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SARS coronavirus papain-like protease up-regulates the collagen expression through non-Samd TGF-β1 signaling

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Short title: Up-regulating collagen by SARS-CoV PLpro

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Highlights

- SARS-CoV PLpro induced TGF-β1-dependent up-regulation of Type I collagen in vitro and in vivo.
- Non-SMAD pathways in TGF-β1 signaling involved in PLpro-induced collagen expression.
- STAT6 activation was required for TGF-β1-dependent collagen up-regulation by PLpro.
Abstract

SARS coronavirus (CoV) papain-like protease (PLpro) reportedly induced the production of TGF-β1 through p38 MAPK/STAT3-mediated Egr-1-dependent activation (Sci. Rep. 6, 25754). This study investigated the correlation of PLpro-induced TGF-β1 with the expression of Type I collagen in human lung epithelial cells and mouse pulmonary tissues. Specific inhibitors for TGF-βRI, p38 MAPK, MEK, and STAT3 proved that SARS-CoV PLpro induced TGF-β1-dependent up-regulation of Type I collagen in vitro and in vivo. Subcellular localization analysis of SMAD3 and SMAD7 indicated that non-SMAD pathways in TGF-β1 signaling involved in the production of Type I collagen in transfected cells with pSARS-PLpro. Comprehensive analysis of ubiquitin-conjugated proteins using immunoprecipitation and nanoLC-MS/MS indicated that SARS-CoV PLpro caused the change in the ubiquitination profile of Rho GTPase family proteins, in which linked with the increase of Rho-like GTPase family proteins. Moreover, selective inhibitors TGF-βRI and STAT6 (AS1517499) ascertained that STAT6 activation was required for PLpro-induced TGF-β1-dependent up-regulation of Type I collagen in human lung epithelial cells. The results showed that SARS-CoV PLpro stimulated TGF-β1-dependent expression of Type I collagen via activating STAT6 pathway.

Keywords: SARS-CoV, papain-like protease, TGF-β1, collagen, STAT6
1. Introduction

Severe acute respiratory syndrome (SARS)-associated coronavirus (CoV), a member of betacoronaviruses in the Coronaviridae family, is identified as the causative agent for the outbreak of SARS in Asia and other countries in 2002-2003. Like other human coronaviruses (HCoVs) HCoV-229E, HCoV-OC43, HCoV-NL63, HCoV-HKU1, and MERS-CoV (Li and Lin, 2013; Huang et al., 2015), SARS-CoV genome is an approximately 30 kb positive-strand RNA consisting of a 5’ cap, a 3’ poly (A) tract, and 14 open reading frames (ORFs). The largest ORFs ORF1a and ORF1ab encode for the polyprotein replicases 1a and 1ab mainly involving in the SARS-CoV replication, as cleaved in cis and in trans by ORF1a-encoded papain-like protease (PLpro) and 3C-like protease (3CLpro). PLpro, a de-ubiquitinating/de-ISGylating enzyme (Barretto et al., 2005; Ratia et al., 2006), has the antagonistic activities of Type I interferon (IFN) via blocking IRF-3 and ERK1 phosphorylation, preventing the IκBα degradation, and de-ubiquitinating the STING-TRAF3-TBK1 complex (Li et al., 2011; Frieman et al., 2009; Sun et al., 2012). Recently, PLpro shows the inhibitory effect on Toll-like receptor 7 (TLR7) mediated cytokine production through removing Lys63-linked ubiquitin chains of TRAF3 and TRAF6 (Li et al., 2016a).

SARS-CoV up-regulates pro-inflammatory cytokines like IFN-γ, IL-18, TGF-β1,
TNF-α, IL-6, IP-10, MCP-1, MIG, and IL-8 (Huang et al., 2005; He et al., 2006), in which recruits immune responder cells into the lungs, triggers acute respiratory distress syndrome (ARDS), and even causes lung fibrosis in the late phase (Huang et al., 2005; He et al., 2006). Among SARS-CoV proteins, the nucleocapsid induces a Smad3-dependent induction of TGF-β1 expression (Zhao et al., 2008); spike protein stimulates the IL-8 up-regulation in lung cells (Chang et al., 2004); NSP1 provokes the expression of CCL5, CXVL10, and CCL3 (Law et al., 2007); PLpro elevates the production of TGF-β1 and pro-fibrotic markers via ubiquitin proteasome, p38 MAPK, and ERK1/2-mediated signaling (Li et al., 2012). Recently, SARS-CoV PLpro notably initiates ROS/p38 MAPK/STAT3 pathway to activate Egr-1 dependent expression of TSP-1, TGF-β1 and vimentin in vitro and in vivo (Li et al., 2016b). Therefore, PLpro becomes a virulent factor in SARS pathogenesis.

