

Articles

MSCs-derived secretome counteracts the effect of apoptosis inhibition by SARS-CoV-2 S protein on Calu-3 cells mediated lowered expression of PTGS2

Shuo Gao¹, Yao Ge¹, Haojia Wang¹, Yang Luo^{1,2,3*}

Abstract

The spike glycoprotein of severe acute respiratory syndrome coronavirus 2 is essential for viral entry, but its direct impact on host cell fate remains incompletely defined. Here we report that purified recombinant spike protein alone significantly suppressed apoptosis in human lung epithelial Calu-3 cells in a dose-dependent manner, even under actinomycin D-induced apoptotic stress. This anti-apoptotic effect was effectively reversed by conditioned medium derived from interferon- γ -primed umbilical cord mesenchymal stem cells (MSC-CM). To explore the underlying molecular changes, we performed whole-transcriptome RNA sequencing and identified hundreds of differentially expressed mRNAs, long noncoding RNAs, and circular RNAs, with the vast majority of these transcripts functionally enriched in inflammatory responses and apoptotic pathways. Among the candidate molecules, prostaglandin-endoperoxide synthase 2 (*PTGS2*) showed the most prominent upregulation upon spike protein stimulation, and its protein level was markedly reduced by MSC-CM treatment, suggesting a possible involvement of *PTGS2* in the antagonistic action of MSC-CM against spike-induced apoptosis resistance. To further assess its clinical relevance, we analyzed public transcriptomic datasets and found that *PTGS2* was consistently upregulated in SARS-CoV-2-infected pulmonary cells and in autopsied lung tissues from fatal COVID-19 cases. Moreover, in a multi-omics Long COVID cohort, *PTGS2* remained persistently elevated and its expression inversely correlated with residual pulmonary function, indicating a potential link to chronic inflammatory sequelae. Collectively, our findings reveal a novel pro-survival function of the spike protein and point to *PTGS2* as a candidate molecule worthy of further functional investigation. MSC-CM, by downregulating *PTGS2* and reinstating apoptosis, may represent a cell-free approach with potential relevance for both acute COVID-19 and Long COVID, though direct causal relationships remain to be established through future mechanistic studies.

Keywords: SARS-CoV-2, Spike protein, Transcriptome, Apoptosis, MSC-derived culture medium (MSC-CM), Prostaglandin endoperoxide synthase 2 (*PTGS2*)

Article history: Submitted 15 January 2026 | Accepted 1 June 2026 | Published 3 July 2026

iCell, Vol. 3, No. 2 (2026): 65-64. DIO: <https://dx.doi.org/10.71373/YMPK2936>

Introduction

COVID-19 is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is a positive sense, single-stranded, enveloped, and non-segmented RNA virus that belongs to the coronaviridae family^[1]. Lung is the dominant infecting tissue of SARS-CoV-2, so the cells derived from lung tissue are often used to investigate the SARS-CoV-2 infecting mechanisms^[2]. Previous studies demonstrated that SARS-CoV-2 infection induced immune response and cytokine storm, which caused serious damages or even mortality of infected individuals^[3,4]. It is important to decipher how the lung cells response to the infection of SARS-CoV-2 and how the following cellular processes are activated.

SARS-CoV-2 genome contains the genetic codes for ten major proteins: six non-structural in addition to four structural proteins. The latter comprise a nucleocapsid- (N), a membrane- (M), Envelope- (E) and a glycosylated spike protein (S). The spike (S

) glycoprotein plays a key role in mediating host cell infection process, which present on the viral surface binds to corresponding receptors from host cells, in particular the angiotensin-converting enzyme 2 (ACE2) receptor^[5]. In this study, we employed a recombinant fragment corresponding to the receptor-binding domain (RBD) of the S1 subunit to investigate its direct signaling effects on lung epithelial cells. S protein comprises two functional subunits responsible for binding to the host cell receptor (S1 subunit) and fusion of the viral and cellular membranes (S2 subunit)^[5]. In terms of the virus transmission, S protein binds to ACE2 through its S1 subunit and then fuses viral and host membranes through its S2 subunit^[6,7]. During the fusion process, the conformation of S protein will change from prefusion into post-fusion, which are the key steps in the virus infection cycle^[8]. One recent study also demonstrated that the D614G variant of S protein supplanted the ancestral virus in human and increased infectivity on human lung cells, indicating that S protein plays crucial roles in the evolution and adaption of SARS-CoV-2^[9,10]. At present, the majority of the approved COVID-19 vaccines rely on the spike protein (S), since antibodies targeting the S protein block the entry of the virus into the target cells and thus, prevent the virus from replicating^[11]. Functional analysis of S protein revealed that SARS-CoV-2 spike promotes inflammation and apoptosis by transfecting spike pseudovirions into human bronchial epith-

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elial and microvascular endothelial cells^[12]. Besides, another study also determined that SARS-CoV-2 can promote the expression of TNF- α and induce TNF- α -dependent apoptosis in HEK293T cells^[13]. However, current studies have revealed that SARS-CoV-2 can upregulate the expression of cellular FADD-like interleukin-1 (IL-1)-converting enzyme-inhibitory protein (cFLIP), inhibiting caspase-8/10 activation^[14]. Hence, it is reasonable speculation that the impeded apoptosis of host cells is advantageous for the viral replication, especially in the early stages of SARS-CoV-2 infection.

Prostaglandin-endoperoxide synthase 2 (*PTGS2*), also known as cyclooxygenase-2, is a key inflammatory mediator that has recently drawn attention in the context of COVID-19 pathogenesis. Previous work has shown that SARS-CoV-2 infection can transcriptionally upregulate *PTGS2* expression, and the downstream prostaglandin E2 pathway has been implicated in modulating host immune responses and viral persistence. Notably, *PTGS2* is also involved in the regulation of cellular stress and apoptosis, with its elevated expression potentially contributing to a pro-survival state that may favor viral propagation. This is supported by transcriptomic evidence from infected pulmonary models and autopsied lung tissues, where *PTGS2* is consistently upregulated. However, whether *PTGS2* serves as a downstream molecular node associated with the anti-apoptotic effect of the spike protein, and whether mesenchymal stem cell-derived factors modulate this node, remains to be explored. In this study, we therefore investigated the potential functional connection between the SARS-CoV-2 spike protein and *PTGS2*, with the aim of uncovering novel mechanisms underlying host cell survival and identifying possible points of therapeutic intervention^[4,14].

Since specific antivirals or efficient therapies are required to combat SARS-CoV-2, other drug-free therapies have also been explored to treat COVID-19^[15]. Mesenchymal stem cells (MSCs) have broad anti-inflammatory and immune-modulatory properties. At present, these provide the greatest focus of human MSCs in pre- or clinical testing, such as arthritis, lupus nephritis, type 1 diabetes, Graft-versus-Host Disease, atherosclerosis, multiple sclerosis, et al^[16-20]. MSCs also have been proven to fight the SARS-CoV-2 infection. Mechanically, cytokine storm inhibition, hyperinflammatory syndrome alleviation and coinstantaneous tissue regeneration are considered for the primary mechanism^[21]. MSCs not only secrete exosomes but also produce and release packed with various cellular content, which terms as extracellular vesicles (EVs). Different EV populations include exosomes, microvesicles, apoptotic/necroptotic bodies, cytokines, growth factors and phagosomes^[22]. EVs represent membranous organelles generated under different physiological and pathophysiological conditions. Given the therapeutic effects of MSC mediated by cell-secreted products, MSC-derived culture medium (MSC-CM) is considered an attractive candidate for stem cell-based therapeutic applications^[23]. Of note, that would contribute importantly to the cell-free therapy. Therefore, due to the vast array of secretome derivatives, MSC-CM is deemed to exerts beneficial effects in treating COVID-19^[24]. Whether the MSC-CM could induce apoptosis of spike protein- stimulated Calu-3 cells, we here preliminary observed that MSC-CM can induce Calu-3 cells apoptosis. Importantly, even when stimulated by spike protein that exerts the inhibitory apoptotic function on Calu-3, non-significantly reduced apoptosis was detected. Meanwhile, proliferation assay was also confirmed that the antiapoptotic effect of spike protein is not related to the proliferation decline, that possibly means an intrinsic manner would be at work.

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Materials and Methods

Cell culture and apoptosis detection

Human airway epithelial Calu-3 cells (Procell Life Science & Technology Co., Ltd., China) were cultured at 37°C with 5% CO₂ in α -MEM with 10% fetal bovine serum (FBS), 100 μ g/mL streptomycin, 100 U/mL penicillin (All supplements were from Gibco). The cells used for the experiments were 5–8 passages.

Calu-3 cells were treated with recombinant SARS-CoV-2 spike protein for 48 hours at various final concentrations (0, 1, 10, 50, 100, and 500 nM). The recombinant protein used in this study (Sino Biological, Cat PSV001, Sino Biological) corresponds to the receptor-binding domain (RBD) of the S1 subunit. It is expressed in HEK293 cells and purified to >95% purity as verified by SDS-PAGE and high-performance liquid chromatography by the manufacturer. This RBD fragment lacks the S2 subunit and the transmembrane domain, and is therefore provided as a monomeric form that does not recapitulate the native trimeric spike structure. All experiments were performed using a single production lot of this recombinant protein to ensure batch-to-batch consistency. After the 48-hour treatment, some wells were additionally exposed to 0.75 μ M actinomycin D (A4262, Sigma) for another 12 hours.

After the indicated treatments, cells were harvested for apoptosis detection with flow cytometry (Cytotflex, Beckman) using double-stained with APC-conjugated Annexin V and 7-aminoactinomycin D (4A Biotech Co. Ltd., Beijing, China) according to the manufacturer's protocol. For all flow cytometry plots, axis limits were uniformly set to include the entire recorded cell population, and the same scaling was applied across all experimental groups to facilitate accurate visual comparison. All flow cytometry experiments were carried out with three independent biological replicates. The apoptosis rate was calculated as the percentage of Annexin V-positive cells (early plus late apoptotic populations) in the total cell population, based on quadrant statistics using the instrument's analysis software.

EDU assay

Calu-3 cells were seeded in 48-well plates, cultured for 48h. Cell proliferation assay was performed using the BeyoClick™ EdU Cell Proliferation Kit (Beyotime, C0081S). Briefly, various concentration of S protein-treated Calu-3 cells were incubated with 10 μ M EdU for 2 hours at 37°C. After fixation, permeabilization and rinse, the cells were exposed to 100 μ L of click reaction cocktail for 30 min. The percentage of EdU-positive cells was analyzed by flow cytometry and defined as the proliferation rate^[25].

