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Apelin as a new therapeutic target for COVID-19 treatment

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Summary

Background: Apelin is an endogenous neuropeptide that binds to the G-protein-coupled receptor (APJ) and participates in a variety of physiological processes in the heart, lungs and other peripheral organs. Intriguingly, [Pyr1]-Apelin-13, a highly potent pyroglutamic form of apelin, has the potential to bind to and be degraded by angiotensin-converting enzyme 2 (ACE2). ACE2 is known to operate as a viral receptor in the early stages of severe acute respiratory coronavirus (SARS-CoV-2) infection.
Aim: This study aimed to determine if apelin protects against SARS-CoV-2 infection by inhibiting ACE2 binding to SARS-CoV-2 spike protein.
Design and methods: To determine whether [Pyr1]-Apelin-13 inhibits ACE2 binding to the SARS-CoV-2 spike protein (S protein), we performed a cell-to-cell fusion assay using ACE2-expressing cells and S protein-expressing cells and a pseudovirus-based inhibition assay. We then analyzed publicly available transcriptome data while focusing on the beneficial effects of apelin on the lungs.
Results: We found that [Pyr1]-Apelin-13 inhibits cell-to-cell fusion mediated by ACE2 binding to the S protein. In this experiment, [Pyr1]-Apelin-13 protected human bronchial epithelial cells, infected with pseudo-typed lentivirus-producing S protein, against viral infection. In the presence of [Pyr1]-Apelin-13, the level of viral spike protein expression was also reduced in a concentration-dependent manner. Transcriptome analysis revealed that apelin may control inflammatory responses to viral infection by inhibiting the nuclear factor kappa B pathway.
Conclusion: Apelin is a potential therapeutic candidate against SARS-CoV-2 infection.

Introduction

Apelin is a small, secreted peptide that binds to the apelin receptor (APJ), which was initially reported as an orphan G protein-coupled receptor.[1] Apelin is a 77-amino acid peptide that is cleaved into short active forms such as apelin-36, apelin-13 and [Pyr1]-Apelin-13 [(Pyr)apelin-13].[2] (Pyr)apelin-13 is a highly potent pyroglutamic form of apelin and the predominant isoform in the cardiovascular system.[3] This small molecule is also detected in the lungs. Its abundance decreases in case of pulmonary diseases such as pulmonary arterial hypertension.[3] Apelin has been shown

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to have beneficial roles in vascular endothelial dysfunction; thus, it has emerged as a therapeutic target in various diseases, such as heart failure, hypertension, obesity and cerebral ischemia.[4,5]

Angiotensin-converting enzyme 2 (ACE2) is not only the key regulator of the renin–angiotensin–aldosterone system but is also the host functional membrane receptor for a new pathogenic virus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).[6] Thus, inhibition of the spike (S) protein of SARS-CoV-2 binding to ACE2 of the host cell membrane has been recognized as a potential therapeutic target for COVID-19.[7]

Intriguingly, the human APJ gene (*APLNR*) encodes a seven-transmembrane protein that closely resembles the angiotensin receptor.[8] Apelin is a catalytic substrate for ACE2 activity.[9] (Pyr)apelin-$13_{(1\text{-}12)}$ is an ACE2 metabolite of (Pyr)apelin-13(3) and has shown beneficial effects, such as protecting acute lung injury by reducing the levels of reactive oxygen species[10] and preventing pulmonary edema development.[11] However, to the best of our knowledge, no previous studies have been conducted to determine the potential role of apelin against SARS-CoV-2 infection.

In this study, we investigated whether (Pyr)apelin-13 could prevent ACE2 binding to the S protein of SARS-CoV-2. In addition, we analyzed the lung transcriptome of COVID-19 patients infected with SARS-CoV-2 to identify the potential role of apelin in the pathogenesis of COVID-19.

Methods

Cell culture

All cell lines were grown in a 37°C, 5% CO_2 environment. Human embryonic kidney 293 cell line (HEK293) and human lung cancer cell line (A549) were obtained from the Korean Cell Line Bank. The human bronchial epithelial cell line (16HBE14o-) was purchased from Merck Millipore. The cells were cultured in Roswell Park Memorial Institute-1640 medium with 0.5 μg/ml puromycin in 10% fetal bovine serum and 1% penicillin/streptomycin.