TGF-β1 plays a crucial mediator of tissue fibrosis in lung, skin, liver, heart, and kidney through modulating the expression of pro-fibrotic proteins including type I collagen, fibronectin, α-SMA, and vimentin (Kubiczkova et al., 2012). In the canonical Smad signalling pathway, TGF-β1 interacts with the TGF-β type I receptor, activates receptor-regulated Smads (Smad2 and Smad3) complexed with Smad4, and then translocate to the nucleus to synthesize TGF-β1-induced transcriptional genes like Type I collagen (Leask and Abraham, 2004; Kubiczkova et al., 2012). In
non-canonical TGF-β signaling pathways, TGF-β1 activates MAPKs (Erk, JNK and p38 MAPK), Rho-like GTPases (RhoA, Rac and Cdc42), PI3K/AKT, WNT/β-catenin, or Ca^{2+} signaling cascades in TGF-β1-induced transcriptional response (Zhang, 2009). The activation of p38 MAPK is responsible for TGF-β-induced epithelial-to-mesenchymal transition of mouse mammary gland epithelial cells under a receptor independent of receptor-mediated Smad activation (Yu et al., 2002). The activation of RhoA in TGF-β-induced EMT response is also independent of Smad2 and/or Smad3 (Bhowmick et al., 2001).

Our prior study demonstrated SARS-CoV PLpro triggering the TGF-β1 production in vitro and in vivo that linked with up-regulating the expression of pro-fibrotic proteins (vimentin and glial fibrillary acidic protein) (Li et al., 2016b). This study assesses possible effects and mechanisms of SARS-CoV PLpro-induced TGF-β1 upregulation on the expression of Type I collagen. The induction ability of SARS-CoV PLpro on in vitro and in vivo expression of Type I collagen was characterized. In addition, subcellular localization of Smad3, Smad7, and STAT6 was performed to elucidate the key factors involved in the induction of Type I collagen by PLpro. The relationship between TGF-β upregulation and the mechanism of Type I collagen induction by PLpro was validated by the specific inhibitors of TGF-β receptor kinase, p38 MAPK and STAT6.
2. Materials and methods

2.1 Cell culture and transient transfection

Human alveolar basal epithelial A549 cells grew in Dulbecco’s Modified Eagle’s Medium (HyClone Laboratories) and were transfected with control vector pcDNA3.1/His C (Invitrogen), or pSARS-PLpro containing SARS-CoV PLpro gene, as described in our prior reports (Li et al., 2011; Li et al., 2012; Li et al., 2016a; Li et al., 2016b). In addition, pSARS-PLpro(H273A) that had the alanine substitution for histidine at position 273 by Ala within PLpro gene was constructed using PCR-based site-directed mutagenesis with a mutated primer pair (5’-GGTAAACTATCAGTGTGGCTTACACTCATATAACTGCTAAG-3’ and 5’-CTTAGCAGTTATATGAGTGTAAGCACCACACTGATAGTTACC-3’). A549 cells transiently expressing recombinant PLpro 2 days post transfection was analyzed using Western blotting, real-time RT-PCR, Sirius staining, and immunofluorescent staining assays.

2.2 Western blotting and immunoprecipitation assays

The lysate of transfected cells was performed by Western blotting with primary antibodies including rabbit anti-TGF-β1 (Cell Signaling), anti-E. coli synthesized PLpro mouse serum, anti-phospho STAT6 (Tyr641) (Cell Signaling),
and anti-β-actin mAb (Abcam), and HRP-conjugated secondary antibodies like goat anti-mouse or anti-rabbit IgG. Immune complexes were detected using enhanced chemiluminescent HRP substrate (Millipore).