Western Blot

First, 40 mg total protein lysate was separated on a 10% polyacrylamide gel and then transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked in 5% skim milk for 1 h, then were incubated overnight at 4°C with antibodies against *PTGS2* and GAPDH (Cell Signaling, Danvers, MA, USA). Second, the membranes were then washed three times with PBST and incubated with the secondary horseradish-peroxidase (HRP)-conjugated anti-rabbit antibody (Boster, Wuhan, China) at room temperature for 2h. Third, protein bands were exposed to autoradiography film for

visualization using enhanced chemiluminescent imaging system (Tanon, Shanghai, China).

RNA extraction, library construction and sequencing

The selected treatment Calu-3 cells were subjected to total RNA extraction using TRIZOL (Ambion). The RNA was further purified with two phenol-chloroform treatments and then treated with RQ1 DNase (Promega, Madison, WI, USA) to remove DNA. The quality and quantity of the purified RNA were determined by measuring the absorbance at 260 nM/280 nM (A260/A280) using Smartspec Plus (BioRad, USA). The integrity of RNA was further verified by 1.5% agarose gel electrophoresis. For each sample, 1 µg total RNAs were used for stranded RNA sequencing library preparation using Ribo-Zero Gold rRNA Removal Kit (human/mouse/rat) (N406-01, Vazyme) and remained RNAs were fragmented and purified, and converted into double strand cDNA. After end repair and A tailing, the DNAs were ligated to VAHTS RNA Adapters (Vazyme). After purification, ligation products corresponding to 200 ~ 500 BPS were digested with heat-labile UDG, and the single strand cDNA was amplified, purified, quantified, and stored at -80°C before sequencing. For high-throughput sequencing, the libraries were prepared for following the manufacturer's instructions and applied to Illumina HiSeq X Ten system for 150 nt paired-end sequencing.

RT-qPCR experiment

To determine the validity of the RNA-seq result, RT-qPCR was performed for selected DEGs and DElncRs. Internal control was selected as the housekeeping gene GAPDH. Primers are designed in transcribed regions, which were presented in Table S1. The detailed protocol was fully described in a previously published study [26].

Process of RNA-seq raw data

The raw reads were trimmed of low-quality bases using a FASTX-Toolkit (v.0.0.13; http://hannonlab.cshl.edu/fastx_toolkit/). Then the clean reads were evaluated using FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>). Quality control statistics, including raw reads, clean reads after filtering, and mapping percentages for each sample, are summarized in Supplementary Table S2.

Reads alignment and differentially expressed gene (DEG) analysis

Clean reads were aligned to the human GRCh38 genome by tophat2[27] allowing 4 mismatches. Uniquely mapped reads were ultimately used to calculate read number and fragments per kilobase of exon model per million mapped fragments (FPKM) for each gene. The expression levels of genes were evaluated using FPKM. The software edgeR[28], which is specifically used to analyze the differential expression of genes, was applied to screen the RNA-seq data for DEGs. The results were analyzed based on the fold change ($FC \geq 1.5$ or ≤ 0.37) and p -value (p -value ≤ 0.01) to determine whether a gene was differentially expressed.

LncRNA prediction and direction

identification

To systematically analyze the lncRNA expression pattern, we used a pipeline for lncRNAs identification similar as previously reported[29], which was constructed based on the cufflinks software[30].

Co-expression and functional enrichment analysis

To explore the regulatory mode between lncRNAs and their host mRNAs, we calculated the Pearson's correlation coefficients (PCCs) between them and classified their relation into three class: positive correlated, negative correlated and non-correlated based on the PCCs value ($|\text{correlation}| > 0.6$, p -value < 0.05).

To sort out functional categories of gene sets, Gene Ontology (GO) terms and KEGG pathways were identified using KOBAS 2.0 server[31]. Hypergeometric test and Benjamini-Hochberg FDR controlling procedure were used to define the enrichment of each term.

Identification and target prediction of differentially expressed circRNAs

Circular RNAs were predicted from our RNA-seq data using find_circ software (version 1.2) with default parameters[32]. Differential expression analysis of circRNAs between paired groups was performed with the TCC package in R[33], employing a negative binomial model to estimate significance and false discovery rate (FDR). CircRNAs with an absolute fold change greater than 2 and an FDR below 0.05 were considered differentially expressed. The TCC analysis was configured with the following parameters: normalization method = "tmm", test method = "edgeR", iterations = 3, and floorPDEG = 0.05. To predict the miRNA targets of these differentially expressed circRNAs, we applied miRanda (v3.3a) with default settings. Candidate circRNA-miRNA interactions were retained only when the miranda score exceeded 500, ensuring a stringent selection for subsequent ceRNA network construction.

miRNA target prediction

The potential miRNA targets were identified using miRanda (v3.3a) and TargetScan (v6.0) with default settings. All predicted target genes were evaluated by scoring system, and the sequences were considered to be miRNA targets only if miranda score was more than 500 or targetscan context + score was less than 0. To reduce the false identification rate, the gene expression profile of putative targets was required to be negatively correlated with the profile of miRNAs. For each paired samples, we identified the set of differentially expressed target genes and their corresponding miRNAs significantly changing in the opposite way. The mRNA-miRNA pairs whose negatively correlated relationship was detected in at least two paired samples were retained further to plot network by Cytoscape.

Phenotyping and differentiation of hUC-MSCs

Human umbilical cord MSCs (hUC-MSCs, passage 2, cat. no. SNP-H198, SUNNCEL) were purchased and cultured in

DMEM-F12 (BI) containing 10% fetal bovine serum (FBS, Gibco) at 37°C in a 5% CO₂ incubator. HLA-DR, CD34, CD45, CD73, CD90, and HLA-ABC (Biolegend) were analyzed by FACS.

Adipogenic and osteogenic differentiation. hUC-MSCs were seeded at a density of 1×10⁵ cells/well in a six-well plate. When the cells reached 100% confluence, the corresponding osteogenic induction solution (hUC-MSC osteogenic differentiation complete medium, HUXUC-90021, Cyagen, China) and adipogenic induction solution (hUC-MSC adipogenic differentiation complete medium, HUXUC-90031, Cyagen, China) were added. The medium was changed according to the operating procedure. After 24 days of induction, Alizarin red staining and Oil Red O staining were carried out for osteogenesis and adipogenesis respectively, then the cells were photographed and observed under a microscope (Olympu, Japan).

Preparation of IFN-γ pre-conditioned MSC-derived culture medium

Passage 3 hUC-MSCs were cultured in six-well plate until subconfluence to obtain culture medium (MSC-CM). Human recombinant IFN-γ (500 U/ml, R & D Systems, Minneapolis, MN, USA) was added in the plate for 12 hours, then culture medium was depleted, and cells were washed twice with HBSS to remove serum and IFN-γ before incubated in 6mL of α-MEM for another 48h. The resultant supernatants were centrifuged at 300 × g for 5 min to remove cells, then at 2,000 × g for 10 min to eliminate cell debris, and finally filtered through a 0.22 μm pore-size filter to ensure sterility and remove any remaining particulate matter. The clarified medium was collected and used immediately. To minimize variation between preparations, we seeded the same number of cells, used identical culture volumes (6 mL per well), and collected the supernatants after the same incubation period (48 h)^[34]. The collected medium was not further concentrated or normalized by protein concentration; it was used directly as the conditioned medium. Three independent batches were prepared, and each experiment was repeated with at least two batches to confirm reproducibility.

Preclinical Model Bioinformatics and Transcriptomic Analysis

To investigate the preclinical cellular mechanisms of SARS-CoV-2, secondary bioinformatics analyses were conducted using published datasets. Raw read counts from GSE147507 were utilized to evaluate *PTGS2* expression in Calu-3 cells subjected to SARS-CoV-2 infection (MOI=2) versus Mock controls at 24 hours. For clinical tissue-level validation, raw count data from GSE150316 were extracted. Raw count matrices for both datasets were processed and normalized using log₂(Counts+1). Statistical significance for differential gene expression between infected/pathological groups and respective baseline controls was determined using the Wilcoxon rank-sum test.

Multi-omics COVID-19 Cohort Analysis and Functional Intersect

Multi-omics data from the Long COVID discovery cohort (GSE150316, GSE147507) were evaluated. Normalized transcriptomic expression values of *PTGS2* were extracted and Liu et al. *icell*, Vol.3a1wrp114(2026) 23 August 2026

stratified to compare Long COVID subjects against healthy and non-Long-COVID (NLC) post-COVID controls (Panel A). To evaluate the relationship between continuous pathological drivers and clinical decline, Spearman rank correlation tests were applied between *PTGS2* expression levels and derived clinical pulmonary function impairment scores (Panel B). Directional network dependencies and multi-omics module intersections were derived strictly from patient-matched data matrices. To map shared biological processes, differentially expressed genes (DEGs) from our in-vitro *PTGS2*-regulatory network (incorporating *IL6*, *CXCL8*, *STAT1*, *PTGS2*, *ICAM1*, and *VCAM1*) were intersected with clinical DEGs from the Long COVID cohort. Intersecting genes were subjected to Gene Ontology (GO) enrichment analysis focusing on biological processes (Benjamini-Hochberg FDR-adjusted $P < 0.05$). All statistical comparisons were two-sided, with multiple testing controlled via Benjamini-Hochberg false discovery rate adjustment where applicable.

Statistical analysis

Principal component analysis (PCA) analysis was performed by R package factextra (<https://cloud.r-project.org/package=factextra>) to show the clustering of samples with the first two components. After normalizing the reads by TPM (Tags Per Million) of each gene in samples, in house-script (sogen) was used for visualization of next-generation sequence data and genomic annotations. The pheatmap package (<https://cran.r-project.org/web/packages/pheatmap/index.html>) in R was used to perform the clustering based on Euclidean distance. Student's t-test was used for comparisons between two groups.

All quantitative data are presented as the mean ± standard deviation (SD) unless otherwise specified. Prior to parametric comparisons, the normality of data distribution was verified using the Shapiro-Wilk test, and homogeneity of variance was assessed utilizing Levene's test. Data conforming to a normal distribution and equal variance were analyzed using appropriate parametric tests (e.g., unpaired Student's t-test for two groups or one-way ANOVA for three or more groups, followed by Tukey's post-hoc test). For non-parametric data, corresponding non-parametric methods (e.g., the Mann-Whitney U test or Wilcoxon rank-sum test) were applied. Statistical analyses were performed using GraphPad Prism 8. A value of $P < 0.05$ was considered statistically significant ($P < 0.05$, * $P < 0.01$, ** $P < 0.001$; ns, not significant).

