Long non-coding RNA THRIL regulates *Helicobacter pylori* CagA induced-inflammation by inhibition of NF-kB translocation

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Long non-coding RNA THRIL regulates *Helicobacter pylori* CagA induced-inflammation by inhibition of NF-kB translocation

Directed by Professor Sang Kil Lee

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degree of Master of Medical Science

Na Keum Lee

June 2015

This certifies that the Master's Thesis

of Na Keum Lee is approved.

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The Graduate School

Yonsei University

June 2015

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Na Keum Lee

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ABSTRACT

Long non-coding RNA THRIL regulates *Helicobacter pylori* CagA induced-inflammation by inhibition of NF-kB translocation

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The most obvious cause of gastritis and gastric cancer is known as *Helicobacter pylori (H. pylori)* infection and cytotoxin-associated gene A (CagA). Nevertheless, less is known about what is causing the *H. pylori* optionally gastric cancer. Recently, long non-coding RNA is interestingly emerged to be associated with regulation of the immune response by pathogen. Moreover, the innate immune response-related lncRNAs have demonstrated to regulate pro-inflammatory cytokines by interacting with RNA binding protein. THRIL (TNFα and hnRNPL related immunoregulatory lincRNA) is first reported to regulate lipopolysaccharide induced tumor necrosis factor alpha (TNFα) by interacting with heterogenous nuclear ribonucleoprotein L (hnRNPL).

We investigated the roles of THRIL in *H. pylori* infection. The expression of THRIL was determined by qRT-PCR in normal gastric cell line GES-1 cell and a variety of human cancer cell lines. Down regulation of THRIL was confirmed by processing two different siRNAs. For pathogen infection, *H. pylori* strains 60190 (CagA+), 8822 (CagA-) and ΔCagA (an isogenic mutant of 60190 lacking CagA)

were used. We measured TNFα secretion by *H. pylori* infection using ELISA analysis. We dissected canonical NF-kB pathway by *H. pylori* infection after treatment with siRNAs targeting THRIL.

When compared to gastric normal epithelial cell, expression of THRIL was up-regulated in various human cancer cell lines. 8822 (CagA-) and ΔCagA did not influence the levels of TNFα, IL-8 and THRIL. Only 60190 (CagA+) induced TNFα and IL-8 in GES-1 cell and inversely down-regulated THRIL. Knockdown of hnRNPL also resulted in significant decrease in TNFα and IL-8 induction caused by CagA-positive *H. pylori* without alteration of THRIL. Down regulation of THRIL by siRNA decreased mRNA expression of TNFα and IL-8 in both basal status and CagA-positive *H. pylori* infection. Also double knockdown of THRIL and hnRNPL attenuated TNFα induction by CagA-positive *H. pylori*.

Taken together, THRIL and hnRNPL regulated CagA-induced inflammation by NF-kB dependent way.

Key words: long non-coding RNA, *Helicobacter pylori*, tumor necrosis factor alpha, nuclear factor-kB

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Ⅰ. INTRODUCTION

Helicobacter pylori (H. pylori) cytotoxin-associated gene A (CagA) is an important bacterial pathogen that inducing chronic gastritis, gastroduodenal ulcers and gastric adenocarcinoma.[1,2] In general, the infection of CagA-positive *H. pylori* was implicated through type IV secretion system and the translocated CagA contributes to gastric inflammation according to be recognized by epithelial cells as host signaling molecules, and undergone tyrosine phosphorylation by host src kinases.[3] In addition, *H. pylori* infection is required for induction of proinflammatory cytokines and chemokines including TNFα, IL-1ß, IL-8 and COX-2 in immune response-related diseases. Also, nuclear factor-kB (NF-kB) by *H. pylori* infection plays important role in many molecular mechanisms including immune response-related disease. In recent year, microRNA as a class of non-coding RNAs has interestingly studied to play an important role in TLR signaling by virulence factor and *H. pylori*-induced inflammation.[4]