2.3 Quantification of mRNA expression of type I collagen, TGF-β1 and vimentin using real-time RT-PCR

To measure the expression of type I collagen, TGF-β1, and vimentin in transfected cells, total RNAs extracted from transfected cells 2 days post transfection and mouse lung tissues were analyzed using two-step real time RT-PCR with SYBR Green I, as described in our prior reports (Li et al., 2016b). Primer pairs included (1) 5’-GTTCGTGACCGTGACCTCG-3’ and 5’-TCTTGTCTTTGGGTTTTCAG-3’ for human type I collagen, (2) 5’-GAGCGGAGAGTACTGGATCG-3’ and 5’-TACTCGAACGGAATCCATCG-3’ for mouse type I collagen, (3) 5’-GGCCTTTTCTGCTTTACTGG-3’ and 5’-CCTTGCTGTACTGCTGTCAC-3’ for human TGF-β1, (4) 5’-TCTTCTGAGGCTCGTGTCCAG-3’ and 5’-CAGGGGTGACGAGGAGACCAG-3’ for human vimentin, (5) 5’-CAGAACAGCCTCCAATGG-3’ and 5’-TGCTACGCTCCTCCATTAC-3’ for human Rac1, (6) 5’-AGCCACATCGCTCAGACAC-3’ and 5’-GCCCAATACGACCAA ATCC-3’ for human GAPDH, and (7)
5’-TGAGGCCGCGTGCTGAGTATGTCG-3’ and
5’-CCACAGTCTTTCTGGTGTCGATG-3’ for mouse GAPDH. Specific PCR product was quantified using the ABI Prism 7900HT Sequence Detection System (PE Applied Biosystems). Relative mRNA levels of indicated genes were normalized relative to GAPDH mRNA.

2.4 Sirius stain assays

For the detection of collagen expression, the tissue sections were stained with Sirius red solution for 2h, and then rinsed 10 times with 0.5% glacial acetic acid in PBS. After dehydrating with ethanol, stained sections were mounted on the glass slides, and then examined using light microscopy (Olympus, BX50).

2.5 Mouse model with a chest injection of recombinant plasmids

The mouse model with a direct chest injection was performed as described in our prior report (Li et al., 2016b). Empty vector pcDNA3.1 or recombinant plasmin pSARS-PLpro (50 μg/100 μl) in 3% sucrose/PBS was injected into the right chest of 5 eight-weeks-old BALB/c male mice using a 1-ml syringe with a 28-gage needle every 2 days. After 15 injections, the mice were sacrificed; the lung tissues were fixed, dehydrated, embedded in paraffin, and cut at 4-5 μm thickness using a rotary microtome. For immunohistochemistry (IHC) staining, mouse lung tissues were
performed with anti-\textit{E. coli} synthesized PLpro serum, as described in our previous report (Li et al., 2016b). For H&E staining, sections were stained with hematoxylin for 3 min, eosin for 3 min, dehydrated in ethanol, and then mounted as slides that were examined and photographed using light microscopy (Olympus, BX50). Sirius staining and SYBR Green real time RT-PCR assays were mentioned above.

2.6 \textit{Immunofluorescence staining assay}

For determining the effects of SARS-CoV PLpro on the nuclear translocalization of SMAD3, SMAD7, and STAT6, A549 cells grew on the glass coverslip in 6-well plates were transfected with pSARS-CoV PLpro or pcDNA3.1, and treated with or without 1 \textmu M kartogenin (Sigma). For testing the role of Rac1 in STAT6 signal, the Rac1 mutant plasmid, pMX-IG-Rac1 T17N provided by Dr. Takehito Uruno (Kyushu University, Japan), was co-transfected into cells. After 2-day incubation, cells were fixed with 3.7\% formaldehyde in PBS for 1 h, blocked with 1\% bovine serum albumin in PBS for 1 h, and then incubated with specific primary antibodies against SMAD3, SMAD7, and STAT6 at 4\degree C overnight. Subsequently, cells were reacted with FITC- or AF546-conjugated secondary antibodies in a dark box for 2 h, Finally, cells were stained with 4’,6-diamidino-2-phenylindole (DAPI) for 10 min. After washing with PBS, stained cells were photographed using the
immunofluorescence microscopy (Olympus, BX50).

2.7 Identification of ubiquitin-conjugated proteins nanoLC-MS/MS

The lysates from PLpro-expressing and empty vector cells were reacted with anti-ubiquitin antibodies for 4 h at 4°C, and then incubated with protein A-Sepharose beads. The ubiquitin-conjugated proteins were collected after centrifugation, washed four times with NET buffer, embedded in SDS-PAGE gel, and then digested in gel. The peptides of ubiquitin-conjugated proteins were recovered for NanoLC-MS/MS spectra. Proteins were identified according to mass spectra obtained were compared to SwissPort database (release 51.0) via MASCOT algorithm (version 2.2.07), as described in our prior reports (Li et al., 2012). Peptides were identified if MASCOT individual ion scores exceeded 30.