Results

SARS-CoV-2 spike protein inhibits apoptosis of Calu-3 cells

Spike glycoprotein (S) is the envelope of coronavirus that mediates host cell infection and a primary determinant of cell tropism and pathogenesis. To systematically explore the signaling functions of the SARS-CoV-2 spike protein, we utilized a commercially purified recombinant RBD fragment of the S1 subunit to treat human lung adenocarcinoma epithelial (Calu-3) cells. Transcriptome sequencing experiment (RNA-seq) was performed to investigate the expressed RNA level changes by spike protein (Fig. 1A). We first examined the effect of S protein treatment on the survival of Calu-3 cells in vitro. As seen in Fig. 1B, with the increase of the concentration of S protein, the apoptotic level of the Calu-3 cells was markedly

decreased. Accordingly, treatment at a higher dose (50 to 500 nM) significantly reduced the apoptosis, which showed a steady dose dependent manner (Fig. 1B and C). To some extent, it suggests that SARS-CoV-2 spike protein could have the ability to inhibit lung epithelial cell apoptosis. Extensive studies have shown that the apoptosis signaling pathway plays a critical role in the progression of SARS-CoV virus infection^[14]. For example, a previous study demonstrated that SARS-CoV activates apoptosis of human primary T lymphocytes in vivo³⁵. In addition, adenovirus-mediated expression of spike protein of SARS-CoV also induced apoptosis in Vero E6 cells in vitro³⁶. However, SARS-CoV-2 is particularly enigmatic, with seemingly contradictory roles in the apoptosis, which was recently found that SARS-CoV-2 virus-like particles (trVLP) can suppressed cell apoptosis^[37]. To further confirm our experimental results, we re-performed the experiment using an additional condition. As actinomycin D (Act D) treatment promoted DNA damage and induced apoptosis in lung cancer cells^[38]. Therefore, Calu-3 cells were firstly infected with S protein for 48h, then Act-D was added for another 12h. As we expected, Act D

clearly increased the Calu-3 cell apoptosis rate (Fig. 1D and E), however, S protein significantly inhibited the pro-apoptotic effect of Act D with a dose dependent manner. In addition, another batched experiment was also performed. Calu-3 cells were pretreated with Act D for 12h, then washed and cocultured with S protein (0, 10, and 100 nM) for 48h. Then, as before, flow cytometry was used for apoptosis detection. Undoubtedly, spike protein still suppressed the Act D-induced apoptosis (Fig. 1F). These results did reveal an interesting phenomenon that SARS-CoV-2 S protein plays an apoptosis-inhibiting role on the human epithelial cells even in the presence of actinomycin D stimulation. Whether the decreased apoptosis was accompanied by an increase in the proliferating rate, EdU proliferation assay was used. As shown in (Fig. S1 A and B), no statistical difference of proliferation rates was observed between control and 1nM, 10nM, 50nM ($p > 0.05$), whereas there was a statistical proliferation difference between control and 100 or 500nM group, converged an intrinsic apoptosis pathway may be involved.

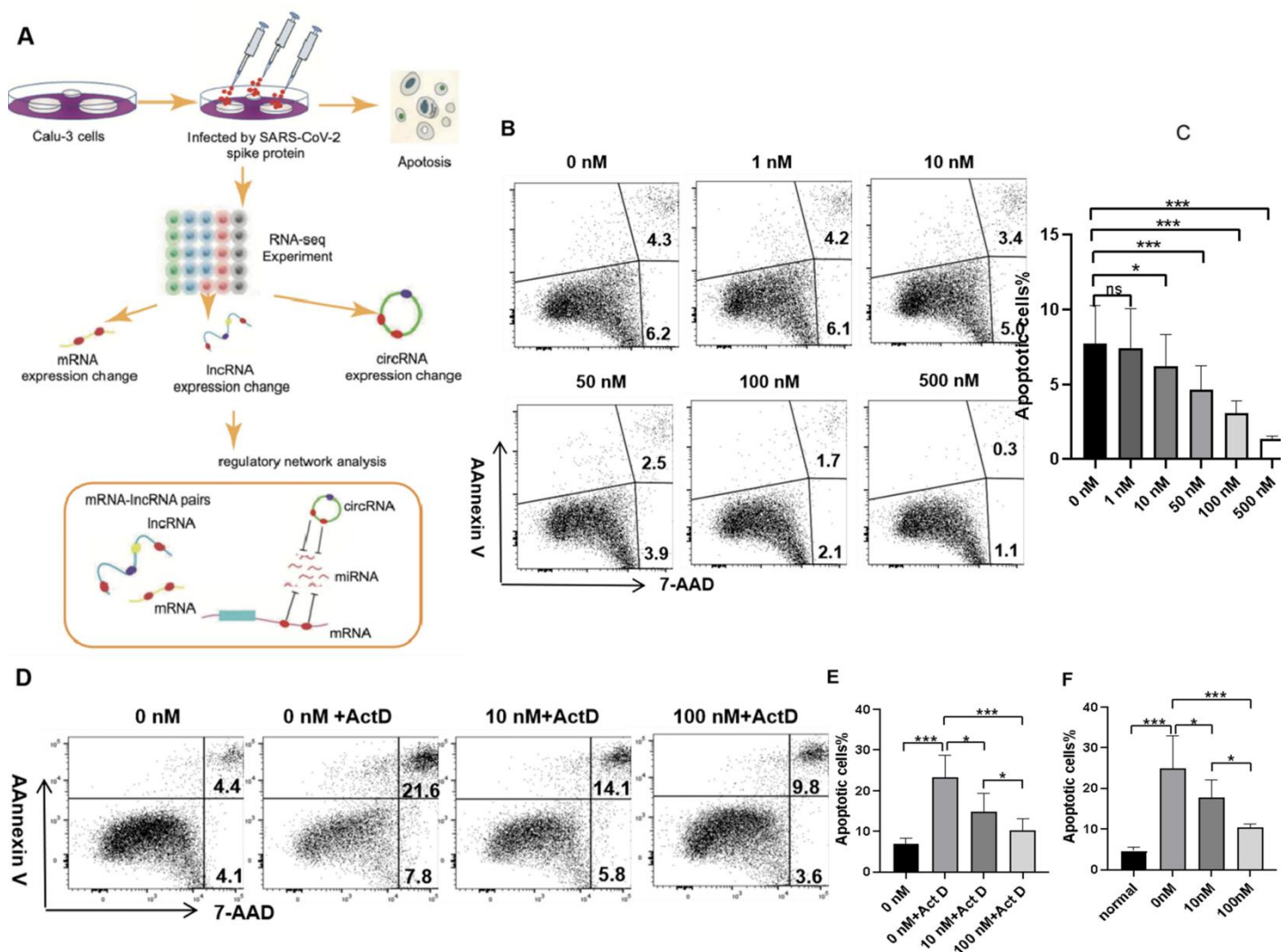


Figure 1. SARS-CoV-2 spike (S) protein inhibits Calu-3 cell apoptosis in a dose dependent manner. Experimental and analysis pipeline to study the cellular effects of SARS-CoV-2 S protein in Calu-3 cells (A). Calu-3 cells were stimulated with various concentration of S protein for 48h, flow cytometric analysis presentation of annexin V assay showed the significantly decreased apoptosis level after S protein treatment (B, C). Bar plot showed the significantly increased apoptotic level after only Act D treatment (left two bars). While decreased apoptotic level was also observed after Act D and S protein co-treatment comparing with only Act D treatment. (right two bars) (D, E). Bar plot showed the decreased apoptotic level of Act D-primed Calu-3 cells after S protein treatment in another experimental batch (F). ns, non-significant; *, p -value < 0.05 ; **, p -value < 0.01 ; ***, p -value < 0.001 ; one way ANOVA analysis.

Spike protein extensively regulates the steady transcriptome profile of Calu-3 cells

The underlying mechanism of inhibition of cellular apoptosis by the S protein is largely unknown. We then performed whole transcriptome sequencing experiment (RNA-seq) for the treated cells by removing ribosomal RNAs (rRNAs) strategy. Following the apoptosis results in Fig. 1B, we selected Calu-3 cells only (control group, SD_0nM) in comparison with 100 nM (SD_100nM) S protein treatment (S-RBD group) both under Act D (0.75 μ M) condition. Three biological replicates for each group were prepared for RNA-seq. After filtering low quality reads and aligning reads to human GRCh38 genome sequences, we obtained the genomic region distribution and expression levels for each gene (fragments per kilobase per million, FPKM) by classifying the uniquely aligned reads onto human genome. Genomic region distribution revealed most of the aligned reads were located into the transcribed regions, including 5'UTR, CDS, intron, 3'UTR, and nc_exon (Fig. 2A). There was no difference between SD_0nM and SD_100nM groups (Fig. 2A).

We then analyzed whether spike protein could alter global gene expression pattern. Principal component analysis (PCA) showed that SD_0nM samples were clearly separated from SD_100nM samples by the first component, which could explain 32.3% of the total variation (Fig. 2B). Following differentially expressed gene (DEG) analysis revealed 223 upregulated and 140 downregulated genes between SD_100nM vs. SD_0nM with criteria p -value < 0.01 and fold change (FC) > 1.5 (Fig. 2C). Hierarchical clustering heat map of the expression levels of DEGs showed up DEGs and down DEGs were clearly separated (Fig. 2D). We then performed functional enrichment analysis for up and down DEGs based on gene ontology (GO) annotation. For upregulated genes, we found they were enriched in inflammatory response, cell adhesion, GTP catabolic process, axon guidance, small GTPase mediated signal transduction, positive regulation of apoptotic process, carbohydrate metabolic process, signal transduction transmembrane transport, and apoptotic process (Fig. 2E). Several genes involved in apoptotic process were upregulated by S protein treatment, including *RHOB*, *IL24*, *PTGS2*, *NGFR*, *UNC5C*, *RRAGC*, and *TMEM102*. Meanwhile, down DEGs were enriched in metabolic process related pathways (Fig. 2E and F).