Recently, non-coding RNAs (ncRNAs) are classified as two subclasses small ncRNAs (<200 nt) and long non-coding RNAs (lncRNAs, >200 nt) based on transcript size. These ncRNAs have expressed

in specific cell type or tissues during development, and are known to be regulated within protein coding gene in *cis*.[5] In particular, lncRNAs can be considered as the major class of RNA derived from genome. To date, lncRNAs have been widely reported to regulate biological processes including genomic imprinting, chromatin remodeling, alternative splicing, cancer and differentiation at the post-transcriptional and transcriptional levels.[6,7] However, the field of lncRNAs is newly emerging in the innate and adaptive immunity as a crucial regulator in immune cells.[5,8] The innate immune response-related lncRNAs have demonstrated to regulate pro-inflammatory cytokines by interacting with RNA binding protein.[9-11]

THRIL (TNFα and hnRNPL related immunoregulatory lincRNA) is first reported to regulate the expression of tumor necrosis factor alpha (TNFα) by interacting with heterogenous nuclear ribonucleoprotein L (hnRNPL), a member of hnRNAs which works on formation, packaging and processing mRNA, in macrophage by Pam3CSK4 stimulation.[11,12] THRIL is sized ~2-2.5 kb in length and located in reverse strand of the BRI3 binding protein (Bri3bp) with overlapping mRNA 3' UTR of protein coding gene. THRIL is diversely expressed in various human tissues, and the expression of THRIL in stomach belongs to high level.[11] This data suggests that THRIL can be associated with gastric-related inflammation or disease.

In *H. pylori*-induced gastric inflammation and adenocarcinoma, the mechanism of lincRNA has not been studied as yet, and is fairly complicated. However, recent studies have demonstrated that lncRNAs could modulate human immune diseases, and these findings suggest they might be considered as therapeutic target.

In our study, we found that the expression of THRIL by CagA-positive *H. pylori* infection was reversely regulated when compared with the expression of TNFα and IL-8 in gastric normal epithelial cell. Also, we investigated that CagA-positive *H. pylori* infection after dysregulation of THRIL could suppress NF-kB translocation into nucleus in GES-1 cell.

Taken together, THRIL regulates TNFα and IL-8 through canonical NF-kB pathway targeting CagA-positive *H. pylori.*

Ⅱ. MATERIALS AND METHODS

1. Cell lines, cell culture and *H. pylori* infection

Human gastric normal epithelial cell line GES-1 was kindly provided by Dr. Nam SW (Catholic University Medical School, Seoul, Korea) and other cell lines were obtained from the Korean Cell Line Bank (KCLB, Seoul National University, Seoul, Korea) and the American Type Culture Collection (ATCC, Rockville, MD, USA). GES-1 was cultured in RPMI-1640 medium (Thermo Scientific, Rockford, IL, USA) supplemented with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin. The GES-1 cell was maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. *H. pylori* strains 8822 (CagA-), 60190 (ATCC 49503, CagA+) and ΔCagA (an isogenic mutant of 60190 lacking CagA) were used as described previously.[13] *H. pylori* strains were cultured on blood agar plates containing 10% horse serum at 37°C in a microaerobic atmosphere using the Campy Container System (BBL, Sparks, MD, USA) as earlier mentioned.[14] The each *H. pylori* strains were infected at multiplicity of infection (MOI) of 500 : 1 at various time points.

2. Small interfering RNA (siRNA) transfection

GES-1 cells were plated in 6-well plates and incubated overnight. The cells were transfected with two siRNAs targeting THRIL, siRNA targeting hnRNPL or negative control siRNA (Invitrogen, Carlsbad, CA, USA) at 50nM using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. The sequences of target siRNAs for THRIL and hnRNPL were listed at Table 1.