Cell-to-cell fusion assay

Transfection of the HEK293 and 16HBE14o- cells was performed using Lipofectamine™ 3000 Transfection Reagent (Invitrogen, #L3000001) when the cell density was above 80%, following the manufacturer's instructions. For the cell-to-cell fusion assay, 16HBE14o- and A549 cells were used as the producer cells and co-transfected with Gluc1 plasmid (pcDNA3.1puro-Bipss-Zip-Glu1-KDEL) and SARS-CoV-2-Spike plasmid (Addgene, #145032). The target cells were co-transfected with the Gluc2 plasmid (pcDNA3.1puro-Bipss-Zip-Glu2-KDEL) and the human ACE2 plasmid (Addgene, #141185) as described above.[12] To inhibit cell fusion, [Pyr1]-Apelin-13 trifluoroacetate salt (Bachem, #4029110), Apelin-17 [Apelin-17 trifluoroacetate salt (Bachem, #4050029)], and Apelin-36 were added to the producer and target cells. The dual-luciferase reporter assay system (Promega, #E1910) was used to measure the luminescence of cell lysates with a luminometer (Centro XS3 LB 960, Berthold Technologies) according to the manufacturer's instructions.

Immunofluorescence staining

HEK293 and 16HBE14o cells were transfected with human ACE2 or apelin plasmids (Origene, #RC205832). The cells were fixed with 2% paraformaldehyde and permeabilized with 0.5% TWEEN20 in phosphate-buffered saline (PBST) for 5 min. After blocking with 5% goat serum for 1 h at room temperature (RT), the cells were incubated with the primary antibodies. Anti-ACE2 antibody (Abcam, #ab15348) and anti-apelin antibody (Abcam, #ab230536) were diluted 1:100 in 0.5% PBST and 5% goat serum mixture and maintained at 4°C for 6 h. Then, secondary antibodies (Alexa 488 anti-goat Immunoglobulin G (IgG) antibody and Alexa 594 anti-rabbit IgG antibody) were incubated at 4°C for 2 h. After washing with PBST, cells were stained with 4',6-diamidino-2-phenylindole (Abcam, #ab104139). Fluorescent images were acquired using a confocal microscope (Carl Zeiss LSM 880 NLO). Image analysis was performed using a confocal microscopy software (Zeiss, py20037.2).

Binding affinity assay for observing the interaction between SARS-CoV-2 spike protein and apelin

Binding affinity was tested using an enzyme-linked immunosorbent assay kit on ACE2-coated plates, following the manufacturer's instructions. The assay was performed in 96-well, flat-bottom plates. The apelin compounds were serially diluted in distilled water. Then, the pre-coated serial dilution compounds were added to the plate and gently shaken at RT for approximately 4 h. The solution was then removed, and the plate was washed four times with wash buffer before adding the detection antibody (anti-apelin antibody). The reaction was performed at RT with gentle shaking for 1 h. The washing procedure was repeated as described above. Horseradish peroxidase-conjugated anti-rabbit IgG was added to each well, and the reaction plate was gently shaken for an additional 30 min at RT. The washing procedure was performed again as described above. Next, 3,3′,5,5′-tetramethylbenzidine (step 1 substrate) was added to each well, and the reaction mixture was incubated for 30 min at RT in dark along with shaking at 180 rpm. The reaction was stopped by adding 50 μl of stop solution. The absorbance at 450 nm was measured using a microplate reader (SpectraMax190, Molecular Devices, Sunnyvale, CA, USA).

Quantitative polymerase chain reaction analysis for pseudovirus inhibition assay

The full-length S gene from the CoV2-Spike D614G plasmid (Addgene, #177960) was cloned into the lentiviral vector (Supplementary Figure S1). The pseudovirus was then produced in the Lenti-X 293 T cell line (Takara Bio, #632180). For the inhibition assay, 16HBE14o- cells were cultured in a 12-well plate and infected with a pseudovirus containing the S protein. To quantify the pseudovirus infection, total RNA from cells was isolated using TRIzol reagent (Invitrogen, #15 596,026) according to the manufacturer's protocol. One microgram of total RNA from each sample was used for cDNA synthesis using the QuantiTect Reverse Transcription Kit (Qiagen, #205311), according to the manufacturer's protocol. The mRNA expression of the target genes was measured using real-time quantitative PCR with a CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad, USA) using s (Gendepot, #Q5600-010).

The primers used for the reaction were as follows: SARS2-Spike forward: GCCTGCCTACACCAATTCCT; reverse, ATCGAA GCGTTTGGTCCCAT; human β-actin forward, CATGTACGTT GCTATCCAGGC; reverse, CTCCTTAATGTCACGCACGAT.

Human database analysis

To investigate the potential role of apelin in the human lung, we downloaded transcriptome data from SARS-CoV-2-infected human lung tissues (GSE150316).[13] We categorized these datasets as normal, low viral RNA and high viral RNA groups. We then analyzed gene expression as described below.