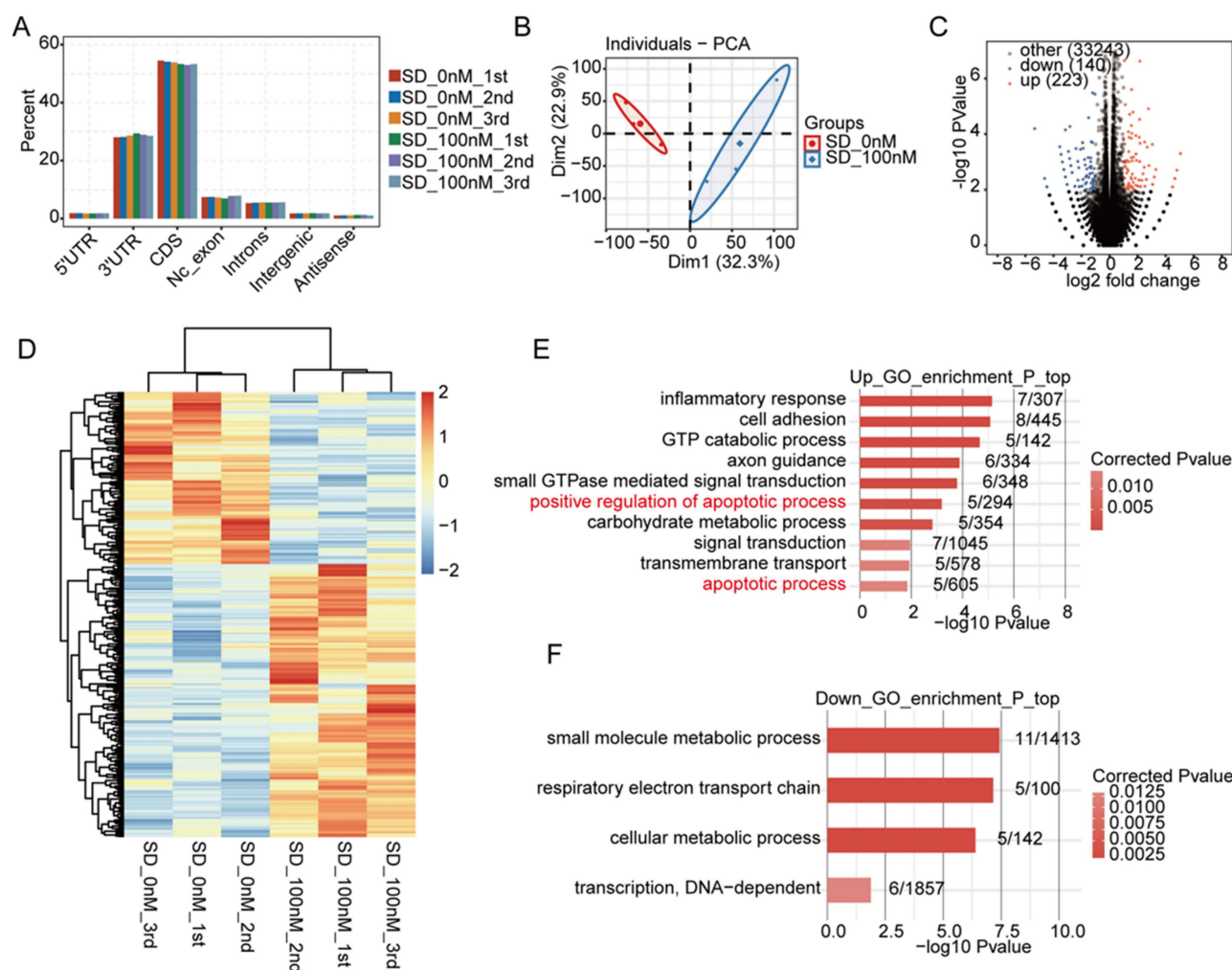


Figure 2. Global transcriptome profile presentation of Calu-3 cells in spike (S) protein treated and control condition under Act D treatment. Calu-3 cells were infected with SARS-CoV-2 Spike RBD-His Recombinant Protein (100 nM, RBD group) for 48h, then Act D (0.75 μ M) were added for another 12h. Control group was that Calu-3 cells cultured for 48h only and then followed by Act D treatment (0.75 μ M, 12h). Bar plot showed the genomic region percentage of mapped reads in the RNA-seq data (A). PCA result presentation showed the clear separation (B) and volcano represented the differentially expressed genes (DEGs) (C) between S protein treated and control samples. Hierarchical clustering heat map represented the expression changes of DEGs between S protein treated and control samples (D). Bubble plot showed the enriched GO BP functions of upregulated DEGs (E) and downregulated DEGs (F) after S protein treatment.

Validation of DEGs associated with apoptosis

To further validate the apoptosis-related DEGs identified from the RNA-seq data, we prioritized genes with high fold changes, significant *p*-values, and documented involvement in inflammatory or apoptotic signaling, then selected several candidates for RT-qPCR confirmation, including both up-regulated and down-regulated genes. Among the down DEGs, mitochondrially encoded genes were observed among the top significant DEGs sorted by *p*-value, including *MT-ND1*, *MT-ND2*, and *MT-ND4*. Previous studies have demonstrated *MT-ND1*, *PTGS2* and GTPase play an important role in SARS-CoV-2 affection^[39-41].

We finally selected four genes for validation, including *RHOB*, *PTGS2*, *RRAGC* and *MT-ND1*, which showed consistent and significant results with the RNA-seq data (Fig. 3A and B), suggesting the authenticity of RNA-seq result. After comparison, we then focused on the *PTGS2*. Network pharmacology and molecular docking have been revealed that *PTGS2* was identified as a potential target in treating COVID-19, which was associated with the suppression of oxidative stress and cell apoptosis^[42]. Moreover, other study also confirmed that *PTGS2* can inhibit cell apoptosis via the ROS-ERK1/2-NF- κ B signaling axis, which enhanced the resistance of non-small cell lung cancer cells against the cisplatin^[43].

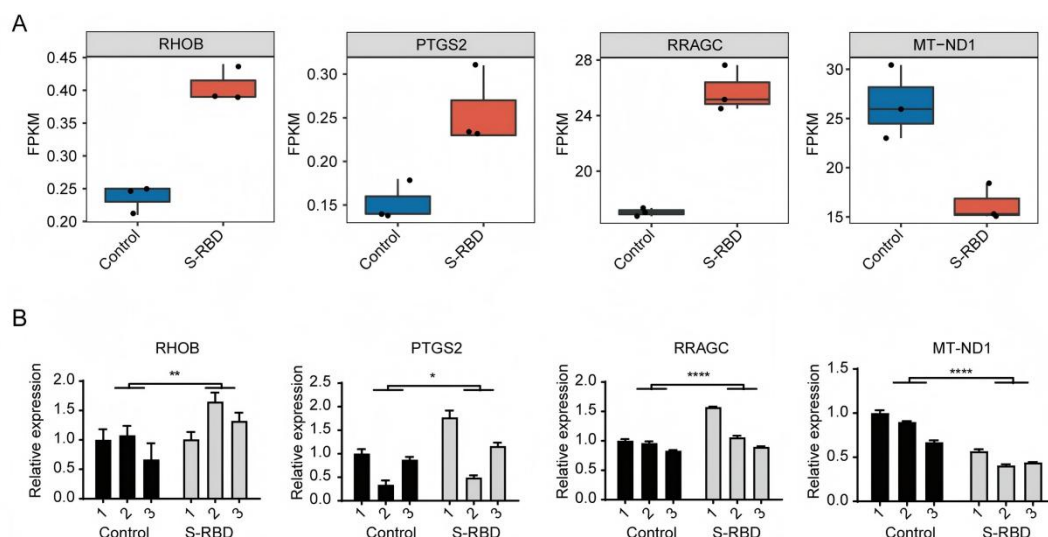


Figure 3. RT-qPCR and WB validation for DEGs associated with apoptosis in spike (S) protein affected Calu-3 cells. Calu-3 cells were divided into control group and RBD group. Box plot represented the expression levels of up DEGs in RNA-seq data. Three genes were included. (*, *p*-value < 0.05; **, *p*-value < 0.01; ***, *p*-value < 0.001; two-way ANOVA test) (A). Total RNA was extracted from the two group, and mRNA were quantified by RT-PCR, β -actin mRNA was used as internal control (B). The data indicates the mean $\pm$ S.E.M (n=3; *, *p*-value < 0.05; **, *p*-value < 0.01; ***, *p*-value < 0.001; ns, not significant).

MSC-CM reverses the inhibition of Calu-3 cell apoptosis by spike protein, correlating with *PTGS2* expression changes

There was research that shows MSCs inhibit the apoptosis of alveolar epithelial cells caused by acute respiratory distress syndrome (ARDS) through CXCL12/CXCR4 axis^[44]. Considering the importance of immunomodulation in COVID-19, MSCs might modulate the apoptosis of SARS-CoV-2 infected cells. Accumulating research findings confirmed that MSCs pretreated with some inflammatory cytokines, such as IFN- γ , exhibit enhanced immunomodulatory functions^[21,45]. However, whether conditioned medium from IFN- γ -stimulated MSCs (MSC-CM) exerts beneficial effects in terms of SARS-CoV-2 infected cells remains unknown. Based on this knowledge, we initially investigated whether MSC-CM have the potential of reversing the anti-apoptotic potent of S protein on Calu-3 cells.

hUC-MSCs were isolated and identified using procedures described in our previous study. These cells had the ability to differentiate into osteoblasts and adipocytes (Fig. S2 A) after culture in osteogenic and adipogenic media followed by Alizarin red and Oil Red-O staining respectively. Meanwhile, the surface markers of CD73 (99.94%), CD105 (99.86%), and

CD90 (99.99%) were positive (>95%), and hematopoietic markers such as CD45 (0.04%), HLA-DR (0.19%) and CD34 (0.12%) were present at low level (Fig. S2 B). MSC-CM was collected from passage 2 hUC-MSCs. Therefore, to determine whether MSC-CM has the ability to attenuate the anti-apoptotic role of S protein, 100 nM concentration of S protein were applied. As we expected, S protein markedly mitigated the apoptosis of Calu-3 cells in normal culture medium. If the cells were cultured in MSC-CM, the apoptotic frequency was significantly increased. More importantly, MSC-CM did diminish the anti-apoptotic effect of S protein (Fig. 4A and B). Additionally, using Act D pretreated Calu-3 cells, we also observed that MSC-CM has a powerful effect of reversing S protein-induced anti-apoptosis ability (Fig. 4C and D). In order to explore the possible mechanism whereby MSC-CM showed strong antagonistic action against S protein, we locked the candidate molecule to *PTGS2*. A similar result was observed that S protein significantly increased *PTGS2* protein level while MSC-CM decreased the *PTGS2* production (Fig. 4E and F). In summary, we here confirmed that conditioned medium from IFN- γ -stimulated MSCs strongly induced Calu-3 cell apoptosis, whereby S protein mediated anti-apoptosis effect was also suppressed by MSC-CM. The downregulation of S protein-induced *PTGS2* expression by MSC-CM correlates with the restoration of apoptosis, positioning *PTGS2* as a candidate molecule for future mechanistic dissection.