Table 1. siRNAs targeting lincRNA THRIL and hnRNPL

Gene	Direction	Sequence (5' to 3')
siTHRIL1	Sense	5'-AAACUUGACUCAAUCUGCCUUUAUU-3'
	Antisense	5'-AAUAAAGGCAGAUUGAGUCAAGUUU-3'
siTHRIL2	Sense	5'-GCACAGAUAAAUUUCUCUUACUGUA-3'
	Antisense	5'-UACAGUAAGAGAAAUUUAUCUGUGC-3'
sihnRNPL	Sense	5'-GUCCAUACCCUUACACUCU-3'
	Antisense	5'-AGAGUGUAAGGGUAUGGAC-3'

3. Total RNA extraction, reverse transcription and quantitative real-time PCR

Total RNA was extracted from GES-1 cells using TRIzol reagent (Invitrogen). cDNA was synthesized using 2.0ug of total RNA through Random Hexamers primers (Applied Biosystems Inc., Carlsbad, CA, USA) and superscript^{TM}Ⅱ reverse transcriptase (Invitrogen) according to manufacturer's instructions. The expression level of THRIL, hnRNPL, NF-kB1 (p50), TNFα and IL-8 was determined by real-time PCR using iQ SYBR Green Supermix (Applied Biosystems) and normalized to GAPDH. The ct value was analyzed by 2-ΔΔct method. The primer sequences for qRT-PCR are shown in Table 2.

Table 2. Primer sequences for qRT-PCR

Gene	Direction	Sequence (5' to 3')
THRIL	Forward	5'-AACTCCTGACCTCAGGTGATCCAT-3'
	Reverse	5'-AAGGGAGTTTCAGAAGGTGTGGCT-3'
TNFalpha	Forward	5'-CAGCCTCTTCTCCTTCCTGAT-3'
	Reverse	5'-GCCAGAGGGCTGATTAGAGA-3'
hnRNPL	Forward	5'-AATGGAGTTCAGGCGATGG-3'
	Reverse	5'-TCACCTTGTCCACTGAGATTG-3'
IL-8	Forward	5'-GCAGCCTTCCTGATTTCTGCAGCTC-3'
	Reverse	5'-ACTTCTCCACAACCCTCTGCACCCA-3'
NF-kB1 (p50)	Forward	5'-CCTCCACAAGGCAGCAAATAG-3'
	Reverse	5'-CTGAGTTTGCGGAAGGATGTCT-3'
GAPDH	Forward	5'-CCGGGAAACTGTGGCGTGATGG-3'
	Reverse	5'-AGGTGGAGGAGTGGGTGTCGCTGTT-3'

4. ELISAs for secreted TNFα

GES-1 cells were transfected with 50nM siRNAs targeting THRIL or siCT in 24-well plates. After 48 hrs, the transfected cells were incubated in serum-free and antibiotics-free RPMI-1640 medium (Thermo Scientific) and infected with *H. pylori* at multiplicity of infection of 500:1 overnight. The culture supernatants were re-plated in Coated Microplate (PeproTech, Rocky Hill, NJ, USA) and incubated following the manufacturer's instructions. TNFα secretion was measured by enzyme-linked immunosorbent assay (ELISA).

5. Western blot

The transfected cells by siTHRILs were infected with *H. pylori* at multiplicity of infection of 500:1 for 1 hr. The cells were harvested and lysed with 1X RIPA buffer (Cell Signaling Technology, Beverly, MA, USA) containing protease inhibitor to extract whole cell lysate. For cytosol/nuclear protein fraction, prepared cells were harvested and lysed with cytosol extraction buffer (10 mM Tris-Cl [pH 8], 60 mM KCl, 1 mM EDTA, 1 mM DTT) and nuclear extraction buffer (20 mM Tris-Cl [pH8], 0.4