Transcriptome analysis

Publicly available transcriptome data were downloaded from the Gene Expression Omnibus database. To compare differentially expressed genes (DEGs) between apelin knockout (*Apln* KO) and wild-type (WT) mice, we analyzed endothelial cell transcriptome datasets (GSE100293).[14] The corresponding data from the 'estimateSizeFactors' function in the DESeq2 package was used to normalize the raw RNA count matrix.[15] For each gene, we built many statistically significant filters in which 50% of the samples had a raw count above three. Heat maps for genome-wide gene expression were created using normalized gene counts that were log2 transformed. The 'DESeqDataSetFromMatrix' function in the DESeq2 package was used to identify DEGs. Volcano plots were characterized using the EnhancedVolcano package, which uses a significant cutoff, a *P* value of <0.01, and a log2 fold change absolute value greater than 0.5. The sorted gene lists were based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) and gene ontology (GO) pathways as determined by the Database for Annotation, Visualization and Integrated Discovery website.[16] The gene set enrichment analysis (GSEA) software was used to perform gene set enrichment analysis.[17] The R software program (version 4.1) was used to process the data, except for the KEGG, GO and GSEA pathways.

Statistical analysis

One-way analysis of variance (and non-parametric or mixed) was used in a program called Prism to evaluate the statistical significance. Asterisks indicate significant differences ($^{*}P < 0.05$; $^{**}P < 0.01$). The R software was used to perform all statistical analyses.

Results

Apelin prevents S protein-mediated cell-to-cell fusion by inhibiting ACE2 binding to SARS-CoV-2 spike protein

To test whether apelin can interfere with SARS-CoV2 spike protein binding to ACE2, we established a cell-to-cell fusion assay system using split luciferase and Gluc1 and Gluc2 plasmids, as described in the Methods section.[18] We tested apelin-36, apelin-17 and [Pyr1]-Apelin-13.

Figure 1 displays the results of the experiment. First, we developed a cell-to-cell fusion assay using HEK293 cells. S proteins on the producer cells (Glu1-SARS2) bound to ACE2 on target cells (Glu2), and this binding induced cell membrane fusion. This fusion increased luciferase activity. When apelin peptides were used in this system, apelin-36 did not interfere with this binding, but apelin-17 and [Pyr1]-Apelin-13 significantly inhibited the fusion between the two cells. We then established a cell fusion assay system using the human lung cancer cell line (A549) and human bronchial epithelial cell line (16HBE14o-). In this system, we tested [Pyr1]-Apelin-13, the most potent apelin isoform in the lung and heart.[19] In both the human lung cell lines, [Pyr1]-Apelin-13 effectively reduced the binding between the spike protein and ACE2 (Figure 1B).

Co-localization of ACE2 and apelin in human cell lines

To explore the colocalization of ACE2 and apelin in lung cells, we used both human A549 and 16HBE14o- cells. First, both cells were co-transfected with a human ACE2 expression plasmid and an apelin expression plasmid. Immunofluorescence images were acquired using a confocal microscope. A549 cells and 16HBE14o- cells co-transfected with ACE2 and apelin are shown in Figure 2A and B. ACE2 (green) was co-localized with apelin (red) in both cell lines. This co-localization (yellow) supports the binding of apelin to ACE2. The enzyme-linked immunosorbent assay-based binding affinity assay also revealed direct binding between apelin and ACE2 (Supplementary Figure S2).

Apelin suppresses infectivity of SARS-CoV-2 S pseudovirus

We then established a pseudovirus-based inhibition assay using recombinant lentivirus pseudotyped with the SARS-CoV-2 spike protein.[20] 16HBE14o cells were then infected with pseudovirus, and the pseudovirus content in the cells was quantified using reverse transcription polymerase chain reaction. The results are shown in Figure 2C. [Pyr1]-Apelin-13 pretreatment effectively reduced the pseudovirus content. This means that apelin can inhibit SARS-CoV-2 invasion of lung epithelial cells.

Transcriptome analysis reveals potential roles of apelin in SARS-CoV-2 infection

Next, to uncover the potential roles of apelin in the pathophysiology of SARS-CoV-2 infection, we performed transcriptome analysis using human lung autopsy data (GSE150316).[13] Based on the viral RNA content, we categorized lung datasets into three groups: a control group from normal lungs, a low viral load group from the infected lungs with low viral RNA content, and a high viral load group from the infected lungs with high viral RNA content. We then investigated the expression of *APLN* (gene that encodes the apelin protein) in these three groups. Figure 3A shows the obtained results. *APLN* expression was low in the low viral load group as compared to the control group, while the high viral load group showed increased *APLN* expression as compared to the low viral load group.

Intriguingly, the high viral load group could be divided into two subgroups based on the level of *APLN* expression: low and high expression groups. We then profiled gene expression in the high viral load group and identified DEGs between the high and low *APLN* expression subgroups. Figure 3B shows a volcano plot of DEGs between the high and low *APLN* expression groups. The top five up-regulated genes based on average fold change were nuclear receptor subfamily 4, group A, member 3 (*NR4A3*), fibrinogen alpha chain (*FGA*), C-X-C motif chemokine ligand 10 (*CXCL10*), proline-rich and Gla domain 3 (*PRRG3*), and apelin (*APLN*), and the top five down-regulated genes were polypeptide N-acetylgalactosaminyltransferase 15 (*GALNT15*), tubulin beta 1 class VI (*TUBB1*), apolipoprotein D (*APOD*), progastricsin (*PGC*) and interleukin 1 receptor type 2 (*IL1R2*).

