammatory conditions, providing new insights into the mechanisms of glucocorticoid action and expanding the understanding of GMD's biological significance. These findings offer fresh perspectives for re-evaluating glucocorticoid signaling and GMD-related regulatory networks.

Glucocorticoids exert their diverse biological effects through the coordinated participation of various biomolecules, including nuclear receptor superfamily number GR[66], transcription factor[67], GRE responsive element[11], non-coding RNA[1,58], and so on. Among these, long non-coding RNA (lncRNA) molecules represent a class of RNA molecules with regulatory functions, that can either promote or inhibit molecular interactions through multiple mechanisms, thereby regulating cellular phenotypes. For example, antioxidant protein FAM129B directly binding to lncRNA NEAT1 and influence RNA stability, therefor increase myotube diameter in glucocorticoid-induced skeletal muscle atrophy[69]. Long non-coding RNA GAS5 interaction between GCs and NF-κB in multi-faceted manner, suggesting that GAS5 could be a potential target in pediatric patients with inflammatory conditions[70]. Furthermore, lncRNAs play crucial roles in maintaining the balance between ROS

generation and clearance. For instance, Elevated lncRNA PVT1 expression drives osteosarcoma development by stimulating the STAT3/GPX4 pathway, thereby inhibiting ferroptosis and altering GSH and ROS balance[71]. LINC00618 binds lymphoid-specific helicase, inhibiting SLC7A11/GSH and increasing lipid ROS to promote ferroptosis, suggesting a novel AML therapy[72]. These studies collectively indicate that lncRNAs serve as important mediators in both glucocorticoid signaling and ROS homeostasis. Our experimental results demonstrate that LINC00605 specifically mediates glucocorticoid-induced mRNA degradation of NOXA1, a key regulator of ROS production. LINC00605 represents the first lncRNA molecule shown to participate simultaneously in both glucocorticoid action and ROS regulation. This finding strongly suggests that glucocorticoid-mediated ROS regulation is not a broad, nonspecific mechanism, but rather a precise process governed by specific lncRNA-mRNA interactions. This novel discovery provides fresh insights for understanding the complex and diverse biological functions and mechanisms of glucocorticoids.

Conclusions

In summary, fibroblast-related pathological conditions such as pulmonary, hepatic, and renal fibrosis, as well as pathological scarring, are relatively common, yet clinically effective anti-fibrotic strategies or drugs remain lacking. Our findings demonstrate that elevated ROS serves as the driving force behind fibroblast activation and phenotypic transformation. Importantly, we confirmed that reducing ROS effectively suppresses excessive fibroblast activation and promotes reversion from myofibroblast to normal fibroblast phenotypes. This provides both theoretical and experimental foundations for developing ROS-targeted anti-fibrotic strategies, with significant potential for clinical translation and therapeutic applications.

Furthermore, contrary to the traditional view that glucocorticoids induce intracellular ROS elevation, we provide the first evidence that glucocorticoids actually suppress ROS levels in fibroblasts under inflammatory conditions. This discovery expands our understanding of the diverse and novel functions of glucocorticoids.

Most significantly, our study reveals a novel LINC00605-dependent GMD mechanism that simultaneously participates in glucocorticoid signaling and ROS regulation, offering fresh insights into glucocorticoid action mechanisms and ROS modulation. However, whether the ROS-suppressive mechanism of glucocorticoids observed in HSFBs similarly applies to fibroblasts in different pathological contexts-such as hepatic, pulmonary, or renal fibrosis-requires further comprehensive investigation. Addressing these questions will advance our understanding of fibroblast dysregulation and contribute to the development of more effective therapeutic interventions for related diseases.

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