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Original Research

Thymosin-α1 binds with ACE and downregulates the expression of ACE2 in human respiratory epithelia

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Abstract

Background: Thymosin-α1 has been implicated into the treatment of novel respiratory virus Coronavirus Disease 2019 (COVID-19), but the underlying mechanisms are still disputable. **Aim**: Herein we aimed to reveal a previously unrecognized mechanism that thymosin-α1 prevents COVID-19 by binding with angiotensin-converting enzyme (ACE), which was inspired from the tool of network pharmacology. **Methods**: KEGG pathway enrichment of thymosin-α1 treating COVID-19 was analyzed by Database of Functional Annotation Bioinformatics Microarray Analysis, then core targets were validated by ligand binding kinetics assay and fluorometric detection of ACE and ACE2 enzymatic activity. The production of angiotensin I, angiotensin II, angiotensin (1–7) and angiotensin (1–9) were detected by enzyme linked immunosorbent assay. **Results**: We found that thymosin-α1 impaired the expressions of angiotensin-converting enzyme 2 and angiotensin (1–7) of human lung epithelial cells in a dose-dependent way ($p < 0.001$). In contrast, thymosin-α1 had no impact on their ACE and angiotensin (1–9) expressions but significantly inhibited the enzymatic activity of ACE ($p > 0.05$). **Conclusion**: The bioinformatic findings of network pharmacology and the corresponding pharmacological validations have revealed that thymosin-α1 treatment could decrease ACE2 expression in human lung epithelial cells, which strengthens the potential clinical applications of thymosin-α1 to prevent severe acute respiratory syndrome coronavirus 2 infection.

Keywords: COVID-19; Thymosin-α1; Angiotensin-converting enzyme 2 (ACE2); Angiotensin-converting enzyme (ACE)

1. Introduction

The novel coronavirus disease 2019 (COVID-19) is caused by the infection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which has swiftly spread around the globe in the past year [1–3]. Although the scientific community is actively exploring several treatments (e.g., remdesivir, immune modulators and COVID-19 vaccinations) that would potentially combat with COVID-19 [4–7], the virus spreading still reached into the billions [8,9]. A retrospective cohort study has analyzed 76 critically ill cases of COVID-19 from General Hospital of the Central Theatre Command and Wuhan Pulmonary Hospital from December 2019 to March 2020. Thymosin-α1 treatment manifested significant reduction in the mortality of severe COVID-19 patients as compared with untreated group (30.00% *vs.* 11.11%, $p = 0.044$) [10]. It thus indicated that thymosin might have the potential therapeutic efficacy for preventing critical COVID-19 infection.

Thymosin-α1, an immune-modulating polypeptide hormone with 28-amino acids which is mainly produced by thymic epithelial cells [11,12], can effectively restore T cells by enhancing their differentiation and inhibiting apoptosis [13,14]. It could also modulate proinflammatory cytokine storm [15] and chemokines production [16]. Therefore, thymosin-α1 has been widely used for anti-virus clinical treatments, which is approved as an immune adjuvant to treat hepatitis B (HBV) [17,18] and hepatitis C (HCV) [19,20]. The latest multicenter retrospective study which has enrolled 334 COVID-19 patients suggested thymosin α1 treatment can markedly decrease 28-day mortality and attenuate acute lung injury in critical type COVID-19 patients (Hazards Ratios: 0.11, 95% confidence interval: 0.02–0.63, $p = 0.013$) [21]. Thymosin-α1 group was also demonstrated with much shorter RNA shedding duration of SARS-CoV-2 (13 days *vs.* 16 days, $p = 0.025$) and hospital stay (14 days *vs.* 18 days, $p < 0.001$) [22]. Nevertheless, the underlying mechanism of anti-viral effect of thymosin-α1 was only interpreted to strengthen immune response by the restoration of lymphocytopenia and reversion of exhausted T cells [23,24]. Further mechanistic targets of thymosin-α1 deserved to be clarified in severe COVID-19 treatment.

In this study, we revealed a novel regulatory mechanism of thymosin-α1 in preventing SARS-CoV-2 infection, which was evidenced from the data of network pharmacological studies and molecular verifications on human lung epithelial cells. It has shed light on the potential underlying

mechanism that thymosin-α1 impairs ACE2 expressions of human lung epithelial cells by binding with ACE, which consequently reduces the host receptors of SARS-CoV-2. This study implicates that therapeutic strategy of thymosin-α1 may help to early prevent COVID-19 diffusion in lung epithelial cells.

2. Materials and methods

Collection of all targets of COVID-19 and thymosin-α1. Based on the available tools of Swiss Target Prediction [25], therapeutic targets of thymosin-α1 were collected by the tool of SuperPred. The above process of targets prediction was carried out in five different species in order to close paralogs and orthologs. Then "smiles" formats of thymosin-α1 were imported into Swiss Target Prediction to predict its putative targets. Of note, these high-probability targets were only limited to *Homo sapiens* and all selected targets were identified in Therapeutic Target Database and Comparative Toxicogenomic Database. Subsequently, the disease-screening tool of GeneCards was employed to harvest COVID-19-associated targets [26]. Ultimately, the identified targets of COVID-19 and thymosin-α1 were further intersected to obtain the shared targets of thymosin-α1 treating COVID-19.

The protein-protein interaction network of all shared targets. 19 shared biotargets were merged to draft a connective network of thymosin-α1 treating COVID-19 by using a STRING tool [27]. Then Cytoscape software was employed to draft the protein-protein interaction (PPI) network of thymosin-α1 treating COVID-19 [28]. Selected from all nodes of the network, we treat those with more than 2-fold of the median degree as the major hubs. They are analysed by several critical topological properties according to the number of node links, the number of shortest paths between pairs of nodes, the sum of the distances of node to all other nodes and K-coreness. Subsequently, KEGG pathway enrichment of the main targets was analysed by DAVID webserver (https://david.ncifcrf.gov/). To obtain a better understanding of the biological function and molecular interaction, the topological parameter of network settings for anti-COVID-19 targets played by thymosin-α1 were identified through the stand-alone software tool FunRich (version 3.0, http://www.funrich.org/download) [29].

Assay of the KEGG pathway enrichment of thymosin-α1 treating COVID-19. Eight therapeutic targets of thymosin-α1 against COVID-19 were uploaded into the online Database of Functional Annotation Bioinformatics Microarray Analysis (DAVID) to search for the top activated and inactivated signaling pathways of thymosin-α1 treating COVID-19. According to the settings of the reported -Log *p* value, the bar graph of activated and inactivated signaling pathways of thymosin-α1 treating COVID-19 were respectively created and illustrated [30].