To identify key features of gene expression between these two subgroups, we performed GO and KEGG pathway enrichment analyses. Figure 3C shows a heat map of the enriched pathways. In the high *APLN* expression group, cellular responses to external stimuli and defense responses to viruses were up-regulated. Several genes related to collagen production, such as collagen type V alpha 1 chain (*COL5A1*) and *COL5A3*, were down-regulated, and the genes related to matrix degradation, such as ADAM metallopeptidase with thrombospondin type 1 motif 4 (*ADAMTS4*) and *ADAMTS9* were up-regulated. Long pentraxin 3, which is a member of the pentraxin family, has a protective effect against pulmonary fibrosis.[21] Pentraxin 3 gene expression was also up-regulated in the high *APLN* expression group (Figure 3D).

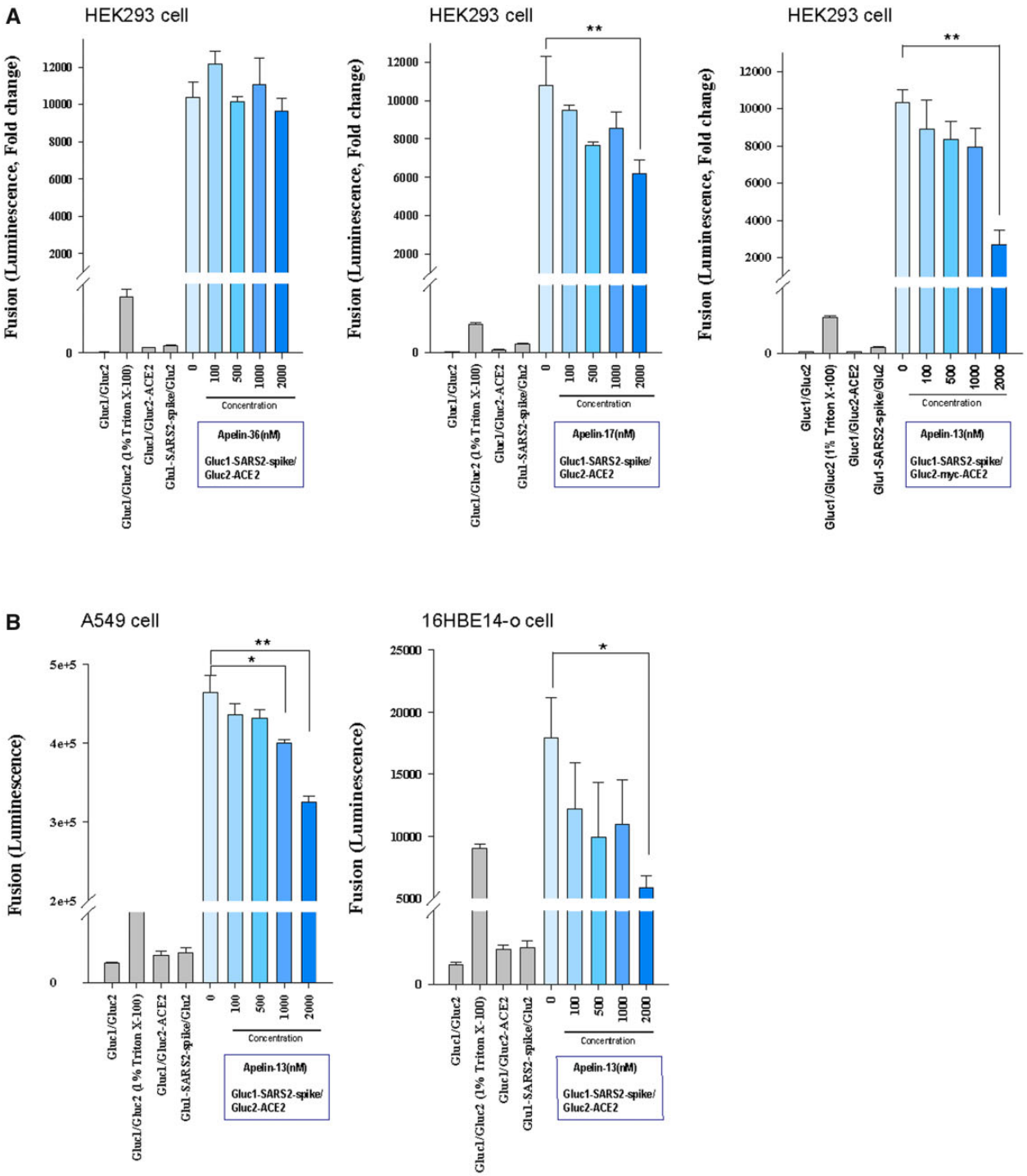
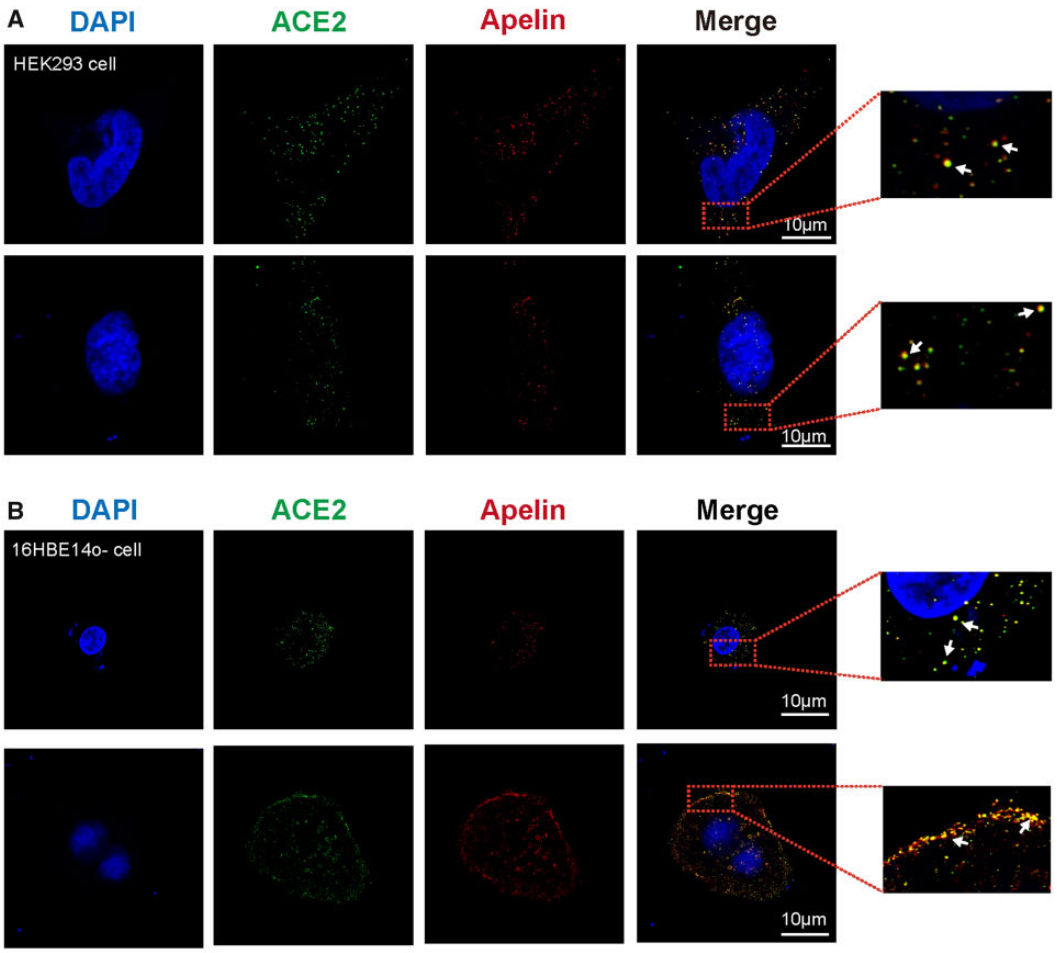


Figure 1. Cell-to-cell fusion assay. (**A**) Apelin-17 and apelin-13 effectively inhibited the binding of SARS-CoV-2 spike protein to angiotensin-converting enzyme 2 (ACE2) in human embryonic kidney 293 cells. (**B**) Apelin-13 effectively reduced the binding of SARS-CoV-2 spike protein to ACE2 in both human lung cell lines (A549, human lung cancer cells; 16HBE14o-, human bronchial epithelial cells). Apelin-13; [Pyr1]-Apelin-13. $^{*}P < 0.05$; $^{**}P < 0.01$.