2.8 Statistical analysis

All data were collected from 3 independent experiments and analyzed using Student’s t-test or χ² test. Statistical significance was considered at p<0.05.

3. Results

3.1 Correlation of SARS-CoV PLpro-induced TGF-β1 production with Type I
collagen up-regulation

To examine the association of SARS-CoV PLpro-induced TGF-β1 production with the collagen up-regulation, A549 lung epithelial cells transiently transfected with pcDNA3.1 and pSARS-PLpro were analyzed the production of TGF-β1 and type I collagen using Western blot, real-time RT-PCR and Sirius red staining assays (Fig. 1). Transfected cells with pSARS-PLpro, but not pcDNA3.1, secreted the active form of TGF-β1, and significantly increased the mRNA and protein expression of Type I collagen. Importantly, SB-431542 (a selective TGF-βRI inhibitor) treatment at 100 nM caused the 4-fold reduction of Type I collagen mRNA in transfected cells with pSARS-PLpro (Fig. 1B). In a mouse model, the expression of Type I collagen in lung tissues from chest injection with pcDNA3.1 or pSARS-PLpro was examined using Sirius staining and quantitative RT-PCR (Fig. 2). The expression of SARS-PLpro in lung tissues of mice was determined using IHC staining with anti-E. coli synthesized PLpro serum, and IHC positivity for PLpro expression within lung tissues was observed in the group infected with pSARS-PLpro (Fig. 2A). H&E and Sirius staining assays indicated that pulmonary inflammation with the infiltration of immune cells and the increase of Type I collagen was identified in the pSARS-PLpro group, but not vector control and solvent groups (Fig. 2A). Real-time PCR confirmed that PLpro triggered the mRNA
expression of Type I collagen in mouse lung tissues in comparison with vector control and solvent groups (Fig. 2B).

To examine the proteolytic enzymatic activity of PLpro on the production of TGF-β1 and type I collagen, the catalytic mutant of PLpro(H273A) was constructed, and then used to investigate whether PLpro(H273A) up-regulated TGF-β1 and type I collagen in vitro (Fig. 3A). Importantly, PLpro(H273A) with the catalytic mutation lose the ability to induce the expression of TGF-β1 and type I collagen in vitro. Since SARS-PLpro had been demonstrated to stimulate p38 MAPK/STAT3-mediated activation of TGF-β1 production (Li et al., 2016b), SB203580 (a specific p38-MAPKs inhibitor), U0126 (a MEK1/2 inhibitor), and Stattic (a small-molecule inhibitor of STAT3 activation) were used to further confirm the correlation between the TGF-β1 production and Type I collagen up-regulation in transfected cells with pSARS-PLpro and pcDNA3.1 (Figs. 3B-3D). SB203580 significantly reduced the mRNA expression of TGF-β1, in which was linked with down-regulation of Type I collagen in transfected cells with pSARS-PLpro in presence of SB203580 or U0126 (Figs. 3B and 3C). Stattic also suppressed the mRNA expression of Type I collagen in transfected cells with pSARS-PLpro (Fig. 3D). Overall, results of the in vitro and in vivo data demonstrated that the proteolytic enzymatic activity was required for SARS-CoV
PLpro-dependent TGF-β1-mediated up-regulation of pro-fibrotic gene Type I collagen.

3.2 Activation of STAT6 was responsible for SARS-CoV PLpro-induced Type I collagen up-regulation

To examine whether SMAD-dependent pathways involve in TGF-β1-mediated up-regulation of Type I collagen in response SARS-CoV PLpro, subcellular localization of receptor-regulated SMAD3 and inhibitory SMAD7 in transfected cells were detected using the immunofluorescent and DAPI staining (Fig. 4). Imaging analysis of transfected cells indicated that SMAD3 localized in the nucleus of pcDNA3.1-transfected cells, but not pSARS-PLpro-transfected cells (Fig. 4A). Moreover, SMAD7 was detected in the nucleus of both transfected cells (Fig. 4B). Interestingly, kartogenin, a stimulator for TGF-β1/Smad3 signal pathway (Wang et al., 2014), was used to verify the inhibitory effect of PLpro on TGF-β1/Smad3 signal in transfected cells (Fig. 4C). After 24 h treatment with kartogenin, Samd3 nuclear translocation was spotted within the nucleus in vector control cells, but not in PLpro-expressing cells. Most Samd3 was in the cytoplasm of pSARS-PLpro transfected cells treated with kartogenin. The result indicated that SARS-CoV PLpro inactivated SMAD-dependent pathways, implying that
Non-SMAD pathways in TGF-β1 signaling for the production of Type I collagen would be initiated by SARS-CoV PLpro.