Binding kinetics assay. The kinetics of thymosin-α1 binding was evaluated through surface plasmon resonance (SPR) using a PlexArray® HT system (Plexera LLC, Woodinville, WA, USA). ACE purified protein (ICA004Mu01, LMAI Bio), SARS-CoV-2 spike glycoprotein (Abcam, ab273063) and Thymosin-α1 (Sigma-Aldrich, T3410, CAS No. 62304-98-7) were purchased from commercial sources. In brief, ACE purified protein was immobilized on a biosensor chip (Graft-to-PCL). The specific interactions of thymosin-α1 with the immobilized ACE purified protein were assessed. Thymosin-α1 was analyzed at a flow rate of 1 μL/min with 0.01 M PBS running buffer and contact time of 200 s. The surface was washed and regenerated with a 0.125% SDS buffer at a flow rate of 2 μL/min for 200 s followed by 30-min waiting time for dissolution after each experiment. The competition binding assay was same as above, except that SARS-CoV-2 spike glycoprotein was fixed on the chip and then dipped into wells containing ACE2 purified protein alone or with doses of thymosin-α1. The analyses were performed in PLEXERA SPR Date Analysis Module (DAM).

Fluorometric detection of ACE and ACE2 enzymatic activity. Purified human ACE protein and human ACE2 protein were purchased from LMAI Bio (ICA004Mu01) and OriGene Technologies (TP720353). Then standard curve preparation and fluorometric substrates measurements were instructed as the standard protocol of Angiotensin I Converting Enzyme Activity Assay Kit (Fluorometric) (BioVision, Catalog # K227-100) and Angiotensin II Converting Enzyme Activity Assay Kit (Fluorometric) (BioVision, Catalog # K897-100).

Western blotting analysis. Proteins were extracted by using RIPA Lysis Buffer (P0013, Beyotime, China) and quantified by using a BCA kit (P0009, Beyotime, China). Twenty micrograms of each protein sample were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membranes were blocked with 5% BSA and incubated with primary antibodies (ACE2, Abcam, ab108252; ACE, GeneTex, GTX54938; Ang I, LifeSpan BioSciences, LS-C301221; Ang II, LifeSpan BioSciences, LS-C299822; angiotensin (1–7), LifeSpan BioSciences, LS-C705843; angiotensin (1–9), LifeSpan BioSciences, LS-C664040; β-actin, A5441, Sigma) for 10 h at 4 °C. The membranes were rinsed five times with PBS containing 0.1% Tween 20 and incubated for 1 h with appropriate horseradish peroxidase-conjugated secondary antibody at 37 °C. Membranes were extensively washed with PBS containing 0.1% Tween 20 for three times. The signals were stimulated with enhanced chemiluminescence substrate (NEL105001 EA, PerkinElmer) for 1 min and detected with a Bio-Rad ChemiDoc MP System (170-8280).

Small interfering RNAs (siRNAs). siRNAs target sequence for human ACE (Genbank No. NM_000789.2) is 5′-GCA TCA CCA AGG AGA ACT AdTdT-3′ and the control siRNA target sequence is 5′-UUC UCC GAA CGU GUC ACG UdTdT-3′, which were both synthesized at Genechem Services (Shanghai, China). BEAS-2B cells

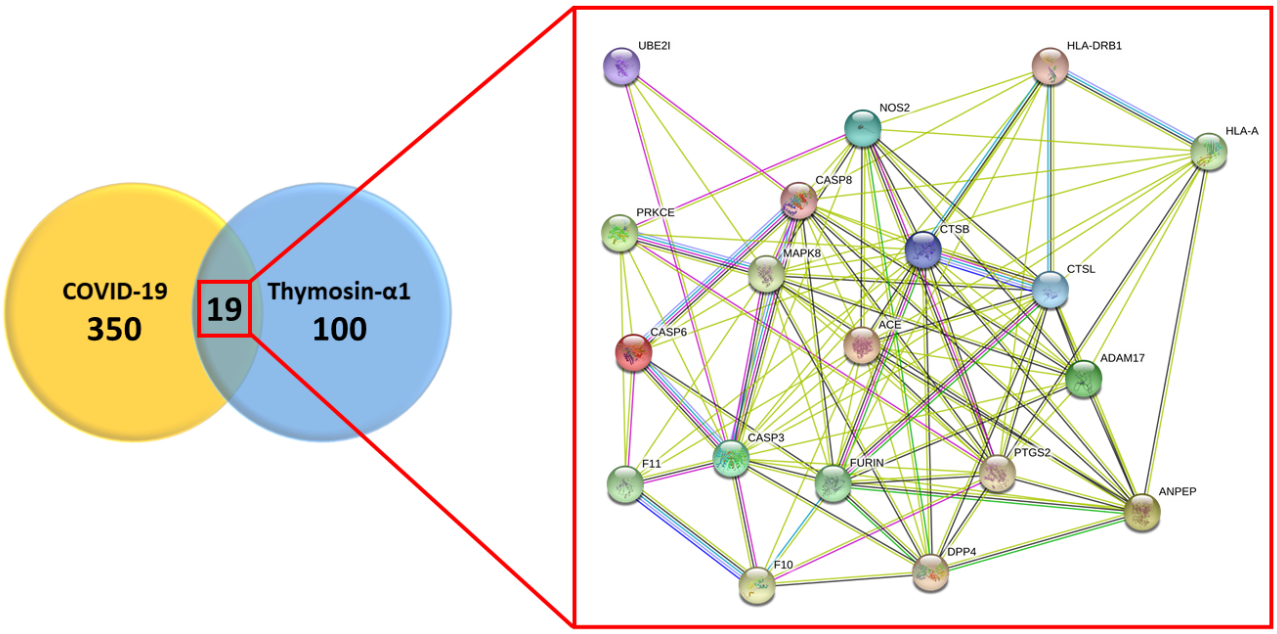

Fig. 1. All targets of thymosin-α1 and COVID-19 were identified and the shared targets of thymosin-α1 treating COVID-19 for drawing the PPI network. COVID-19, Corona Virus Disease 2019; PPI, protein-protein interaction; CASP8, Caspase 8; UBE2I, Ubiquitin Conjugating Enzyme E2 I; HLA-A, Histocompatibility Complex, Class I, A; ANPEP, Alanyl Aminopeptidase; PRKCE, Protein Kinase C Epsilon; CASP3, Caspase 3; CASP6, Caspase 6; ACE, Angiotensin I Converting Enzyme; CTSB, Cathepsin B; CTSL, Cathepsin L; MAPK8, Mitogen-Activated Protein Kinase 8; FURIN, Paired Basic Amino Acid Cleaving Enzyme; NOS2, Nitric Oxide Synthase 2; DPP4, Dipeptidyl Peptidase 4; PTGS2, Prostaglandin-Endoperoxide Synthase 2; F10, Coagulation Factor X; HLA-DRB1, Major Histocompatibility Complex, Class II, DR Beta 1; F11, Coagulation Factor XI; ADAM17, ADAM Metallopeptidase Domain 17. CASP8; UBE21; HLA-A; ANPEP; PRKCE; CASP3; CASP6; ACE; CTSB; CTSL; MAPK8; FURIN; NOS2; DPP4; PTGS2; F10; HLA-DRB1; F11; ADAM17.