Dysregulated immune responses by low apelin in COVID-19

To explore the direct role of apelin at the cellular level, a bioinformatic analysis was conducted using endothelial cell transcriptomes of *Apln* KO and WT (GSE100293). From this dataset, we identified key DEGs and conducted functional enrichment analyses using these DEGs. Figure 4A shows a volcano plot of DEGs between *Apln* KO and WT mice. The top five up-regulated genes based on average fold change were sodium voltage-gated channel alpha subunit 5 (*Scn5a*), fibrinogen like 2 (*Fgl2*), apolipoprotein D (*Apod*), fibronectin leucine-rich transmembrane protein 3 (*Flrt3*) and pleiotrophin (*Ptn*); the top five down-regulated genes were apelin (*Apln*), platelet-derived growth factor receptor-beta (*Pdgfrb*), killer cell lectin-like receptor, subfamily



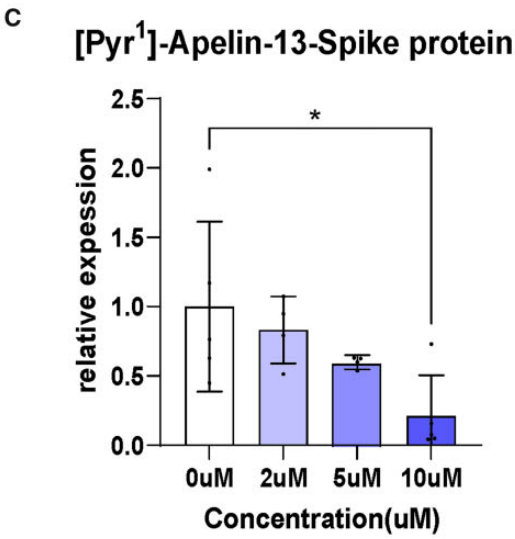


Figure 2. Co-localization of angiotensin-converting enzyme 2 and apelin. Representative images of co-localization of apelin and angiotensin-converting enzyme 2 in human cell human embryonic kidney 293 (HEK293) cells (**A**) and human bronchial epithelial cells (**B**). Apelin (red), GFP (green) and 4',6-diamidino-2-phenylindole (blue) signals can be seen in the images. The overlap of red and green displays as yellow (arrow). (**C**) Pseudovirus inhibition assay using lentivirus containing SARS-CoV-2 spike protein. [Pyr1]-Apelin-13 significantly suppressed SARS-CoV-2 entry into HEK293 cells.