Non-Smad pathways in TGF-β1 signaling include MAP kinase, Rho-like GTPase, and phosphatidylinositol-3-kinase/AKT pathways (Zhang, 2009). To examine the possible pathways involved in TGF-β1-dependent up-regulation of Type I collagen by SARS-CoV PLpro, the profiles of ubiquitin-conjugated proteins in transfected cells with vector control and pSARS-PLpro were determined using immune-precipitation and nanoLC-MS/MS. Interestingly, several proteins of the Ras GTPase family were identified (Table 1), in which indicated the ubiquitination of these Ras family GTPase proteins was influenced by SARS-CoV PLpro. Fig 5A represented the mass spectrum of Rac1 identified by LC-MS/MS. Rac1, a Rho GTPase, had an increase of the ubiquitination in PLpro-expressing cells compared to vector control cells. Real-time RT-PCR and Western blotting assays revealed the elevation of Rac1 mRNA and protein expression in transfected cells with pSARS-PLpro compared to vector control (Figs. 5B and 5C). Rho GTPases were identified in the regulation of G protein-coupled receptor signaling through activation of Jak/STAT and modulated STAT-dependent gene expression (Pelletier et al., 2003). In addition, STAT-6-dependent collagen production has been demonstrated in human skin fibroblasts and mouse airway fibroblasts in response to
bovine milk and platelet-derived growth factor, respectively (Kippenberger et al., 2015; Lu et al., 2014). For analyzing the correlation between PLpro-induced Rac1 up-regulation and STAT6 activation, co-transfection of pcDNA3.1 or pSARS-PLpro plus the Rac1 mutant plasmid pMX-IG-Rac1 T17N was performed, and the STAT6 activation in co-transfected cells was measured using immunofluorescence staining with anti-STAT6 and FITC-conjugated secondary antibodies. PLpro expression caused the STAT6 expression and induced the translation of STAT6 in the nucleus. Remarkably, the Rac1 mutant slightly affected the STAT6 up-regulation by PLpro, but significantly reduced PLpro-induced STAT6 nuclear translocation (Fig. 5D). Later, the functional activity of STAT6 in TGF-β1-dependent collagen up-regulation was further characterized in vector control and PLpro-expressing cells (Figs. 6 and 7). Nuclear localization and phosphorylation of STAT6 was observed in transfected cells with pSARS-PLpro, but not vector control cells (Figs 6 and 7A). Importantly, SB-431542, a selective TGF-βR inhibitor, significantly reduced the entry of STAT6 in the nucleus of PLpro-expressing cells (Fig. 6). The result indicated that Rho GTPases/STAT6 was responsible for one of non-SMAD pathways in SARS PLpro-induced TGF-β1 signals. Furthermore, a STAT6 inhibitor AS1517499 markedly suppressed TGF-β1-dependent collagen expression in PLpro-expressing cells (Fig. 7). The results demonstrated that STAT6 activation was required for
SARS PLpro-induced TGF-β1-dependent production of Type I collagen.