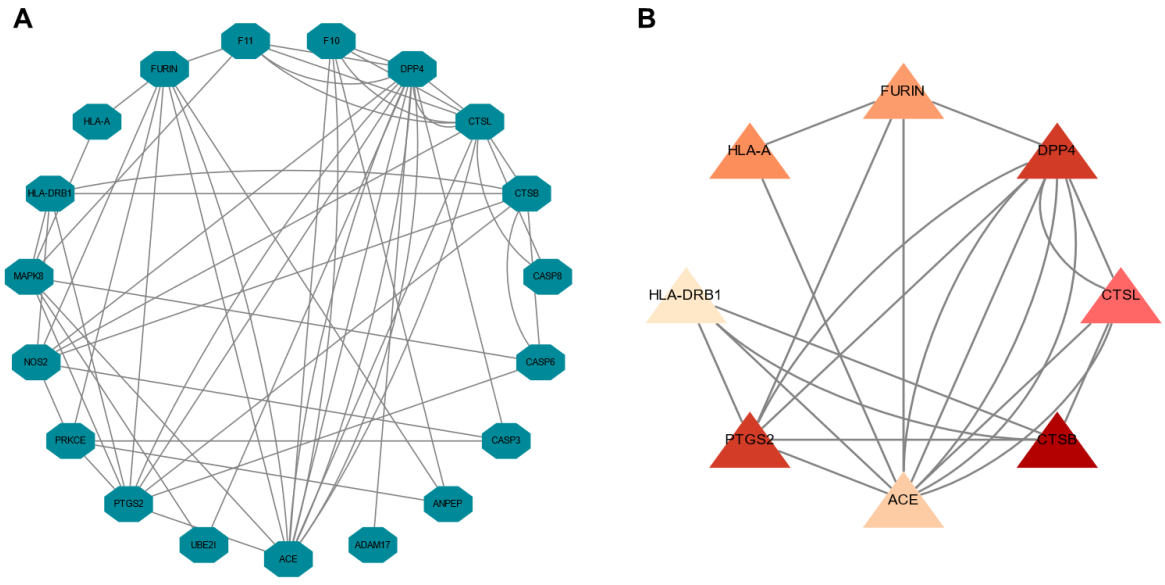

Fig. 2. Selection and identification of core targets in thymosin-α1 treating COVID-19. (A) 19 candidate biotargets of thymosin-α1 and COVID-19 were identified, respectively. Then a PPI network from these biotargets was illustrated by Cytoscape software, which showed all possible molecular interactions. (B) Eight core biotargets of thymosin-α1 treating COVID-19 were identified according to bioinformatics analysis using network pharmacology, including HLA-A, FURIN, DPP4, CTSL, CTSB, ACE, PTGS2 and HLA-DRB1. COVID-19, Corona Virus Disease 2019; PPI, protein-protein interaction.

and BEP-2D cells were cultured with growth medium without BSA one day before transfection. Then we diluted 20 pmol siRNA oligomer in 50 μL Gibco™ Opti-MEM™ and mix with Lipofectamine 2000 for 6 hours incubation. Culture medium was replaced as the BSA-containing medium and gene knockdown was detected by the assay of western blotting and RT-qPCR.

RT-qPCR. Total RNA was extracted with Trizol (Invitrogen, USA) and cDNA synthesis was performed by using the ReverTra Ace qPCR RT Kit (Toyobo, Japan). Real-time quantitative PCR was performed using PowerUpTM SYBRTM Green Master Mix kit for specific amplification (Thermo Fisher, USA). Melting curve analysis confirmed the formation of individual desired PCR products. Real-time PCR for each gene was performed in three replicate trials and normalized to GAPDH expression values.

Enzyme linked immunosorbent assay (ELISA). BEAS-2B siACE cells and BEP-2D siACE cells as well as their control siRNA cells (3×10^4) were seeded to incubate overnight for their cell adherence. Then they were all treated with thymosin-α1 (10 μM, 20 μM and 40 μM) and collected after 48 hours of thymosin-α1 treatment. Ang-1 and Ang-2 levels were analyzed by the Quantikine human Ang-1 ELISA kit (R&D Systems, Minneapolis, MN) and the Quantikine human Ang-2 ELISA kit (R&D Systems), respectively. Angiotensin (1–7) and angiotensin (1–9) were detected by Ang 1–7 and Ang 1–9 ELISA kit (CUSABIO Biotech). These ELISA assays were performed as recommended by the respective manufacturer. All experiments were performed in triplicate.

Statistical analysis. Statistical analysis was conducted by GraphPad Prism 8. After checking data for normal distribution and variance homogeneity, continuous data were compared using multiple Student t tests or two-way ANOVA. All p values are two-tailed, and p values < 0.05 are considered significant ($*p < 0.05$, $**p < 0.01$ and $***p < 0.001$). The data are represented as mean $\pm$ S.E.M. or the median with 10 and 90 percentiles.

3. Results

Biological targets and PPI network of thymosin-α1 treating COVID-19. First of all, a total of 350 COVID-19-associated genes were identified according to GeneCards database (score from 0.24 to 28.74). Meanwhile, 100 functional genes of thymosin-α1 were harvested from Swiss Target Prediction database (probability rate from 6.42% to 14.01%), prior to further screening of 19 shared biological targets of thymosin-α1 and COVID-19 (Fig. 1, left panel). Then these 19 biotargets were used to further plot an optimal STRING PPI network of thymosin-α1 treating COVID-19 (Fig. 1, right panel).

Selection and identification of core targets in thymosin-α1 treating COVID-19. All interceptive targets in Venn diagram were imported into Cytoscape software for detecting and analyzing the topological parameters of anti-COVID-19 targets played by thymosin-α1 and function-related PPI network (Fig. 2A). By calculating the interactions of 19 shared targets, the median degree of freedom of the targets was identified as 3.2, and the maximum degree of freedom was identified as 18.3, followed by the core target screening conditions ranged from 8.1 to 16.7. Herein, a total of eight core targets were obtained involving HLA-A, FURIN, DPP4, CTSL, CTSB, ACE, PTGS2 and HLA-DRB1 (Fig. 2B).