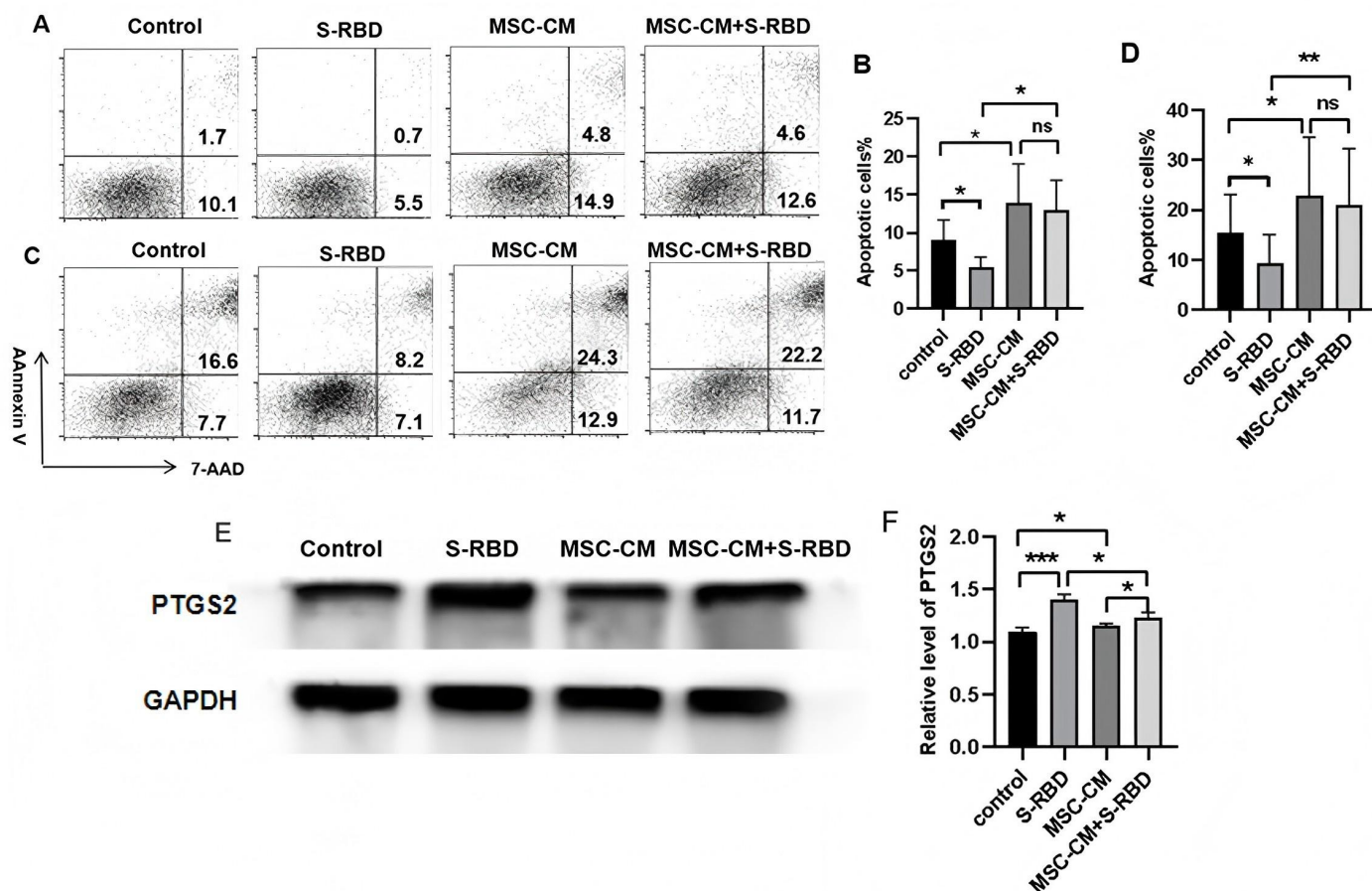


Figure 4. MSC-CM reverses the inhibition of Calu-3 cell apoptosis by spike (S) protein possibly via *PTGS2*-dependent mechanism. Calu-3 cells with S protein in vitro stimulation for 48h (S-RBD group) contained a lower ratio of apoptosis compared with the control cells (Control group). In contrast, the ratio of apoptotic Calu-3 cells was higher in the cells that received MSC-CM (MSC-CM group). MSC-CM also reversed the anti-apoptotic capacity of S protein in Calu-3 cells (A, B). Calu-3 cells were pretreated with Act D (0.75 μ M) for 12h, then washed and followed by the figure 4A process, apoptosis was examined by flow cytometry (C, D). Western blot analysis of *PTGS2* protein level and normalized with GAPDH (E, F). Data are presented as mean $\pm$ SEM of 3 independent experiments. (*, p -value < 0.05; **, p -value < 0.01; ***, p -value < 0.001; ns, not significant). One-Way ANOVA followed by Tukey's multiple comparison test.

Long noncoding RNAs (lncRNAs) were dysregulated by spike (S) protein

Long noncoding RNAs (lncRNAs) are emerging vital regulators in most aspect of cellular functions^[46]. We then investigated the expression profile of lncRNAs by S protein infection. After predicting novel lncRNAs and identifying expressed lncRNAs, we found the two groups shared a large number of detected lncRNAs, including 1810 co-detected lncRNAs. We also detected 217 and 172 lncRNAs were specifically expressed in SD_{100nM} and SD_{0nM} samples, respectively (Fig. 5A). Differentially expressed lncRNAs (DELncRs) analysis revealed 125 upregulated and 58 downregulated lncRNAs in SD_{100nM} group (Fig. 5B). We observed that both more lncRNAs and mRNAs were detected in S protein treated samples, implying that S protein may stabilize the RNA levels. Hierarchical clustering heat map of the expression levels of DELncRs also showed up lncRNAs and down lncRNAs were clearly separated (Fig. 5C), although the expression pattern among replicates was

less consistent than that of mRNAs because of the lower expression levels of lncRNAs. LncRNAs have the ability to regulate gene expression in cis- or trans-manner^[47], so we performed lncRNA-mRNA co-expression analysis to predict the potential targets of DELncRs. By calculating the correlated mRNA numbers and statistical significance of co-expressed lncRNAs, we detected several lncRNAs exhibiting more co-expressed mRNAs and significant false discovery rate (FDR), including RP13-14G15.4, AC004988.1, AC003092.1, GAPLINC, RP11-265F19.1, and RP11-459E10.1 (Fig. 5D). Functional analysis of the mRNA that were co-expressed with DELncRs revealed they were highly enriched in inflammatory response, respiratory electron transport chain, cell adhesion, GTP catabolic process, and cellular metabolic process pathways (Fig. 5E). We selected five lncRNAs that were upregulated in SD_{100nM} samples and highly correlated with mRNAs to show their expression pattern (Fig. 5F). To make a more definite expression changes of these lncRNAs after spike protein treatment, we selected these five lncRNAs for RT-qPCR experiment. The result showed consistent expression changes with RNA-seq result (Fig. 5G).

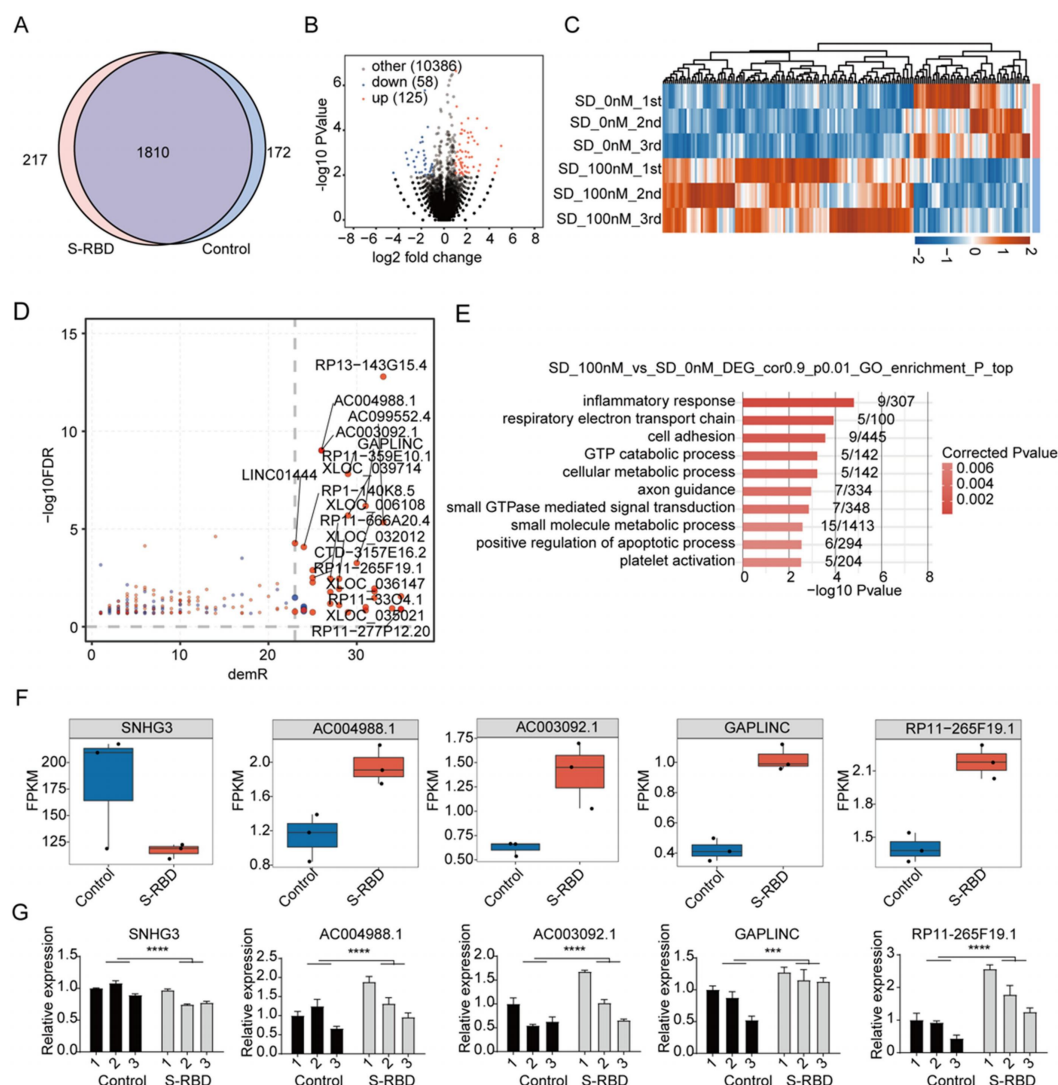


Figure 5. Spike (S) protein affects the global expression pattern of long noncoding RNAs. Venn diagram (A) represented the detected lncRNAs and volcano plot (B) represented the differentially expressed lncRNAs (DElncRs) between control and S-RBD group. Hierarchical clustering heat map showed the expression changes of DElncRs between control and S-RBD samples (C). Scatter plot showed lncRNA-mRNA co-expression network, highlighting several significant lncRNAs. X-axis represented the co-expressed mRNA numbers of DElncRs and Y-axis represented the significant FDR values (-log10FDR) (D). Bar plot showed the enriched GO BP functions of targets of upregulated DElncRs and downregulated DElncRs after S protein treatment (E). Box plot showed the expression changes of five selected DE lncRNAs in the RNA-seq result (F). Bar plot showed the significant expression changes of five lncRNAs in (F) using RT-qPCR experiment (G). (*, p -value < 0.05; **, p -value < 0.01; ***, p -value < 0.001; two-way ANOVA test).