M NaCl, 1.5 mM MgCl2, 1.5 mM EDTA, 1 mM DTT) containing protease inhibitor. Extracted the samples were separated by 8–10% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidenedifluoride membrane (GE Healthcare, Piscataway, NJ, USA). Primary antibodies were used: NF-kB p65 (1:200, polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA, USA), IkB-α (1:1000, polyclonal, Cell Signaling), phospho-IkBα (Ser32/36, 1:1000, monoclonal, Cell Signaling), IKKα (1:200, polyclonal, Santa Cruz), IKKß (1:200, polyclonal, Santa Cruz), Phospho-IKKα/ß (Ser176/180, 1:1000, monoclonal, Cell Signaling), CagA (1:200, polyclonal, Santa Cruz), hnRNPL (1:1000, monoclonal, Abcam Inc., Cambridge, MA, USA), Flag (1:1000, monoclonal, Sigma-Aldrich, St Louis, MO, USA), Lamin B (1:200, polyclonal, Santa Cruz), HDAC1 (1:200, polyclonal, Santa Cruz), alpha-Tublin (1:1000, polyclonal, AbFrontier, Seoul, Korea). The membrane was reacted in ECL solution (GenDEPOT, Barker, TX, USA) and exposed to an Image Quant LAS 4000 bio-molecular imager for 10 sec – 6 min.

6. Immunofluorescence

GES-1 cells were plated on glass coverslips in 6-well plates. The cells were transfected with 5onM siTHRILs or siCT for 48 hrs and infected with *H. pylori* at multiplicity of infection of 500:1 for 1 hr. After incubation, the cells were washed with PBS, fixed with 4% paraformaldehyde for 20 min at room temperature and permeabilized in 0.3% Triton-X 100 in PBS for 30 min. The cells were re-washed with PBS and blocked with 5% BSA in PBS for 1 hr. Primary antibody NF-kB p65 (1:300, monoclonal, Cell Signaling) diluted in 2% BSA in PBS was incubated for overnight 4°C. The glass coverslips were washed with PBS and incubated with FITC-conjugated anti Rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) or Cy^{TM}3-conjugated anti Rabbit IgG (Jackson ImmunoResearch Laboratories) for 1 hr in the dark. The coverslips were mounted with DAPI vecta shield (Vector Laboratories, Burlingame, CA, USA). The mounted slides were photographed using a Zeiss LSM 700 confocal microscope (Carl Zeiss, Oberkochen, Germany).

7. Expression vectors and co-immunoprecipitation analysis

The p3x-Flag-empty or p3x-Flag-CagA wt (wild type) plasmid vector was kindly provided by Dr. Yook JI (Yonsei University College of Denstry, Seoul, Korea). For IP of endogenous RNP complex, the cells were lysed with nuclear isolation buffer (1.28 M sucrose, 40 mM Tris-HCl [pH7.5], 20 mM $MgCl_2$, 4% Triton X-100) and resuspended in RIP buffer (150 mM KCl, 25 mM Tris-HCl [pH7.4], 5 mM EDTA, 0.5 mM DTT, 0.5% NP40, 1U/ul RNAase inhibitor, protease inhibitor). The shearing of chromatin was mechanically conducted through a dounce homogenizer with 15-20 strokes. After centrifugation, antibody (2ug) was added to supernatant (600-800ug) and incubated for 2 hrs at 4°C with gentle rotation. After incubation, Magna Chip protein G magnetic beads (20ul) (Merck Millipore, Darmstadt, Germany) was added and incubated for 2 hrs at 4°C with gentle rotation. The pellet was washed with RIP buffer and repeated for a total of three RIP washes. After wash, the samples were eluted in SDS and analyzed on western blot.

Ⅲ. RESULTS

1. Long non-coding RNA THRIL is diversely expressed in various cancer cell lines

Long non-coding RNA THRIL was reported universally expressed in 20 different human tissues.[11] Stomach was the one of the organ that THRIL was highly expressed. We hypothesized that THRIL is likely to be associated with gastritis or *H. pylori* induced-inflammation. Prior to this, we measured expression of THRIL in 8 human cancer cell lines including THP1 monocyte cell (Figure 1). The level of expression of THRIL was higher in THP-1 cell which is a human monocytic cell line. The level of expression of THRIL in gastric cancer cell line including AGS and MKN 28 was comparable to colorectal cancer and breast cancer cell lines. When compared to gastric cancer cells lines, GES-1 cell, nonmalignant gastric epithelial cell, has relatively reduced expression of THRIL. Even though expression level of THRIL in GES-1 cell was lower than in gastric cancer cells, it could consider that being appropriate for study to *H. pylori* induced-inflammation.