4. Discussion

SARS-CoV PLpro has been demonstrated to trigger the TGF-β1 production in human promonocytes, human lung epithelial cells, and pulmonary tissues in mouse models (Li et al., 2012; Li et al., 2016b). SARS-CoV PLpro induced the ROS-mediated p38 MAPK and STAT3 activation of Egr-1 expression, in which Egr-1 specifically bound to the TGF-β1 promoter region between -175 to -60, resulting in the increase of TGF-β1 production. This study indicated that SARS-CoV PLpro stimulated the production of Type I collagen in vitro and in vivo (Figs. 1-3). SARS-CoV PLpro-induced collagen deposition in pulmonary tissues was associated with lung inflammation and pulmonary fibrosis in mice injected with pSARS-PLpro (Fig. 3). The specific inhibitor for TGF-β receptor, SB-431542, blocked the up-regulation of Type I collagen in transfected cells with pSARS-PLpro. Moreover, the inhibitors for p38 MAPK and STAT3 that involved in Egr-1-dependent TGF-β1 production significantly reduced the expression of TGF-β1 and Type I collagen. Overall, the results revealed that SARS-CoV PLpro elicited TGF-β1-dependent up-regulation of Type I collagen. TGF-β1-dependent profibrotic response was considered as the crucial character in SARS-CoV
PLpro-induced pathogenesis. An elevated level of pro-inflammatory cytokines including TGF-β1 was detected in SARS-CoV-infected cells in autopsy tissues from died SARS patients (He et al., 2006), in which implied the association of pro-inflammatory cytokines with the severity and mortality of SARS. The increase of serum TGF-β1 concentration during the early phase of SARS could be associated with lung infiltration (Beijing Group of National Research Project for SARS, 2003). Meanwhile, the decrease of serum TGF-β1 concentration markedly lowered in the severity of SARS patients (Zhang et al., 2004).

In canonical pathway, TGF-β1 interacts with type I and II TGF-β receptors and causes the activation of a serine/threonine kinase domain in TGF-β receptors and the recruitment and phosphorylation of SMAD2 and SMAD3. The phosphorylated SMAD2/3 complexed with SMAD4 was trans-localized into nucleus to regulate the target gene expression (Kubiczkova et al., 2012). Subcellular localization analysis demonstrated that SMAD3 was predominant in cytoplasmic, but not in the nucleus in transfected cells with pSARS-PLpro compared to vector control (Fig. 4), revealing that canonical Smad-dependent signaling pathway was not involved in PLpro-induced TGF-β1-dependent up-regulation of Type I collagen. TGF-β also activates non-canonical non-SMAD pathways, including MAPKs (ERK1/ERK2, JNK and p38), PI3K kinases, AKT/PKB, mTOR, and Rho-like GTPase family
proteins (Ras, RhoA, Rac1, and Cdc42) (Zhang, 2009; Kubiczkova et al., 2012). Particularly, activation of RhoA pathway was required for TGF-β-mediated process of epithelial-to-mesenchymal trans-differentiation in a dominant-negative Smad3 cells (Bhowmick et al., 2001). In addition, Rac1 activation promoted TGF-β-dependent collagen expression in mesangial cells (Hubchak, et al., 2009). LC-MS/MS analysis demonstrated that SARS-CoV PLpro influenced the ubiquitination status of Ras-related C3 botulinum toxin substrate 1 (Rac1), Ras GTPase-activating-like protein IQGAP1, Ras-related protein Rab (Rab5C), putative Ras-related protein Rab (Rab1C), and Ras GTPase-activating protein-binding protein (G3BP1) (Table 1). Real-time RT-PCR and Western blotting assays indicated the increased expression of Rac1 in transfected cells with pSARS-PLpro compared to vector control (Fig. 5). Since activation of Jak/STAT pathway in a Rac-dependent manner was identified in response to the agonist of protein-coupled receptors (Pelletier et al., 2003), STAT-dependent signals were investigated in this study. The TGF-βRI inhibitor SB-431542 significantly attenuated the phosphorylation and nuclear localization of STAT6 in transfected cells with pSARS-PLpro (Fig. 6). Meanwhile, the STAT6 inhibitor AS1517499 meaningfully reduced TGF-β1-dependent up-regulation of Type I collagen in PLpro-expressing cells (Fig. 7). The results indicated that STAT6 activation was required for PLpro-induced
TGF-β1-dependent expression of Type I collagen in human lung epithelial cells and mouse lung tissues. The result was accordant with the previous reports in that STAT6-dependent collagen production has been demonstrated in human skin fibroblasts and mouse airway fibroblasts (Kippenberger et al., 2015; Lu et al., 2014). SARS-CoV PLpro affected on the ubiquitination and expression profile of Rho-like GTPase family proteins that could link with the activation of STAT6 signaling in TGF-β1-dependent collagen expression, in which suggested that a new GTPase proteins/STAT6 pathway might play a critical role in TGF-β1-dependent collagen production in PLpro-expressing cells.