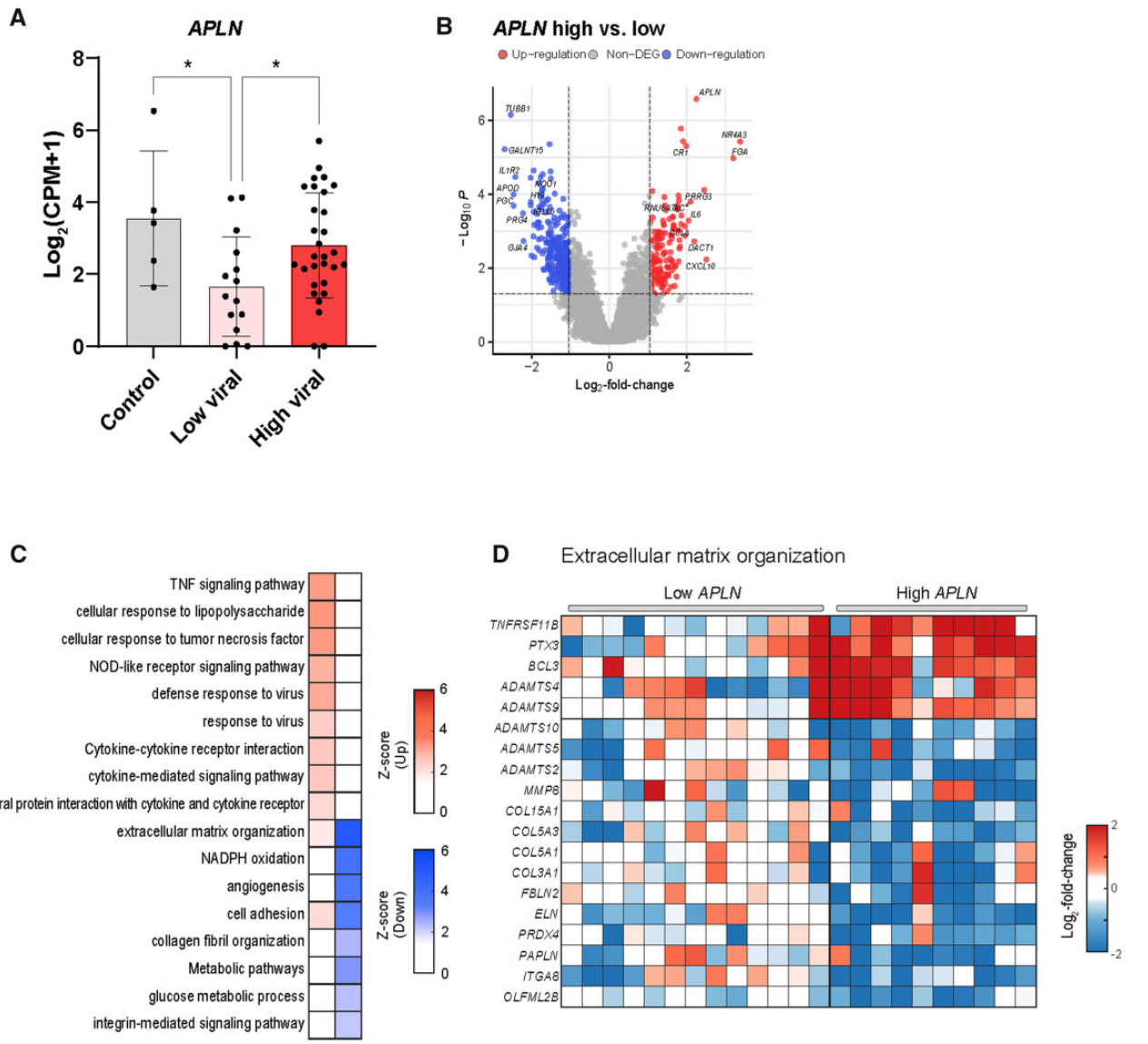


Figure 3. Profiling of SARS-CoV-2 infected human lung transcriptomes. (**A**) Apelin (APLN) expression in human lungs infected with SARS-CoV-2. Low viral: low SARS-CoV-2 virus and high viral: high SARS-CoV-2 virus content groups can be seen. (**B**) Volcano plot using differentially expressed genes (DEGs) between high *APLN* and low *APLN* expression groups. (**C**) Heatmap for the enriched pathway analysis using DEGs. (D) Heatmap for enriched gene-related extracellular matrix organization. *$P < 0.05$.

A, member 4 (*Klra4*), nuclear RNA export factor 3 (*Nxf3*) and spondin 1 (*Spon1*).

Figure 4B shows a heatmap of the enriched pathways of DEGs between the *Apln* KO and WT mice. Functional enrichment assays indicated that multiple pathways related to the immune/inflammation response, such as positive regulation of protein kinase B signaling, cytokine production, and inflammatory response were up-regulated. Metabolic pathways such as glycolysis/gluconeogenesis, cellular glucose homeostasis and response to hormones were down-regulated (Figure 4B). The nuclear factor kappa B (NF-κB) signaling pathway, which is a key mediator of lung inflammation and carcinogenesis, was also positively enriched in the *Apln* KO transcriptome (Figure 4C).

Discussion

In this study, we tested the potential of apelin to be used for the prevention and treatment of COVID-19. In *in vitro* assays using human cell lines, including HEK293, A549 and 16HBE4o- cells, apelin directly bound to ACE2 and successfully prevented SARS-CoV-2 spike protein from binding to ACE2. The virus infectivity assay revealed the preventive effects of apelin. Pretreatment with apelin significantly reduced the amount of SARS-CoV-2 spike pseudovirus in the infected cells.

These results proposed apelin as a possible inhibitor for preventing SARS-CoV-2 binding to host cell ACE2. Transcriptome analysis has suggested that activation apelin/APJ signaling also can be an effective therapy for the delayed and/or long-term complications of COVID-19 such as lung fibrosis.[22] In the lung infected with SARS-CoV-2, the high *APLN* expression group showed increased immune response pathways such as defense response to virus, and decreased pathways related to lung fibrosis formation, including extracellular matrix formation. *APLN*-null epithelial cells showed increased inflammation-related pathway activity, including NF-κB signaling, apoptotic process and decreased pathway activity related to energy metabolism.