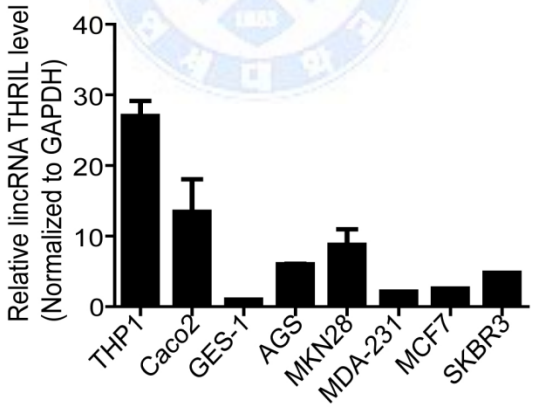


Figure 1. LincRNA THRIL is widely expressed in human cancer cell lines. The expression of lincRNA THRIL was determined by qRT-PCR in various human cancer cell lines. Results are mean ± SD of duplicate wells.

2. Long non-coding RNA THRIL regulates proinflammatory cytokines induced by *H. pylori*

We investigated how THRIL and proinflammatory cytokines were modulated by *H. pylori* infection in GES-1 cell. To induce gastric inflammation, GES-1 cells were infected with *H. pylori* at multiplicity of infection (MOI) of 500 : 1, and observed to 8 hrs.

As we expected, the tumor necrosis factor alpha (TNFα) mRNA was induced by *E. coli* Lipopolysaccharides (*E. coil* LPS) and CagA-positive *H. pylori* 60190 increased mRNA level of from 3 hrs after treatment, but not CagA-negative *H. pylori* 8822 and isogenic mutant of 60190 lacking CagA (Figure 2A). Also, TNFα protein secretion was induced by *E. coil* LPS and CagA-positive *H. pylori* 60190 at 24 hrs after *H. pylori* infection (Figure 2B).

At the same experiment, we measured THRIL simultaneously. The level of THRIL expression was decreased by *E.coli* LPS and CagA-positive *H. pylori* 60190 from 3 hrs after treatment, but not CagA-negative *H. pylori* 8822 and isogenic mutant of 60190 lacking CagA (Figure 2C). The amplitude and duration of THRIL reduction was significantly striking in treatment of CagA-positive *H. pylori* 60190. TNFα and THRIL moved in opposite direction with respect to infection of CagA-positive *H. pylori* 60190. This finding was correlated with previous finding that TNFα and THRIL went toward opposite direction with respect to treatment of *E.coli* LPS.[11] Oppositional change of each TNFα and THRIL was specific phenomenon of CagA.

Furthermore, we carried out kinetics to analyze the relation among lincRNA THRIL, TNFα and IL-8 by CagA-positive *H. pylori* 60190 infection. The decreased expression of lincRNA THRIL was detected in 1 hr after CagA-positive *H. pylori* 60190 infection while inductionof TNFα and IL-8 mRNA was presented in 1 hr and peaked at 8 hrs after treatment (Figure 2D). The modulation of lincRNA THRIL by CagA started as a little faster or stated with induction of with TNFα and IL-8.

Taken together, this finding suggested that lincRNA THRIL modulated TNFα and IL-8 expression by CagA through negative feedback regulation.