In summary, SARS-CoV PLpro induced p38 MAPK/STAT3-mediated TGF-β1-dependent up-regulation of Type I collagen, causing pulmonary pro-fibrotic responses. PLpro diminished the nuclear localization of SMAD3, changed the expression profiling of Rho-like GTPase family proteins, and activated STAT6-mediated TGF-β1-dependent production of Type I collagen. The results let us conclude that SARS-CoV PLpro induced non-SMAD signals including STAT6 activation in TGF-β1-dependent pulmonary pro-fibrotic responses.

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Figure caption

Fig. 1. Analysis of TGF-β1 and type I collagen levels in transiently transfected A549 cells with vector control and pSARS-PLpro. For determining active forms of TGF-β1 in transfected cells, the lysates from transfected cells with pcDNA3.1 or pSARS-PLpro were analyzed using Western blot with anti-TGF-β1 antibodies (A). To detect the mRNA expression of type I collagen, total RNAs extracted from transfected cells treated with or without a TGF-β1 inhibitor (SB-431542) were further examined the mRNA levels of type I collagen using quantitative real-time PCR. The relative fold mRNA levels were normalized by GAPDH mRNA, presented as the relative ratio (B). For verifying the protein levels of type I collagen, transfected cells with serial doses of indicated plasmids were stained using Sirius red staining kit (C). **p value < 0.01 by student’s t-test.

Fig. 2. Immunohistochemistry, H&E, Sirius staining and real-time PCR assays for the lung tissue of mice injected with pSARS-PLpro. After 15-times of chest injection with solvent, control vector or pSARS-CoV PLpro, the mice were sacrificed; the lung tissues were collected, and embedded. The tissue sections were stained using IHC with anti-PLpro sera (left panel), H&E (middle panel), and Sirius (right panel) staining, and were analyzed by light microscopy (A). To detect the mRNA expression of type I collagen, total RNAs extracted from lung tissues were examined the mRNA levels of type I collagen using quantitative real-time PCR. The relative fold mRNA levels were normalized by GAPDH mRNA, presented as the relative ratio (B). **p value < 0.01 by student’s t-test.

Fig. 3. Analysis of TGF-β1 and type I collagen levels in transiently transfected
cells in response to the inhibitors for p38 MAPK and STAT3. To detect the mRNA expression of TGF-β1 or type I collagen, total RNAs extracted from transfected cells treated with or without the Rac1 mutant plasmid (A), an inhibitor for p38 MAPK (SB203580) (C) or STAT3 (Stattic) (D) were examined the mRNA levels of type I collagen using quantitative real-time PCR. The relative fold mRNA levels were normalized by GAPDH mRNA, presented as the relative ratio (A, B). For verifying the protein levels of type I collagen, transfected cells treated with or without an inhibitor for p38 MAPK (SB203580) or MEK (U0126) were stained using Sirius red staining kit (C). ** p value < 0.01 by student’s t-test

**Fig.4. Subcellular localization of SMAD3 and SMAD7 in transfected lung epithelial cells.** For analyzing subcellular location of SMAD3 (A, C) and SMAD7 (B), A549 cells transfected pcDNA3.1 or pSARS-PLpro in the presence (C) or absence (A, B) of 1 µM kartogenin were washed, fixed, and reacted with primary antibodies against SMAD3 and SMA7, followed by FITC- (A, B) or AF546-conjugated (C) secondary antibodies. After staining with DAPI for 10 minutes, imaging was analyzed by immunofluorescent microscopy.

**Fig. 5. Analysis of Rac1 expression in vector control and PLpro-expressing cells.** Representative LC-MS/MS spectra of Rac1 identified from one of up-regulated ubiquitinated proteins in PLpro-expressing cells was shown with the calculated molecular weight (m/z values) along x-axis and relative intensity along y-axis (A). Relative mRNA levels of Rac1 were normalized by GAPDH mRNA (B). Rac1 protein level in transfected cells were detected by Western blotting (C). For analyzing STAT6 nuclear translocation (D), cells was co-transfected pcDNA3.1 or pSARS-PLpro with pMX-IG-Rac1 T17N for 24 h, washed, fixed, reacted with anti-STAT6 antibodies,
followed by FITC-conjugated antibodies. Imaging was taken by immunofluorescent microscopy. **p value < 0.01 compared to the vector control by student’s t-test.