Circular RNAs acts as competitive endogenous RNAs to participate in Calu-3 cellular functions

Circular RNAs (circRNAs) are emerging as a new class of non-coding RNAs with important molecular functions [48]. Recent study demonstrated the important roles of circRNAs during Middle East respiratory syndrome coronavirus (MERS-CoV) infection [49]. We used rRNA depletion method to prepare RNA-seq library, enabling us to investigate the circRNA expression profile in our samples. By predicting circRNAs using FindCirc software [50], a total of 5281 circRNAs were obtained and there was no obvious difference of circRNA numbers for the six samples (Fig. 6A). We then used normalized back-splicing reads number to perform differential expression analysis of circRNAs, and finally obtained 39

upregulated and 38 downregulated circRNAs by spike protein treatment (Fig. 6B). Although the differentially expressed circRNAs (DECircRs) only account for 1% of the detected circRNAs, hierarchical clustering heat map showed a high consistency between biological replicates (Fig. 6C). Most of the circRNAs are generated from exon regions of genes by abnormal splicing, which perhaps could affect the expression of their host genes. We then performed functional analysis for the host genes of DECircRs. For upregulated circRNAs, their host genes were enriched in metabolic pathway, lysosome, and several other glycosphingolipid related pathways (Fig. 6D). Host genes of downregulated circRNAs were enriched in ubiquitin mediated proteolysis, hippo signaling pathway, propanoate metabolism and RNA polymerase pathways (Fig. 6E). Two genes involved in lysosome were *HEXB* and *ATP6V1H*, which were also upregulated by spike protein. On canonical function of circRNAs is to function as competitive

endogenous RNA by sponging miRNAs in cells 48. We then constructed a competitive endogenous RNA network for these dysregulated circRNAs by predicting their binding miRNAs and associated mRNAs. Eight circRNAs (red points), 34 mRNAs, and 14 miRNAs participated in this network (Fig. 6F). One

circRNA originated from *FAM178A* could be served as potential target of eight miRNAs (Fig. 6F). Functional analysis of the involved mRNA genes showed they were enriched in metabolism related pathways (Fig. 6G).

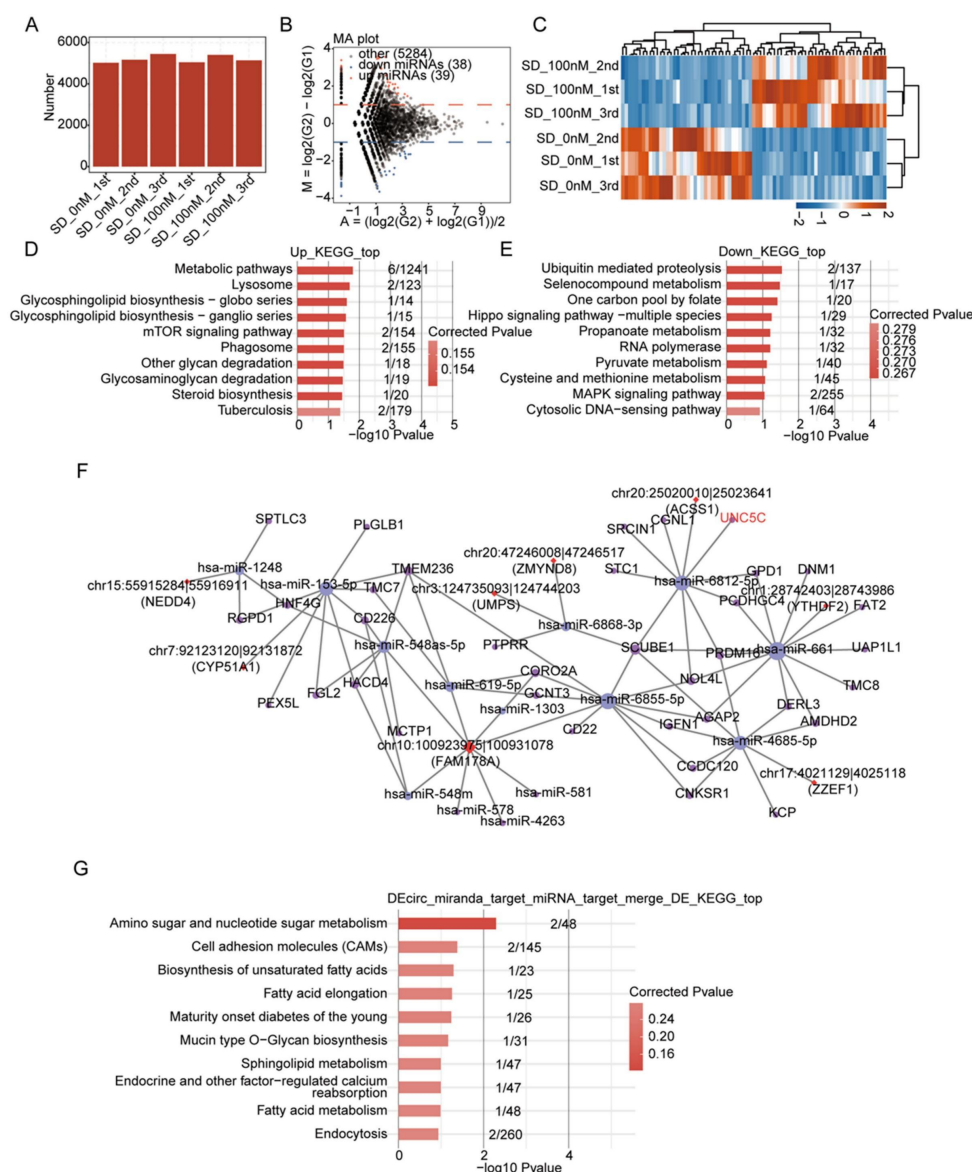


Figure 6. Circular RNAs were dysregulated in spike (S) protein treated Calu-3 cells. Statistics of circRNAs detected in this study (A). Volcano plot represented the differentially expressed circRNAs (DECircRs) (B), and hierarchical clustering heat map showed the expression changes of DECircRs between control and S-RBD group (C). Bar plot showed the RT-qPCR validation of DECircRs in this study (D). Bubble plot showed the enriched GO BP functions of targets of upregulated DECircRs and downregulated DEIncRs after S protein treatment (E). CircRNA-mRNA regulatory network presentation (F). Bar plot showed the enriched GO BP functions of mRNA genes involved in the circRNA-mRNA regulatory network (G).

PTGS2 expression across preclinical models and fatal COVID-19 tissues

To validate the clinical relevance of *PTGS2*-mediated apoptosis inhibition, we interrogated public datasets (GSE147507, GSE150316). Transcriptomic analysis confirmed that *PTGS2* is significantly upregulated in Calu-3 cells upon SARS-CoV-2 infection (Figure 7A). This upregulation was consistently

observed across various pulmonary cell lines, including NHBE and A549-ACE2, highlighting a robust cellular response (Figure 7B). Furthermore, examining autopsied lung tissues from COVID-19 fatalities revealed markedly higher *PTGS2* expression compared to healthy controls (Figure 7C). These findings suggest that the S protein-induced *PTGS2* axis is a conserved pathological feature in infected lung tissue, reinforcing its role as a potential driver of viral persistence and tissue-level apoptosis resistance.

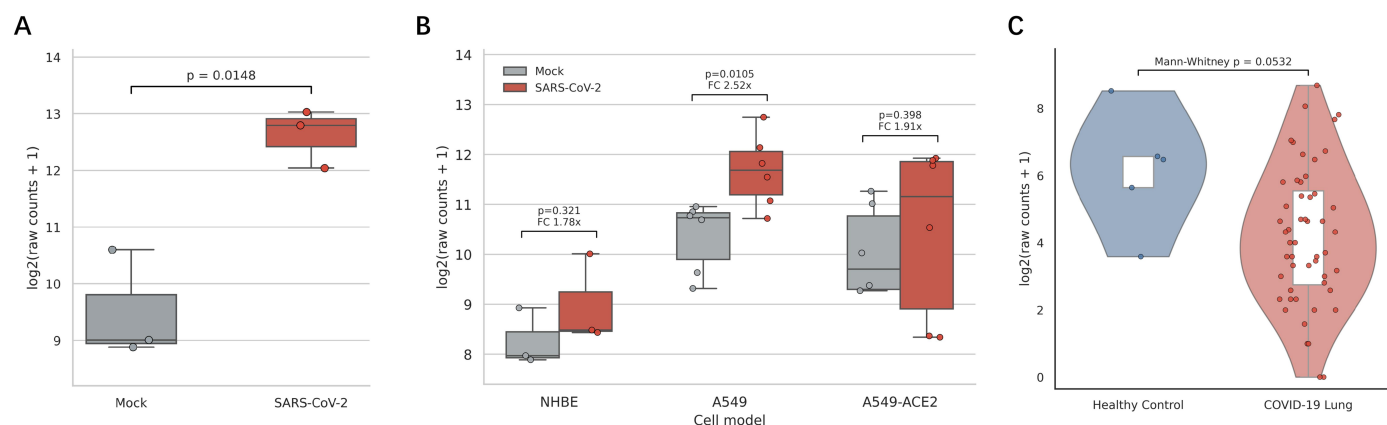


Figure 7. Public transcriptomic validation of *PTGS2* expression in SARS-CoV-2-related models and lung tissues. Panel A shows *PTGS2* expression in Calu-3 cells under Mock and SARS-CoV-2 infection conditions, displayed as $\log_2(\text{raw counts} + 1)$ using boxplot with jittered points; Welch's t-test was used, $p = 0.01477$ (A). Panel B compares *PTGS2* expression between Mock and SARS-CoV-2 infection groups across NHBE, A549, and A549-ACE2 cell models, with Welch's t-test applied within each model (B). Panel C shows *PTGS2* expression in COVID-19 lung tissues and NegControl samples using violin plot, boxplot, and jittered points; Mann-Whitney U test was used, $p = 0.0532$ (C). Data sources: GSE147507 for Panels A–B, and GSE150316 for Panel C.