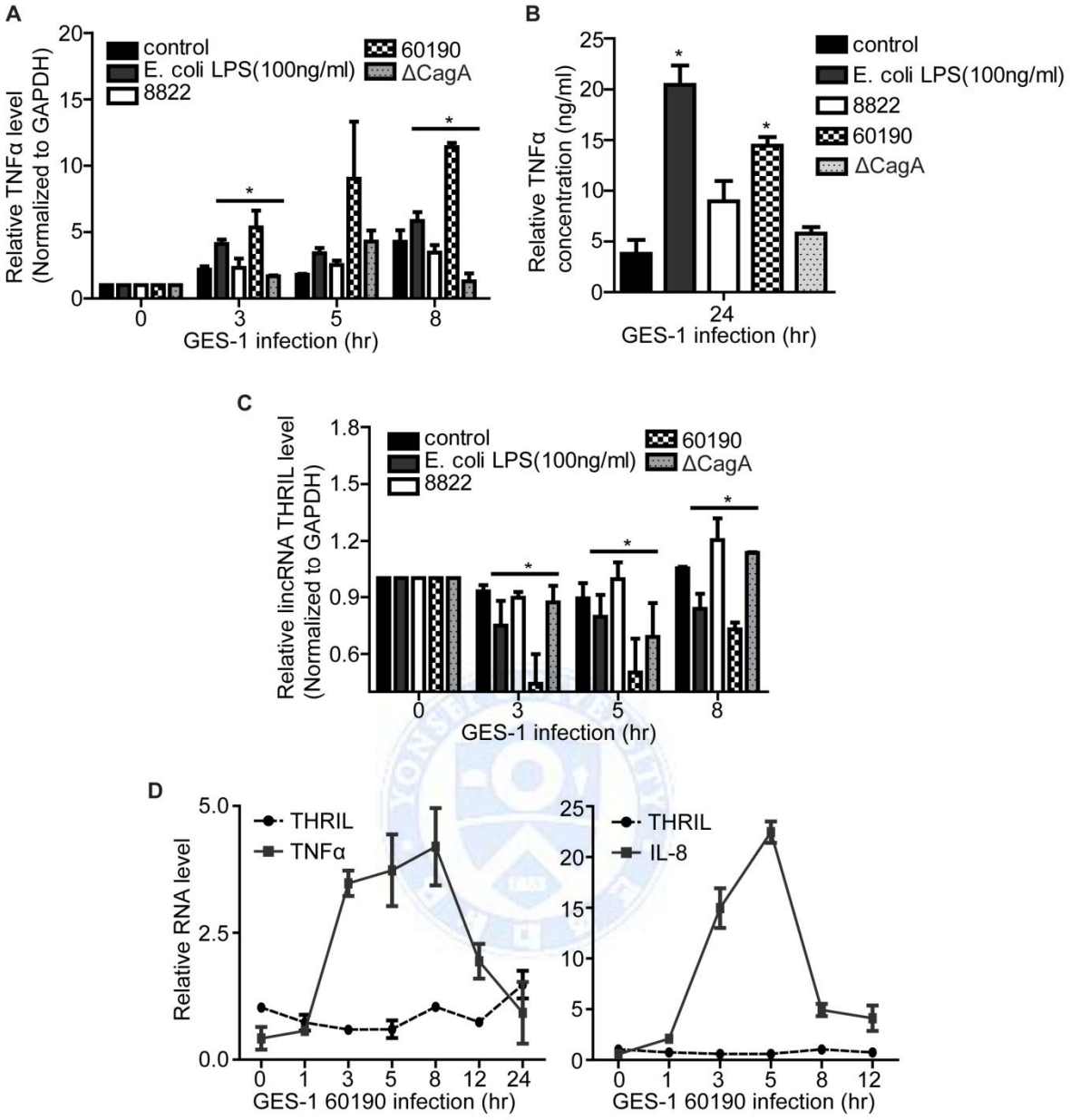


Figure 2. LincRNA THRIL and proinflammatory cytokines are reversely regulated by *H. pylori* infection. In GES-1cell, expression of TNFα (A) and lincRNA THRIL (C) was measured by qRT-PCR after *H. pylori* infection at different time points. Results are mean ± SD of two independent experiments (B) TNFα protein secretion by *H. pylori* infection (D) Kinetics of lincRNA THRIL, TNFα and IL-8 in CagA-positive *H. pylori* 60190 infection. Data are the mean of three independent experiments ± s.e.m. The asterisk represents a statistically significant difference compared with

control (*$P \leq 0.05$).