Fig. 6. Nuclear localization of STAT6 in transfected lung epithelial cells in response to SB-431542. For analyzing the effect of SB-431542 on nuclear localization of STAT6, transfected cells were treated with or without SB-431542 for 2 days, washed, fixed, and reacted with primary antibodies against phospho-STAT6, followed by FITC-conjugated secondary antibodies. After staining with DAPI for 10 minutes, imaging was analyzed by immunofluorescent microscopy.

Fig. 7. Analysis of STAT6-mediated PLpro-induced production of Type I collagen. To determine the phosphorylation of STAT6, transfected cells with 0.5, 1, 2, 5 or 10μg of pcDNA3.1 or pPLpro were harvested 2 days post transfection; the cell lysates were examined using Western blotting with anti-phospho-STAT6 (Tyr641) or anti-β actin antibodies (A). For analyzing mRNA levels of Type I collagen (B) and vimentin (C), transfected cells were treated with or without AS1517499 for 4 h, and then their mRNA levels of type I collagen and vimentin were measured by quantitative PCR. Relative mRNA levels were normalized by GAPDH mRNA, presented as relative ratio. **p value < 0.01 by student’s t-test.
Table 1. Increased ubiquitination of small GTPase-related proteins in PLpro-expressing cells identified by immunoprecipitation with anti-ubiquitin antibody followed by LC-MS/MS

<table>
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<th>Protein</th>
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<th>Gene ID</th>
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<th>Matches</th>
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<td>Ras-related C3 botulinum toxin substrate 1</td>
<td>RAC1_HUMAN</td>
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<td>Ras GTPase-activating-like protein IQGAP1</td>
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<td>Ras-related protein Rab</td>
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<td>Putative Ras-related protein Rab</td>
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<td>Ras GTPase-activating protein-binding protein</td>
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<td>14916572</td>
<td>52132</td>
<td>2</td>
<td>0.031842</td>
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</tr>
</tbody>
</table>
A. Vector  pSARS-PLpro

- TGF-β1
- SARS PLpro
- β-actin

B. Relative type I collagen mRNA levels

<table>
<thead>
<tr>
<th></th>
<th>pcDNA3.1</th>
<th>pSARS-PLpro</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 nM SB-431542</td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
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<tr>
<td>100 nM SB-431542</td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
</tr>
</tbody>
</table>

C. Plasmid 0 1 5 10 ug

- pcDNA3.1
- pSARS-PLpro
A. | IHC stain | H&E stain | Sirius stain |
---|---|---|---|
**Solvent**

**pcDNA3.1**

**pSARS-PLpro**

B. 

![Graph showing relative mRNA levels across different treatments]

- **Solvent**
- **pcDNA3.1**
- **pSARS-PLpro**
A. Relative mRNA levels of TGF-β1 and Type I collagen.

B. Relative TGF-β1 mRNA levels.

C. Images showing the expression of pSARS-PLpro compared to pDNA3.1 and Mock.

D. Relative type I collagen mRNA levels with Stattic treatment.
A. SMAD3
pcDNA3.1

pSARS-PLpro

B. SMAD7
pcDNA3.1

pSARS-PLpro

C. SMAD3
pcDNA3.1 + Kartogenin (1µM)

pSARS-PLpro + Kartogenin (1µM)
A. Phospho-STAT6

pcDNA3.1

pSARS-PLpro

B. SB-431542 treatment

pcDNA3.1

pSARS-PLpro
A. 

<table>
<thead>
<tr>
<th>pcDNA3.1</th>
<th>pSARS-PLpro</th>
</tr>
</thead>
<tbody>
<tr>
<td>0µg</td>
<td>0µg</td>
</tr>
<tr>
<td>0.5µg</td>
<td>0.5µg</td>
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<td>1µg</td>
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<td>2µg</td>
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</tr>
<tr>
<td>5µg</td>
<td>5µg</td>
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<tr>
<td>10µg</td>
<td>10µg</td>
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</tbody>
</table>

β-actin

p-STAT6(Tyr641)

B.

![Graph showing relative type I collagen mRNA levels for pcDNA3.1 and pSARS-PLpro in Mock and AS1517499 conditions.]

C.

![Graph showing relative vimentin mRNA levels for pcDNA3.1 and pSARS-PLpro in Mock and AS1517499 conditions.]

**Highlights:**

- Increased relative type I collagen mRNA levels in pSARS-PLpro compared to pcDNA3.1.
- Reduced relative vimentin mRNA levels in AS1517499 compared to Mock.