Revelation of biological functions and pathways from the above 8 core targets. All essential KEGG pathways of the above 8 core targets were obtained through the DAVID database and Cytoscape software to draw a core target-related pathway interaction network (Fig. 3). As results, the top 20 KEGG pathways (minor overlap is 5 and p value cut-off is 0.01) were related to hepatitis B, influenza A Chagas disease (American trypanosomiasis), apoptosis, cytokine-cytokine receptor interaction, Epstein Barr virus infection, JAK-STAT signaling pathway, amoebiasis, NF-kappa B signaling pathway, chemokine signaling pathway amyotrophic lateral sclerosis (ALS), apoptosis-multiple species, prion diseases, allograft rejection, epithelial cell signaling in helicobacter pylori infection, TGF-beta signaling pathway, longevity regulating pathway, transcriptional misregulation in cancer, microRNAs in cancer, Alzheimer's disease (Fig. 4A). Furthermore, DAVID database analysis also showed that pivotal biological processes of these core targets were involved in negative regulation of pathways in chondroitin sulfate degradation, MAPK signaling pathway, inflammatory mediator regulation of TRP channels, pertussis, signaling pathways regulating renin-angiotensin system (RAS), pluripotency of stem cells, p53 signaling pathway, oxytocin signaling pathway, complement and coagulation cascades, steroid hormone biosynthesis, arachidonic acid metabolism and negative regulation of phospholipase D signaling pathway (Fig. 4B).

Study of binding kinetics between thymosin-α1 and ACE. It was reported that thymosin-α1 binds to N-domain of ACE with the binding energy of –22.87 kcal/mol by molecular docking, which clarified the potential mechanism of thymosin-α1 treatment in antioxidant and anti-hypertensive effect [31]. In this study, the binding kinetics between thymosin-α1 and ACE were investigated by the assay of surface plasmon resonance (SPR), and the changes in refractive index on a chip coated with the protein were measured to evaluate ligand binding (Fig. 5A). The dissociation constants (K_D) in the low nanomolar range were obtained for thymosin-α1 upon titrating over immobilized ACE (K_D value was 17.4 nM). The slow dissociation rates of inhibitors from the target protein are considered to be beneficial for drug efficacy and selectivity *in vivo* due to the high concentration of the drug near the target, such as EGFR inhibitor lapatinib [32,33]. The remarkably slow K_{off} measured for thymosin-α1 (1.01×10^{-3} s^{-1}), corresponds to a residence time of 16.7 min and denotes its slow

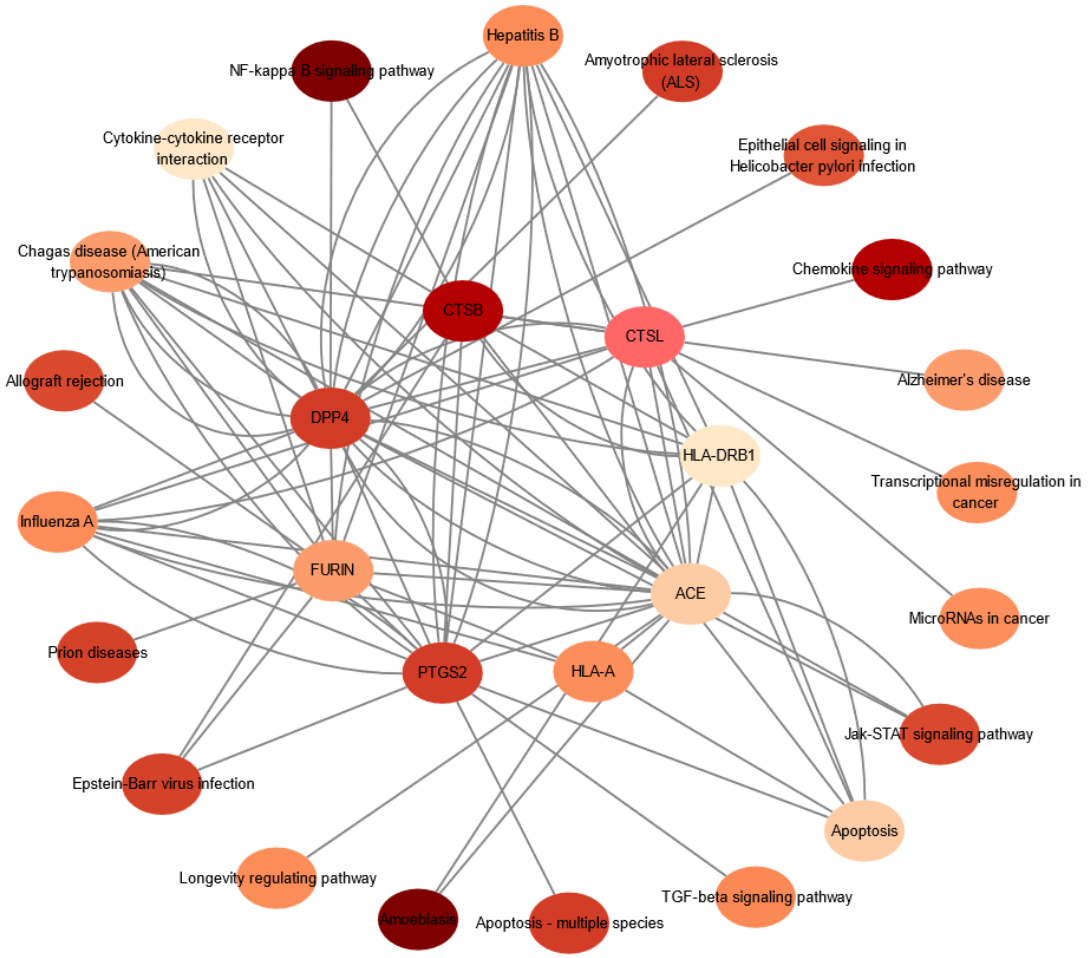

Fig. 3. Cluster assays showed a PPI network from the core targets connected with top 20 activated signaling pathways in thymosin-α1 treating COVID-19. COVID-19, Corona Virus Disease 2019; PPI, protein-protein interaction.

dissociation rate from ACE (Fig. 5A). Then we performed competition SPR assay traces of SARS-CoV-2 spike glycoprotein between ACE2 and thymosin-α1. SARS-CoV-2 spike glycoprotein was fixed on the chip and then dipped into wells containing ACE2 purified protein alone or with doses of thymosin-α1 (50 nM, 100 nM and 200 nM). It showed that increased doses of thymosin-α1 could impair the binding between SARS-CoV-2 spike glycoprotein and ACE2 (Fig. 5B). Subsequently, purified ACE protein and ACE2 protein were treated with doses of thymosin-α1 (10 μM, 20 μM and 40 μM). Their enzymatic activities were analyzed as the Relative Fluorescence Unit (RFU) of the fluorometric substrates (o-aminobenzoyl peptide for ACE and Mca-AlaPro-Lys (Dnp)-OH for ACE2). It showed that increased doses of thymosin-α1 directly suppressed the enzymatic activity of ACE but not ACE2 (Fig. 5C).