3. Dysregulation of THRIL induces reduction of TNFα and IL-8 in basal

We next asked whether knockdown of THRIL affects expression of TNFα and IL-8 in both basal and *H. pylori* infection. Li z et al. reported that hnRNPL, which is necessary for Pam-stimulated TNFα induction, specifically binds to lincRNA THRIL and the functional lincRNA THRIL and hnRNPL complex regulates transcription of TNFα during innate immunity of THP1 macrophages.[11] We designed siRNAs targeting THRIL or hnRNPL respectively and transfected GES-1 cell with them for 48 hrs. To check the effect of siRNAs targeting THRIL and hnRNPL, transfected cells were harvested and analyzed by qRT-PCR in basal level.

The two different siRNA (siTHRIL1 and siTHRIL2) for THRIL reduced THRIL expression to 40% and 60% (Figure 3A, left panel). siTHRIL1 and siTHRIL2 reduced basal level of TNFα and IL-8 respectively (Figure 3A, the second and fourth panel from the left), but did not affect the level of expression of hnRNPL (Figure 3A the third panel from the left). hnRNPL is reported to regulate gene expression of TNFα by binding lincRNA THRIL and play dual roles in the cytosol and nucleus.[11]

In our results, sihnRNPL relatively inhibited TNFα and IL-8 mRNA level in basal in common with result by siTHRIL (Figure 3B, the second and fourth panel from left). However, sihnRNPL also did not affect expression of THRIL (Figure 3B, the first panel from the left). In addition, siTHRIL2 of siRNAs targeting THRIL efficiently activated in reduction of mRNA level and it was shown a synergistic effect by activating with sihnRNPL (Figure 3A and C).

To demonstrate the mechanism by lincRNA THRIL and hnRNPL complex, we performed double knockdown of lincRNA THRIL and hnRNPL in basal. The TNFα and IL-8 mRNA level was significantly decreased by double knockdown of lincRNA THRIL and hnRNPL in basal (Figure 3C). These findings indicated lincRNA THRIL and hnRNPL complex plays a crucial role to maintain basal transcription of TNFα in basal.