PTGS2 as a persistent inflammatory node in Long COVID

Expanding our clinical validation to the post-acute sequelae of COVID-19, we analyzed a multi-omics Long COVID cohort. *PTGS2* expression remained persistently elevated in Long COVID patients relative to controls (Figure 8A). Spearman correlation analysis demonstrated a trend where increased *PTGS2* levels inversely correlate with pulmonary function

markers, suggesting its involvement in impaired tissue repair (Figure 8B). Crucially, functional enrichment of the intersection between our in vitro *PTGS2*-regulatory network and Long COVID differentially expressed genes highlighted pathways encompassing cytokine signaling, inflammatory response, and prostaglandin biosynthesis (Figure 8C). Collectively, these clinical signatures suggest that the *PTGS2*-driven inflammatory microenvironment, reversible by MSC-CM in our model, remains a critical therapeutic target for mitigating Long COVID-related chronic immune dysfunction.

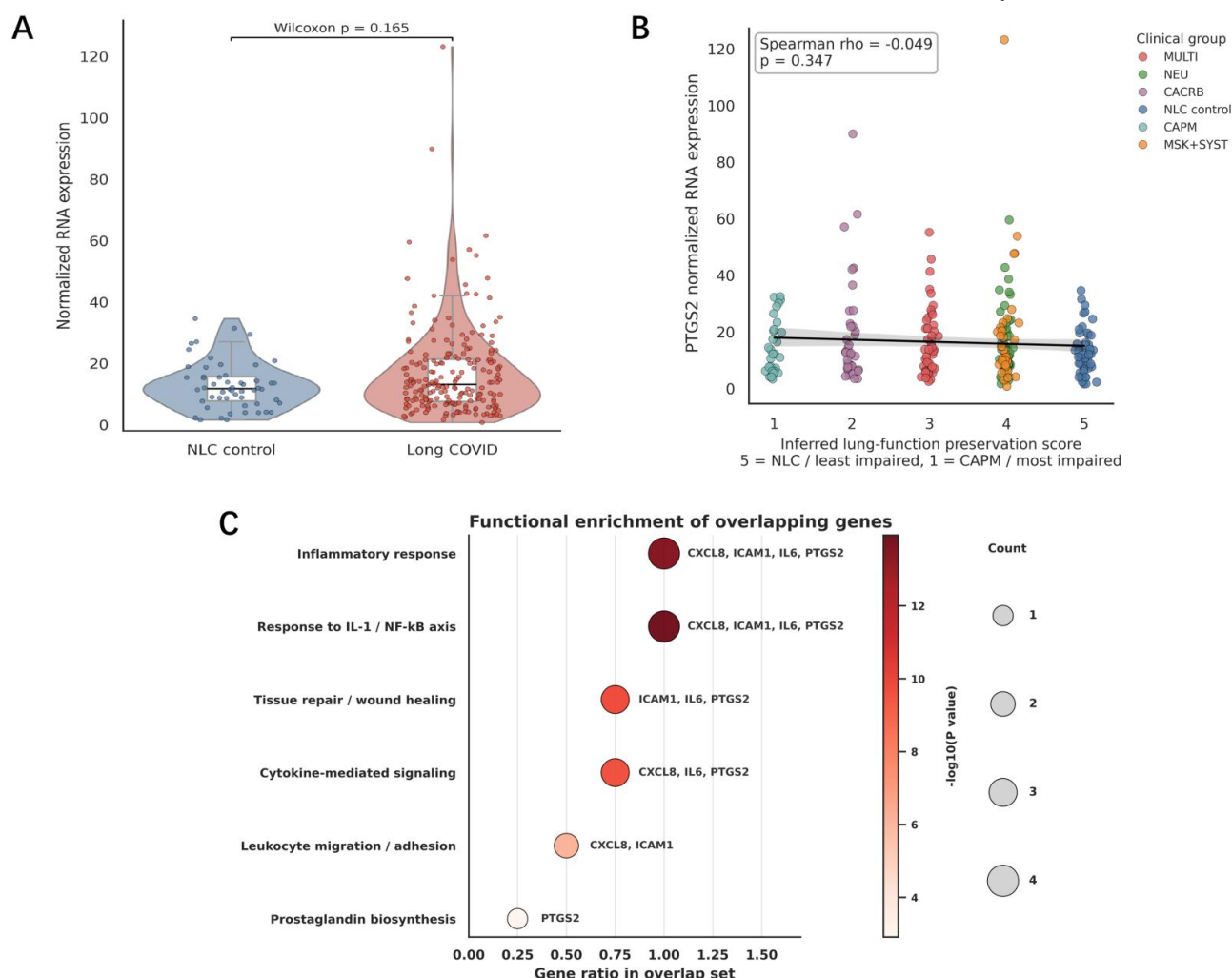


Figure 8. Long COVID cohort validation and pathway-level analysis of *PTGS2*. Panel A shows *PTGS2* normalized RNA expression in Long COVID patients and Non-long COVID controls. *PTGS2* showed higher mean expression in Long COVID samples; Welch's t-test, $p = 0.00525$, while Wilcoxon rank-sum test was not significant, $p = 0.16476$ (A). Panel B shows the association between *PTGS2* expression and an inferred lung-function preservation score derived from clinical subgroup annotation. Spearman correlation showed a weak negative trend, $\rho = -0.059$, $p = 0.347$ (B). Panel C shows the overlap between the in vitro *PTGS2* regulatory network and Long COVID clinical DEGs, identifying *CXCL8*, *ICAM1*, *IL-6*, and *PTGS2*; enrichment was evaluated by hypergeometric testing and highlighted inflammatory response, cytokine signaling, leukocyte migration, IL-1/NF- κ B axis, tissue repair, and prostaglandin biosynthesis pathways (C). Data source: raw data deposited under NGDC accession HRA007867.

Discussion

The COVID-19 pandemic has led to alarming numbers of infections and deaths worldwide. The successful development of vaccines against SARS-CoV-2 has been tested to curb the spread of COVID-19^[51]. Although the peak of the outbreak has passed, it is also very important to decipher the molecular mechanisms of SARS-CoV-2 infection. In this study, we explored the biological functions of spike glycoprotein by identifying its regulated transcriptome profile in human Calu-3 cells. We used a concentration range of 1-500 nM recombinant RBD to establish a clear dose-response curve and identify a saturating concentration for mechanistic studies. While these levels are higher than the circulating spike protein concentrations reported in patient plasma, they are within the range commonly used in in vitro functional studies and reflect the fact that local concentrations at the viral-host cell interface during infection are likely much higher than systemic levels. Nevertheless, we acknowledge that the exact physiological concentration of free RBD in the lung microenvironment remains unknown, and our findings should be interpreted with this limitation in mind. The results provide novel aspects to understand the outcomes of SARS-CoV-2 infection in human lung cells.

One astonishing discovery was that spike glycoprotein infection could significantly inhibit the apoptotic level of Calu-3 cells. Several cellular level studies on SARS-CoV-2 suggested that S protein could induce inflammatory cell death^[12,52]. One previous study showed that MERS CoV S glycoprotein suppressed the production of TNF α , IL-6 and augmented the production of IL-10^[53]. Initial studies revealed SARS-CoV-2 infection can upregulate the cellular FADDlike interleukin-1 (IL-1)-converting enzyme-inhibitory protein (cFLIP) expression, which modulate death effector domains to inhibit the activation of caspase-8/10 apoptotic signaling^[14]. Subsequent study also provided evidence that SARS-CoV-2-encoded membrane glycoprotein M triggers apoptosis with the assistance of N glycoprotein, therefore induce caspase-dependent apoptosis. However, but interestingly, in this study we firstly observed a novel function of SARS-CoV-2 S protein, which play an anti-apoptotic effect on human Calu-3 cells, even in the presence of Act D stimulation. One obvious difference is that we used a recombinant RBD fragment of the S1 subunit to treat Calu-3 cells, whereas other studies have used pseudovirions, full-length S expression constructs, or different subunits. It is important to note that our RBD construct lacks the S2 fusion machinery and does not form the native trimeric spike conformation, which may contribute to the divergent observations regarding apoptosis regulation. It is demonstrated that SARS-CoV-2 has a much higher transmissibility compared with SARS-CoV or MERS-CoV, while the mortality induced by SARS-CoV-2 was lower than the latter two^[54]. A possible interpretation, supported by previous reports that SARS-CoV-2 upregulates anti-apoptotic proteins such as cFLIP and MCL-1^[14,37], is that the S protein might protect host cells from

apoptosis, potentially facilitating viral persistence or replication during early infection. However, our current study did not directly measure viral replication, so this interpretation remains speculative. Nevertheless, we did not directly examine whether this anti-apoptotic effect influences the expression of viral replication-associated factors, as our model used a recombinant protein rather than infectious virus. Future studies using pseudotyped viruses or replicon systems are needed to determine whether the observed apoptosis resistance actually enhances viral progeny production or persistence. The possible reason for the totally different results should be considered that: on the one hand, SARS-CoV-2 could utilize apoptosis inhibition to avoid elimination, so as to help itself to replicate faster and infect other cells at the early stage. On the other hand, the virus induces apoptosis in affected cells resulting in a large amount of tissue damage and loss of function, that could accelerate the development of disease and even death.