Inhibition on ACE2 expressions of human lung epithelial cells by thymosin-α1. In order to characterize the anti-COVID-19 actions of thymosin-α1 based on the above network pharmacological findings, we performed western blotting assay to assess the effects of increased doses of thymosin-α1 (10 μM, 20 μM and 40 μM) on ACE and ACE2 expressions of human lung epithelial cells (BEAS-2B cells and BEP-2D cells), respectively. Interestingly, thymosin-α1 rendered both BEAS-2B cells and BEP-2D cells with significantly downregulated ACE2 expressions in a dose-dependent way ($p < 0.001$), but not for their ACE expressions (Fig. 5D). Then mRNA levels of ACE and ACE2 were detected by RT-qPCR in BEAS-2B cells and BEP-2D cells, which also demonstrated the decreased ACE2 and floating ACE transcription levels after the treatment of thymosin-α1 (10 μM, 20 μM and 40 μM) (Fig. 5E).

Thymosin-α1 significantly affected the synthesis of angiotensin (1–7) in human lung epithelial cells. The synthesis of inactive angiotensin (1–9) from angiotensin I (Ang I) and the catabolism of angiotensin II (Ang II) to produce angiotensin (1–7) represent the main functions of ACE2. To further confirm the effect of decreased ACE2 expression by thymosin-α1, we thus analyzed the synthesis of angiotensin (1–7) and angiotensin (1–9) induced by doses of thymosin-α1 (10 μM, 20 μM and 40 μM) in BEAS-2B^{siACE} cells and BEP-2D^{siACE} cells. The efficiency of ACE knockdown was detected by western blotting and RT-qPCR, respectively (Fig. 6A). Then ELISA assay re-

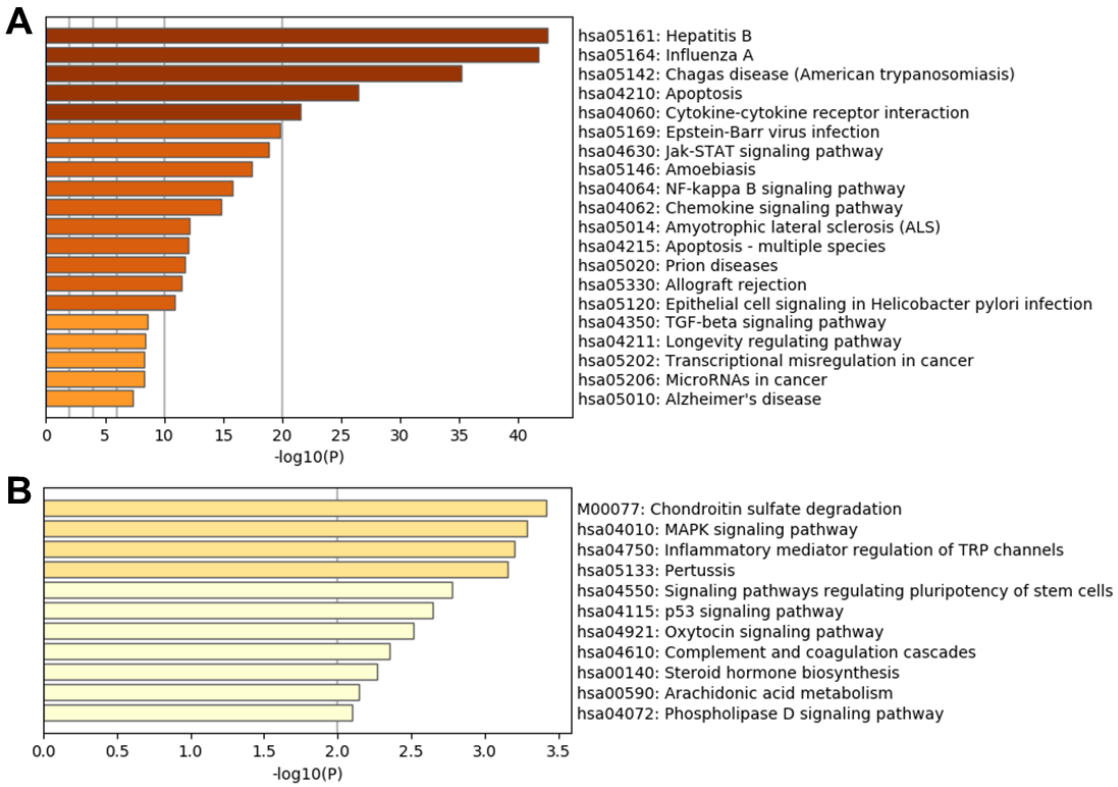

Fig. 4. Enrichment analyses showed the top 20 activated (A) and 11 inactivated (B) functional processes, molecular mechanism of thymosin-α1 treating COVID-19. COVID-19, Corona Virus Disease 2019.

vealed that increased thymosin-α1 could upregulate the expressions of Ang I and angiotensin (1–7), but downregulate the expression of Ang II in both BEAS-2B cells and BEP-2D cells ($p < 0.05$). In contrast, the synthesis of angiotensin (1–9) remained unaffected (Fig. 6B). The expressions of Ang I and angiotensin (1–7) were significantly increased by higher dose of thymosin-α1, while Ang II decreased with higher dose of thymosin-α1 and Angiotensin (1–9) was not obviously changed. But for BEAS-2B^{siACE} cells and BEP-2D^{siACE} cells, increased levels of Ang I and angiotensin (1–7) were significantly impaired, indicating that thymosin-α1-modulated angiotensinogen-renin system was mainly attributed to ACE (Fig. 6B). Ang (1–7) is mostly synthesized from Ang I by three known enzymes, including neprilysin (NEP), thimet oligopeptidase (TOP) and prolyl oligopeptidase (POP). ACE2 also mediates the conversion of Ang II into the enzymatic product Ang (1–7). Herein, as shown in Fig. 5C, the enzymatic activity of ACE was significantly inhibited by thymosin-α1, even more remarkable than ACE2. Increased Ang I thus accumulated to be converted into Ang (1–7), leading to increased expression of Ang (1–7) (Fig. 7).