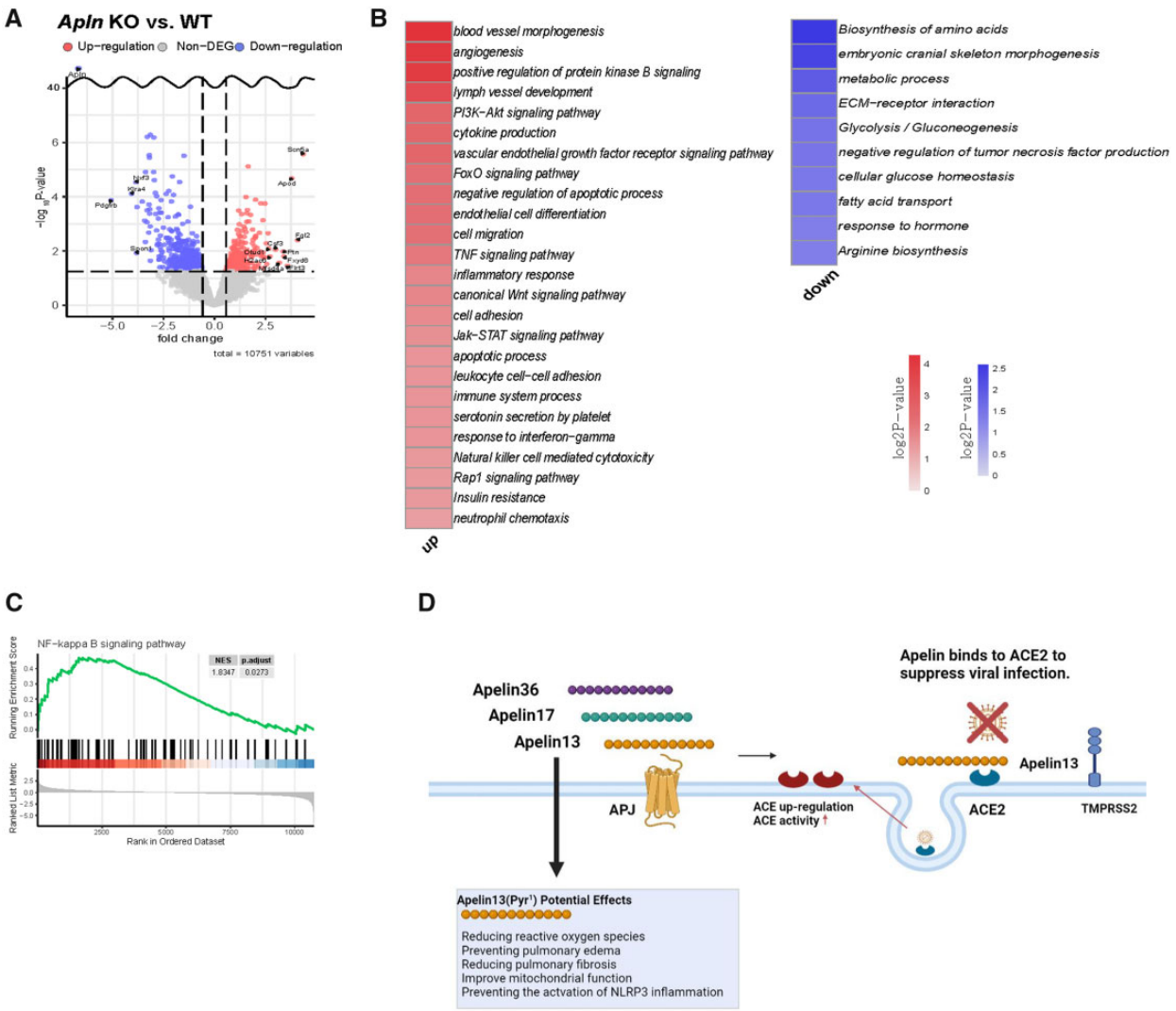


Figure 4. Transcriptome analysis of apelin-null endothelial cells. (**A**) Volcano plot using differentially expressed genes (DEGs) between apelin knock out (KO) and wild type (WT) mouse endothelial transcriptome. (**B**) Heatmap for the enriched pathway analysis using DEGs. (**C**) Enrichment plot of nuclear factor kappa B (NF-κB) signaling. (**D**) Overview of proposed beneficial roles of apelin in the lungs in SARS-CoV-2 pathogenesis.

As a peptide hormone, apelin/APJ signaling has been demonstrated to have beneficial effects on pulmonary diseases.[11,23,24] Apelin treatment improved lung structure and function in a rat model with hyperoxic lung injury by reducing inflammation, fibrin deposition and right ventricular hypertrophy.[23] Apelin-null mice showed severe pulmonary hypertension due to decreased endothelial nitric oxide synthase.[24] In an acute lung injury model, apelin suppressed activation of the NLRP3 inflammasome and NF-κB signaling.[11]

The apelin/APJ signaling has also been found to be advantageous in cardio-cerebrovascular disease,[4,25–27] which are common complications of COVID-19. This signaling regulates vascular tone in animal models and reduced circulating apelin levels were correlated with essential hypertension in human study.[25] In ischemic stroke, apelin protects cellular damage by preventing neuronal apoptosis and excitotoxicity.[4] Apelin also modulates renin–angiotensin system by regulating ACE2 expression and activity in the cardiovascular system.[27] This means that apelin/APJ signaling might be possible therapeutic options for the COVID-19 severe consequences such as heart attack and stroke.[28]

Our study had several limitations. First, the experiments were performed using in *vitro* models. Second, we did not use the SARS-CoV-2 whole virus in our study. Therefore, we need more concrete evidence to support our investigation. Further research is required to validate these positive effects of apelin and to utilize this protein in *in vivo* animal systems against all other viruses.

In conclusion, our study suggests a new and potent therapeutic target for COVID-19. It can be said that apelin can suppress SARS-CoV-2 infection, and activation of apelin/APJ signaling may restore lungs damaged by viral infection (Figure 4D).

Supplementary material

Supplementary material is available at *QJMED* online.

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Conflict of interest: None declared.

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