Another exciting discovery was that we deeply investigated whole transcriptome changes by S protein infection under Act-D stimulation. Apparently, S protein extensively regulates stability and degradation of hundreds of RNAs, including mRNAs, lncRNAs, and circRNAs. Functional analysis of these dysregulated RNAs showed they were highly related to inflammatory and apoptotic pathways, demonstrating their participation in these processes. We recognize that the 100 nM S protein concentration also induced a modest increase in cell proliferation, which could potentially influence the transcriptomic profile. However, because the enriched pathways were predominantly inflammatory and apoptotic rather than cell cycle-related, we believe the observed transcriptome changes largely reflect anti-apoptotic signaling rather than proliferation. Future studies using cell cycle synchronization or lower concentrations may help further dissect these effects. Nevertheless, our current co-expression and ceRNA network analyses did not directly connect specific lncRNAs or circRNAs to *PTGS2*. Some upregulated lncRNAs, such as GAPLINC, were co-expressed with apoptosis-related mRNAs, but whether they regulate *PTGS2* directly or through other mediators is unclear. Similarly, the circRNA-miRNA-mRNA network included several mRNAs involved in inflammatory and apoptotic responses, yet none directly targeted *PTGS2*. Thus, the potential involvement of these non-coding RNAs in *PTGS2*-mediated anti-apoptosis remains speculative and requires targeted functional studies. Meanwhile, we also analyzed the circRNA expression pattern from the RNA-seq data. Metabolic pathways were significantly enriched for host genes of DE circRNAs. CircRNAs have regulatory functions to influence expression of their host genes or other genes by acting as miRNA sponge^[48], their biological functions are also emerged in multiple diseases^[55]. We highlight that circRNAs are another important molecules involved in S protein infection. Therein, four genes involved in apoptotic process were selected and further verified that they are strongly regulated in the process of S protein infection, including *RHOB*, *PTGS2*, *RRAGC*, and *MT-ND1*. Meanwhile, down DEGs were enriched

in metabolic process related pathways. Furthermore, our integrated analysis mapped a complex circRNA-mRNA regulatory network, highlighting functions primarily tied to inflammatory responses, cell adhesion, and apoptotic pathways. However, certain limitations warrant consideration. In this study, differentially expressed circRNAs were mapped into a unified global network rather than being segregated into strictly upregulated and downregulated subnetworks. While this integrated approach captures the holistic landscape of S-protein-induced transcriptomic remodeling, it may obscure specific directional regulatory axes, where significantly dysregulated circRNAs act as precise epigenetic controllers or miRNA sponges. Future studies incorporating baseline-stratified and expression-direction-segregated ceRNA networks will be essential to delineate the exact stimulatory or inhibitory epigenetic circuits driving these host responses.

Due to the anti-inflammatory effects and immune-modulating capacity as well as the ability to induce tissue regeneration^[56], MSCs have been proved to be a promising tool in the treatment of SARS-CoV-2 pneumonia^[15,21]. According to the literatures, several pre-clinical non-SARS-CoV-2 models propose that MSCs-derived secretome could have efficiency against SARS-CoV-2 virus infection. Khatri. et al, observed in a pig model that the administration of MSCs-derived exosome (MSCs-Exo) attenuated influenza virus-induced acute lung injury via inhibiting influenza virus replication^[57]. IFN- γ as a key player in strengthening the immunosuppressive function of MSCs, at least in part, by enhancing IDO, CD39/CD73 activity^[58,59]. Secretome derived from IFN- γ -pretreated MSCs increased miR-125a and miR-125b, which had therapeutic efficacy for colitis^[60]. In addition, IFN- γ -pretreated MSC-derived EVs, containing TNF-stimulated gene 6 protein (TSG-6) and A20, inhibited T-cell proliferation^[61]. Recent study showed that SARS-CoV-2 nucleocapsid (N) protein transcriptionally drives the expression of the host COX-2 (also known as *PTGS2*) gene and stimulates COX-2 expression^[62]. Under inflammatory milieu, COX-2 can be converted to several different bioactive prostaglandins (PGs). Prostaglandin E2 (PGE2) is one of the most abundant PGs, which performs diverse roles, such as regulating immune responses, gastrointestinal barrier integrity, hematopoiesis and carcinogenesis. The COX-2/PGE2 pathway is pro-viral for viruses such as porcine sapovirus, as PGE2 inhibits nitric oxide production, thus permitting viral replication^[63]. Nonsteroidal anti-inflammatory drugs (NSAIDs), the COX-2 inhibitor may modulate multiple stages of the SARS-CoV-2 life cycle^[63]. Network pharmacology and molecular docking analysis on molecular targets revealed *PTGS2* is one of the major molecules of Huashi Baidu formula (HSBDF) that developed to treat the patients with severe COVID-19^[64]. Identically, compared to *MT-ND1*, *RRAGC* and *RHOB*, we found *PTGS2* expression was markedly elevated after spike protein stimulation in Calu-3 cells, and much decreased when the culture medium was replaced by MSC-CM, suggesting that *PTGS2* and its subsequent pathways potentially mediated the SARS-CoV-2 infection. Similarly, *PTGS2* is potentially associated with the functional activity of mesenchymal stem cells (MSCs), whose conditioned medium targets SARS-CoV-2-infected cells and induces apoptosis, thereby terminating viral replication. We also recognize that our analyses were limited to *PTGS2* mRNA and protein levels, and we did not assess its enzymatic activity or products such as PGE2. Whether S protein alters *PTGS2* subcellular localization or whether necroptosis (e.g., -MLKL, p-RIPK3) or pro-inflammatory cytokines (e.g., IL-6, IL-1 β) contribute to the observed effects remains unknown.

Thus, we cannot distinguish whether MSC-CM acts primarily through anti-inflammatory or pro-apoptotic mechanisms. Future studies are needed to clarify these aspects. Additionally, our analyses were limited to *PTGS2* mRNA and protein levels, and we did not assess its enzymatic activity or its products such as PGE2. Moreover, whether S protein alters the subcellular distribution of *PTGS2*, for example its translocation from the endoplasmic reticulum to the nuclear membrane, and whether such changes contribute to apoptosis resistance, remain open questions. Additionally, we did not measure necroptosis markers such as phosphorylated MLKL or RIPK3. Thus, we cannot exclude that some of the observed effects, particularly those related to cell survival, might be mediated through necroptotic signaling. Future experiments using specific inhibitors or phospho-specific antibodies are needed to determine whether necroptosis contributes to the phenotypic changes reported here.

In summary, our study explored the biological functions of a purified recombinant RBD fragment of the SARS-CoV-2 S1 subunit in lung Calu-3 cells and decipher the underlying molecular changes using RNA-seq method. The exciting discovery is that S protein alone could inhibit apoptotic process, which may be completed by stimulating the inflammatory response and lysosomal proteases pathways. Further, we demonstrated that MSC-CM induces apoptosis of SARS-CoV-2 spike protein-infected Calu-3 cells, which *PTGS2* signaling possibly involved. To further corroborate the clinical and pathological relevance of the S protein-*PTGS2* axis, we interrogated publicly available multi-cohort transcriptomic and proteomic datasets (GSE147507, GSE150316). Transcriptional profiling confirmed that *PTGS2* is robustly induced by SARS-CoV-2 across various pulmonary cellular models and is prominently overexpressed in the autopsied lung tissues of severe COVID-19 patients. Integrating these insights with independent time-course proteomics (e.g., Grossegessse et al.) and spatial lung heterogeneity mapping reveals that prolonged host-cell survival pathways parallel active viral protein translation and tissue residency. Moreover, multi-omics characterization of post-acute sequelae (Long COVID) cohorts shows that *PTGS2* remains persistently elevated, correlating inversely with residual pulmonary function. This suggests that S-protein-driven apoptosis resistance not only facilitates short-term viral persistence but may also seed chronic inflammatory microenvironments. While the current study primarily establishes this regulatory mechanism in vitro, ongoing follow-up studies utilizing specific pathway inhibitors are being conducted to quantitatively map viral replication kinetics and definitively confirm the functional link between localized apoptosis inhibition and viral progeny output.

While our current findings establish a correlative link between *PTGS2* expression and the therapeutic efficacy of MSC-derived conditioned medium in SARS-CoV-2-infected cell models, the precise mechanistic role of *PTGS2* remains to be elucidated. To address this gap, loss-of-function and gain-of-function experiments are planned using siRNA mediated knockdown and plasmid based overexpression of *PTGS2* in MSCs. These approaches are designed specifically to determine whether *PTGS2* serves as a critical effector that modulates the paracrine capacity of MSCs. It is not intended to act directly on target cells or viral components. Conditioned media will be harvested from *PTGS2*-modified MSCs and then applied to SARS-CoV-2 permissive cell lines. Evaluation will be performed for apoptosis induction, viral replication kinetics, and inflammatory mediator profiles. It is hypothesized that if *PTGS2* functionally contr-

tributes to the antiviral property of MSC secretome, reduced bioactivity will be observed in media from knockdown MSCs, whereas enhanced effects will be seen in media from over-expressing MSCs. Conversely, if conditioned medium activity is not significantly altered by *PTGS2* modulation, *PTGS2* might be suggested as a surrogate marker of MSC activation rather than a direct functional executor. We emphasize that, in the absence of such loss-of-function experiments, our current data do not prove that *PTGS2* is causally required for either the anti-apoptotic effect of the S protein or the counteractive effect of MSC-CM. Any mechanistic interpretation of *PTGS2* in this study should be further considered preliminary and hypothesis-generating.

Additionally, given its role in prostaglandin biosynthesis, downstream metabolites such as PGE2 will be examined in the modified media. Should these cellular assays yield promising results, subsequent validation in appropriate animal models will be considered. Collectively, these experiments are considered as a logical next step to translate our current correlative observations into a mechanistic understanding of how *PTGS2* shapes the functional landscape of MSCs in the setting of SARS-CoV-2 infection.

In conclusion, our study possibly demonstrated a novel function of SARS-CoV-2 S protein in lung Calu-3 cells, which will be helpful to understand the underlying molecular mechanisms during SARS-CoV-2 infection and the development of drugs and vaccines in future. We acknowledge that our findings were obtained exclusively in the Calu-3 cell line and have not been validated in primary human airway epithelial cells or in animal models. Therefore, any direct clinical interpretation or therapeutic application derived from these results must be made with caution until further validated in more physiologically relevant systems.

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Acknowledgments

Not applicable.

Funding Statement

This work was supported by the National Natural Science Foundation of China (81960293); the Natural Science Foundation of Gansu Province (20JR5RA3); Lanzhou Science and Technology Bureau special project (2020-XG-47); Special project on COVID-19 in Gansu Province; the Fundamental Research Funds for the Central Universities, SCUT (lzujbky-2019-kb21, lzujbky-2020-sp26); the Lanzhou Chengguan District Science and Technology Planning Project (2020JSCX0019), and the Innovation Ability Improvement Project of Colleges and Universities in Gansu Province (2019B-003).

Competing interests

None of the authors have any conflicts of interest to report. Also, we thanks Pro. Xun Li from the first hospital of lanzhou university for the investigation of the COVID-19 project.

Authorship contribution statement

Shuo Gao: Methodology, Data curation, Formal analysis, Writing original draft. Yao Ge: Formal analysis, Methodology, Data curation. Haojia Wang: Conceptualization, Formal analysis, Supervision. Yang Luo: Conceptualization, Project administration, Funding acquisition, Supervision, Writing – review & editing. All authors critically revised and approved the final version of this manuscript.

Ethics statement

Not applicable.

Availability of data and materials

Data will be made available on request.