4. Discussion

It is well known that thymosin-α1 enhances the immune response by restoration of lymphocytopenia and reversion of exhausted T cells, but we do not know whether thymosin-α1 could affect the targets of SARS-CoV-2 infection. In this study, we provided novel insights into the mechanism of thymosin-α1 in preventing COVID-19. The major discoveries mainly include: (1) HLA-A, FURIN, DPP4, CTSL, CTSB, ACE, PTGS2 and HLA-DRB1 are identified as the core targets in thymosin-α1 treating COVID-19; (2) Thymosin-α1 could strongly bind with ACE in the low nanomolar range; (3) Treatment of thymosin-α1 effectively downregulates ACE2 expression, which impairs the synthesis of angiotensin (1–7) in human lung epithelial cells. In essence, this study indicates thymosin-α1 treatment could decrease ACE2 expression in human lung epithelial cells, which implicates that thymosin-α1 might be the potential therapeutic regimen to prevent COVID-19.

Angiotensin-converting enzyme-2 (ACE2) has been reckoned as the functional host receptor for COVID-19 [34,35], which is widely expressed in a variety of human organs [36,37]. Physiologically, ACE2 is a pivotal counter-regulatory enzyme to ACE by breaking down angiotensin II, functioning as the core of the renin-angiotensin-aldosterone system [38,39]. It is concerned that ACE inhibitors could theoretically upregulate ACE2 expression in the lungs, increasing the risk of acquiring SARS-CoV-2 infection. However, no data till now has demonstrated that ACE inhibitors could definitively increase lung ACE2 expression in animals or humans. Sim-

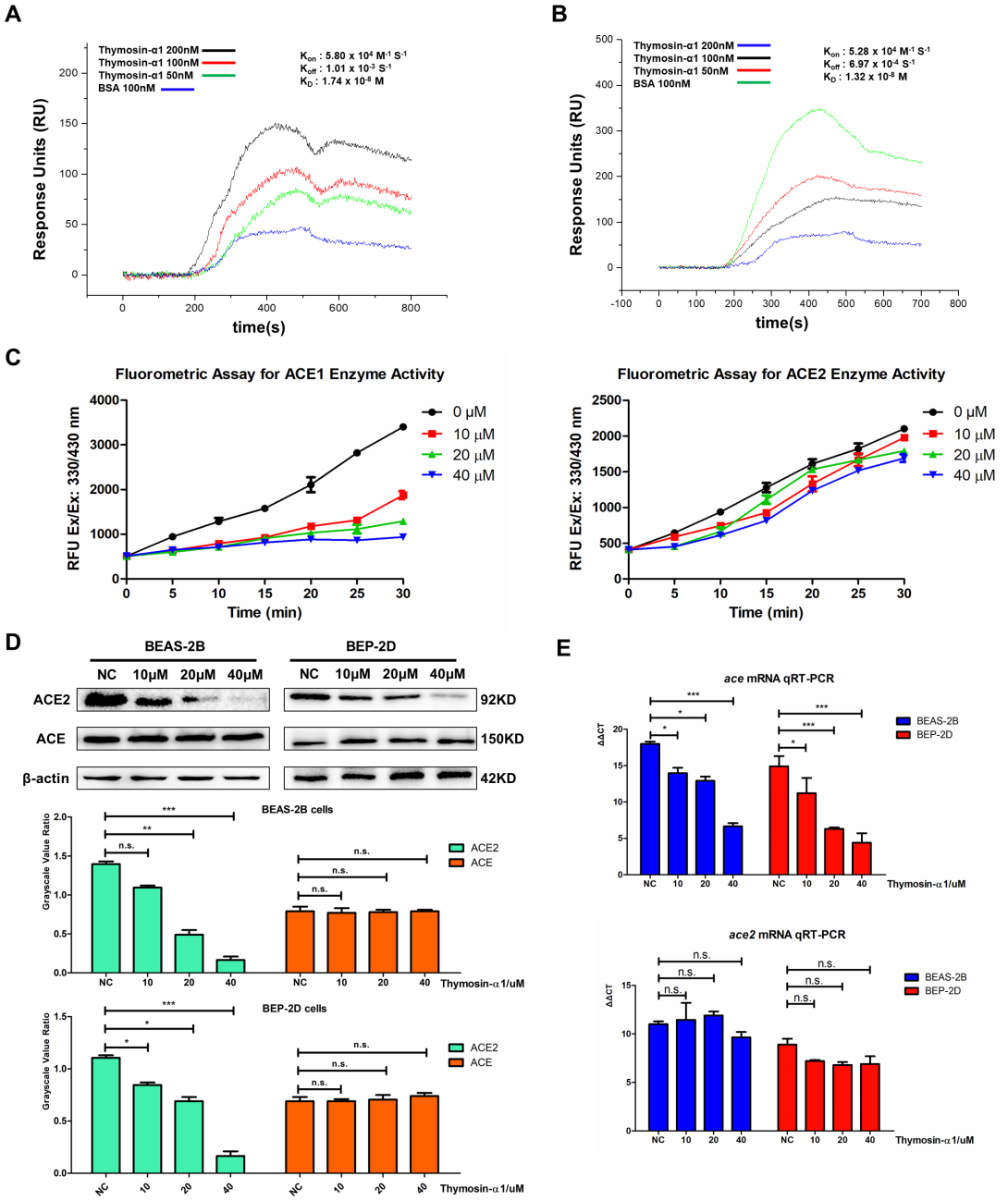

Fig. 5. Thymosin-α1 downregulates ACE2 expressions of human lung epithelial cells by binding with ACE. (A) Surface plasmon resonance (SPR) analysis between thymosin-α1 and ACE. The 2-fold serial dilution of thymosin-α1 was made starting from 50 nM in duplicate. Bull serum albumin (BSA) was used as the negative control. (B) Competition SPR assay traces. SARS-CoV-2 spike glycoprotein was fixed on the chip and then dipped into wells containing ACE2 purified protein alone or with doses of thymosin-α1. The 2-fold serial dilution of thymosin-α1 was also made starting from 50 nM in duplicate. Bull serum albumin (BSA) was used as the negative control. (C) Kinetic activity curves of ACE and ACE2 using different doses of thymosin-α1 (10 μM, 20 μM and 40 μM) for 30 min. Enzymatic activities of purified ACE protein and ACE2 protein were determined by fluorometric substrates assay, respectively. Fluorescence (Ex/Em = 320/420 nm) of each point was measured by the standard curve. (D) Treated with increased doses of thymosin-α1 (10 μM, 20 μM and 40 μM) for 48 hours, the expressions of ACE and ACE2 of BEAS-2B cells and BEP-2D cells were determined by western blotting assay, respectively. The ratios of their corresponding grayscale values to β-actin were statistically illuminated in the bottom panels. (E) Treated with increased doses of thymosin-α1 (10 μM, 20 μM and 40 μM) for 48 hours, mRNA levels of ACE and ACE2 in BEAS-2B cells and BEP-2D cells were determined by RT-qPCR, respectively. $\Delta\Delta CT = (CT_{target\ gene} - CT_{housekeeping\ gene})_{treatment} - (CT_{target\ gene} - CT_{housekeeping\ gene})_{nontreatment}$. Data are presented as mean $\pm$ SEM. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ (Student's t-test).

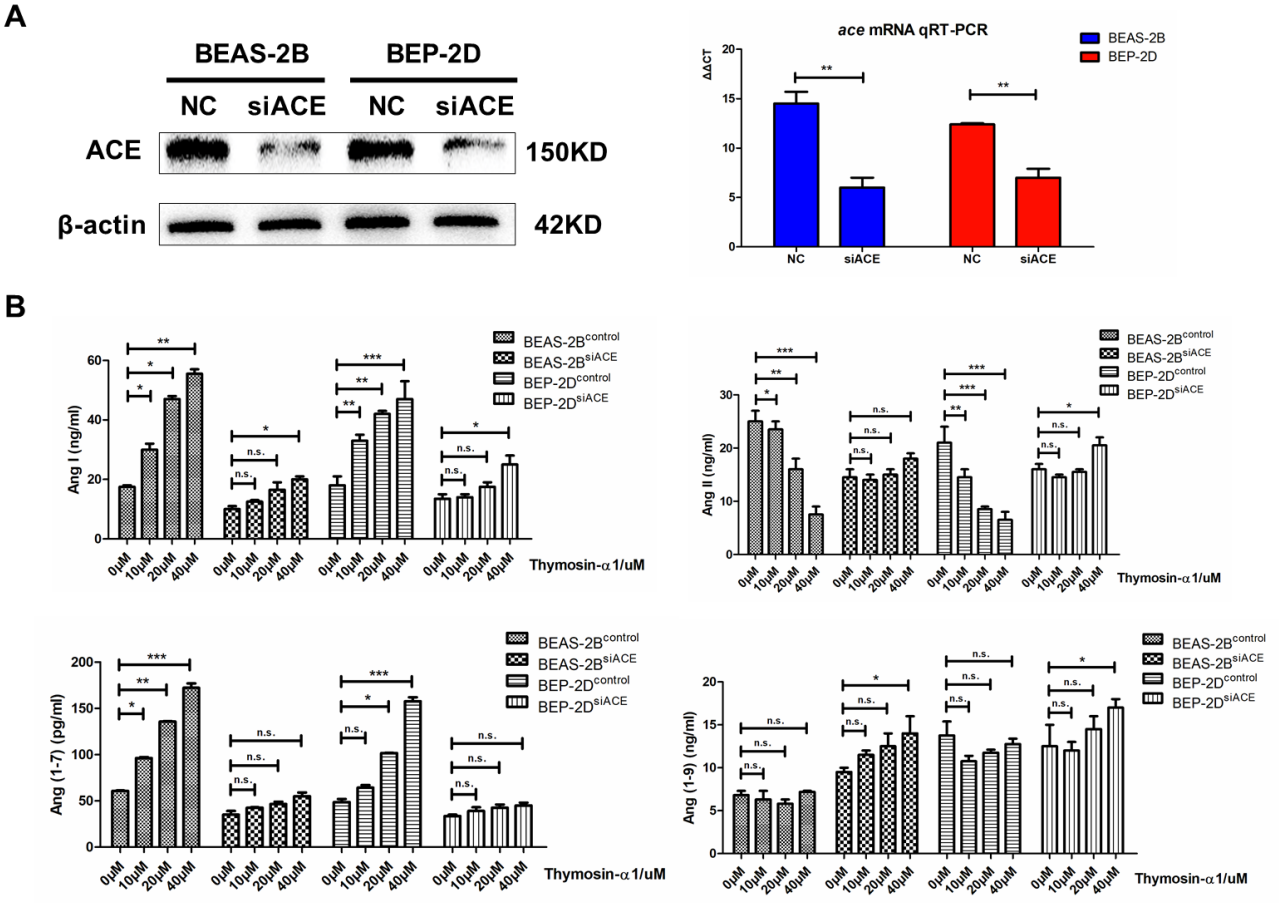

Fig. 6. Thymosin-α1 significantly affected the synthesis of angiotensin (1–7) in human lung epithelial cells. (A) The efficiency of ACE knockdown was detected by western blotting (left panel) and RT-qPCR (right panel). (B) ELISA assay was used to determine Ang I, Ang II, Ang (1–7) and Ang (1–9) of BEAS-2B^{siACE} cells and BEP-2D^{siACE} cells as well as their control cells under the exposure of thymosin-α1 (10 μM, 20 μM and 40 μM) for 48 hours, respectively. Experiments were performed in triplicate and are expressed as mean $\pm$ SEM. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ (Student's *t*-test).

ilarly, downstream effects of these agents hardly increase viral infectivity or virulence. Captopril, the known ACE inhibitor, was reported to downregulate ACE2 expression in osteoporotic rats. Thus, captopril was clinically identified to activate ACE2-dependent Mas receptor signaling in restoring bone metabolism [40]. For cases of type II diabetic patients, renal ACE2 were transcriptionally decreased after Losartan treatment [41]. Administration of pioglitazone along with ACEi Enalapril were performed in the SARS-CoV-2 case-control study, which also indicates to block the overexpression of ACE2 [42]. In this study, we showed that thymosin-α1 could downregulate ACE2 expression but negligibly affect ACE expression in human lung epithelial cells (BEAS-2B cells and BEP-2D cells), which further indicated that ACE and ACE2 expressions seem not to be inherently connected.

SARS-CoV-2 infection cycle includes 5 steps: virus entry by endosomes or plasma membrane fusion, translation of viral replication machinery, replication, translation of viral structure proteins and virion assembly. For the entry of SARS-CoV2, transmembrane protease serine 2 (TMPRSS2) and Paired basic Amino acid Cleaving Enzyme (PACE or FURIN) are also important to the proteolytic activation of SARS-CoV-2 [43], which has been enriched in 8 potential targets of thymosin-α1 (Fig. 1). Obviously, this study manifests that thymosin-α1 is mainly implicated into the first step, virus entry by ACE2-mediated plasma membrane fusion. However, both ACE and ACE2 exist as the membrane-bound and soluble form, with the former one predominately distributed [44]. In this study, targets prediction and *in vitro* verification of this study is limited to focus on the expression of membrane-bound ACE2 in human respiratory epithelia. *In vivo* study could further help to clarify whether thymosin-α1 affected soluble ACE and ACE2 expressions in the blood. Moreover, this study is lack of competing relationship between thymosin-α1 and SARS-CoV-2 spike in the infected models, with the condition of laboratory biosecurity. In addition, thymosin-α1 was owned with pleiotropic functions interact with SARS-CoV-2, which cannot be simplified by ACE inhibition. Ac-

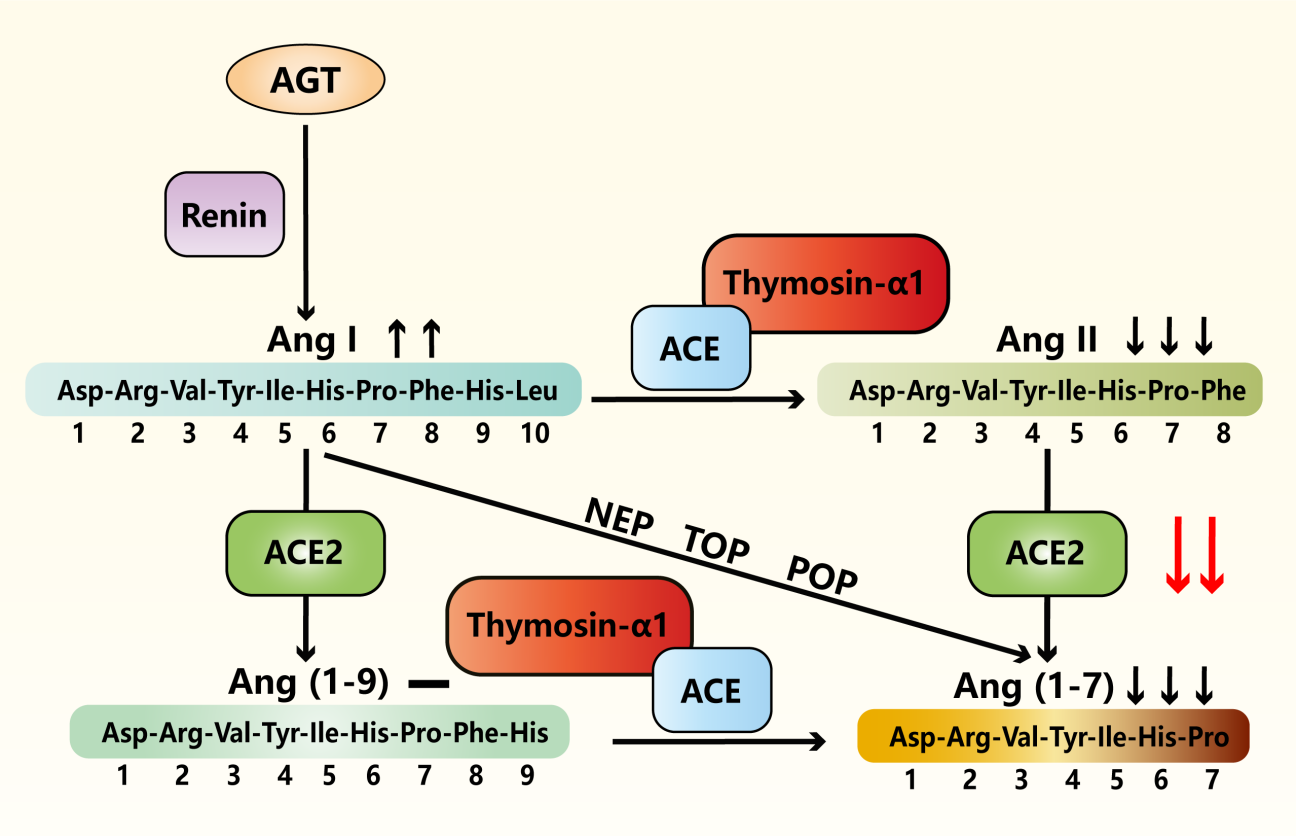

Fig. 7. Schematic representation of Thymosin-α1 modulating renin-angiotensin system. Thymosin-α1 impaired the levels of angiotensin-converting enzyme (ACE2) and angiotensin (1–7) of human lung epithelial cells. In contrast, thymosin-α1 had no impact on their ACE and angiotensin (1–9) expressions, but directly inhibited the enzymatic activity of ACE. Angiotensinogen (AGT) Neprilysin (NEP), Thimet oligopeptidase (TOP) and Prolyl oligopeptidase (POP).

cording to the bioinformatic analysis of 8 core targets, contributions of other modulating factors also deserve further experimental verification such as HLA-DR, the important immune activation factor that was found to be significantly decreased in thymosin-α1-treated COVID-19 patients at peripheral blood [45].

At present, no evidence has proved that patients who are taking ACE inhibitors to treat hypertension, cardiovascular disease, chronic kidney disease are at higher risk of SARS-CoV-2 infection or more severe COVID-19. However, several ongoing clinical trials are implemented, which aimed to inform these decisions by investigating the impact of withdrawal versus continuation of ACE inhibitor medications in patients with COVID-19 (https://www.clinicaltrials.gov; Unique identifier: NCT04338009). In fact, to avoid the serious consequences of stopping ACE inhibitors, many societies of hypertension, cardiovascular and kidney have stated to continually use these important medications of ACE inhibitors until the solid evidence emerges [46].

5. Conclusions

In this study, we suggested that thymosin-α1 impairs ACE2 expressions of human lung epithelial cells by binding with ACE, thus indicating that therapeutic strategy of thymosin-α1 may help to early prevent COVID-19. Ongoing and planned research studies should provide COVID-19 patients with safe ACE2 upstream regulators and further explore the regulatory mechanisms behind ACE and ACE2. Future research into renin-angiotensin-aldosterone system in patients diagnosed with COVID-19, is urgently needed as there remain significant knowledge gaps.

Author contributions

YMC and BZ conceived the project. YHZ, WYW, CZW and HZ performed the experiments of molecular mechanisms. XCP and ZW performed all statistical analysis. YHZ wrote the manuscript, which was edited by all authors.

Ethics approval and consent to participate

Not applicable.

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Conflict of interest

The authors declare no conflict of interest.

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