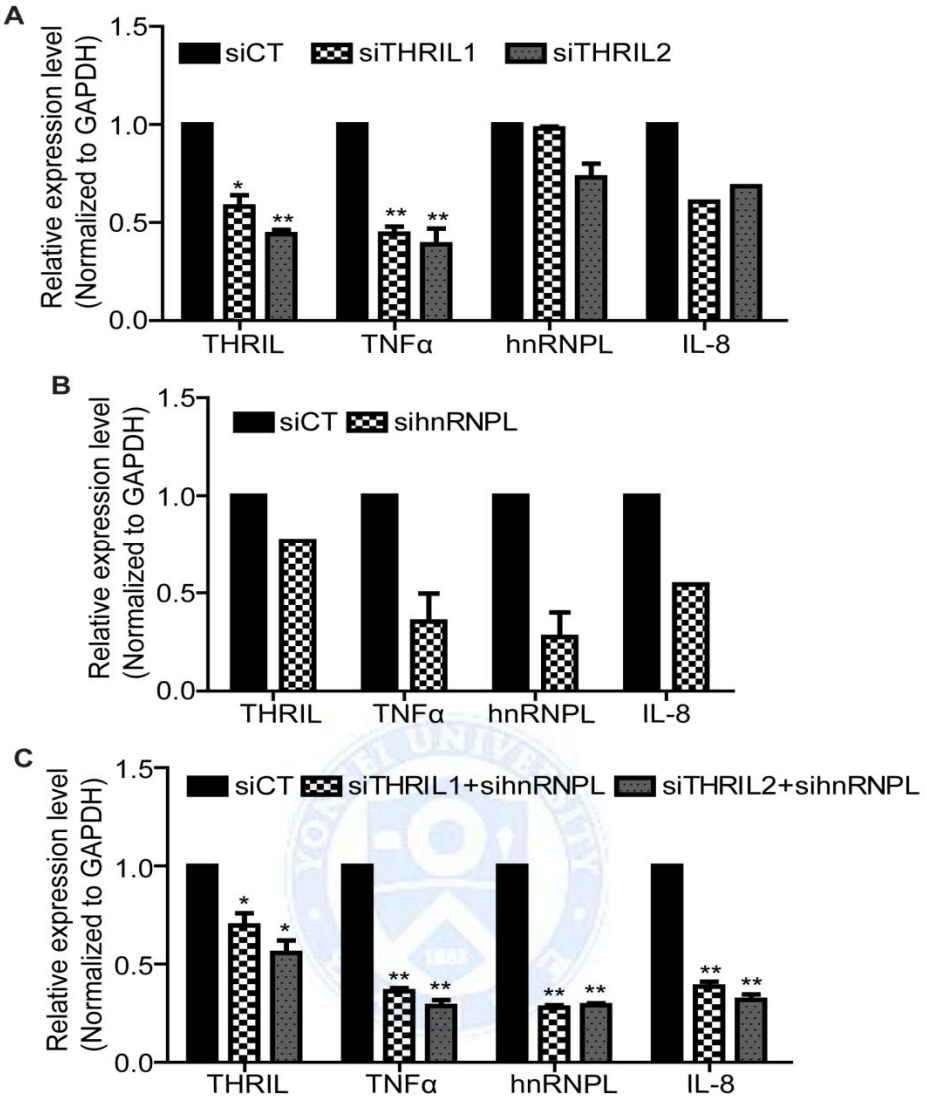


Figure 3. Knockdown of lincRNA THRIL and hnRNPL induces reduction of TNFα and IL-8 mRNA level in basal. (A) Knockdown of lincRNA THRIL (B) Knockdown of hnRNPL (C) Knockdown of lincRNA THRIL and hnRNPL. Data are the mean of three independent experiments ± s.e.m. The asterisk represents a statistically significant difference compared with scrambled control (*$P \leq 0.05$; **$P \leq 0.01$).

4. Dysregulation of THRIL inhibits mRNA and protein expression of TNFα and IL-8 induced by CagA-positive *H. pylori* 60190

We next asked whether knockdown of lincRNA THRIL affects *H. pylori* infection inducing TNFα and IL-8. If lincRNA THRIL could regulate proinflammatory cytokines by *H. pylori* infection, it thought to be a novel biomarker for diagnosis of *H. pylori* infection. First, we confirmed that down regulation by siTHRILs in *H. pylori* infection reduced the expression lincRNA THRIL (Data not shown). Pretreatment of siRNAs for THRIL significantly attenuated induction of TNFα and IL-8 mRNA by CagA-positive *H. pylori* 60190 compared to siCT (Figure 4A and B) respectively.

Moreover, TNFα protein secretion was also concentrated in supernatant by *H. pylori* infection for 24 hrs after lincRNA THRIL silencing. When compared with none-stimulated control, TNFα secretion concentration was respectively increased approximately 4-8 times in CagA-positive *H. pylori* 60190 stimulated-siCT (Figure 4C and D). On the other hand, siTHRILs significantly decreased TNFα secretion in CagA-positive *H. pylori* 60190 compared to siCT (Figure 4C).

In addition, double knockdown of siTHRIL and sihnRNPL were significantly shown decreased TNFα secretion in CagA-positive *H. pylori* 60190 compared to siCT, but did not affect ΔCagA (an isogenic mutant of 60190 lacking CagA) (Figure 4D). These results suggest that lincRNA THRIL and hnRNPL could modulate TNFα as proinflammatory cytokine by CagA-dependent activation.

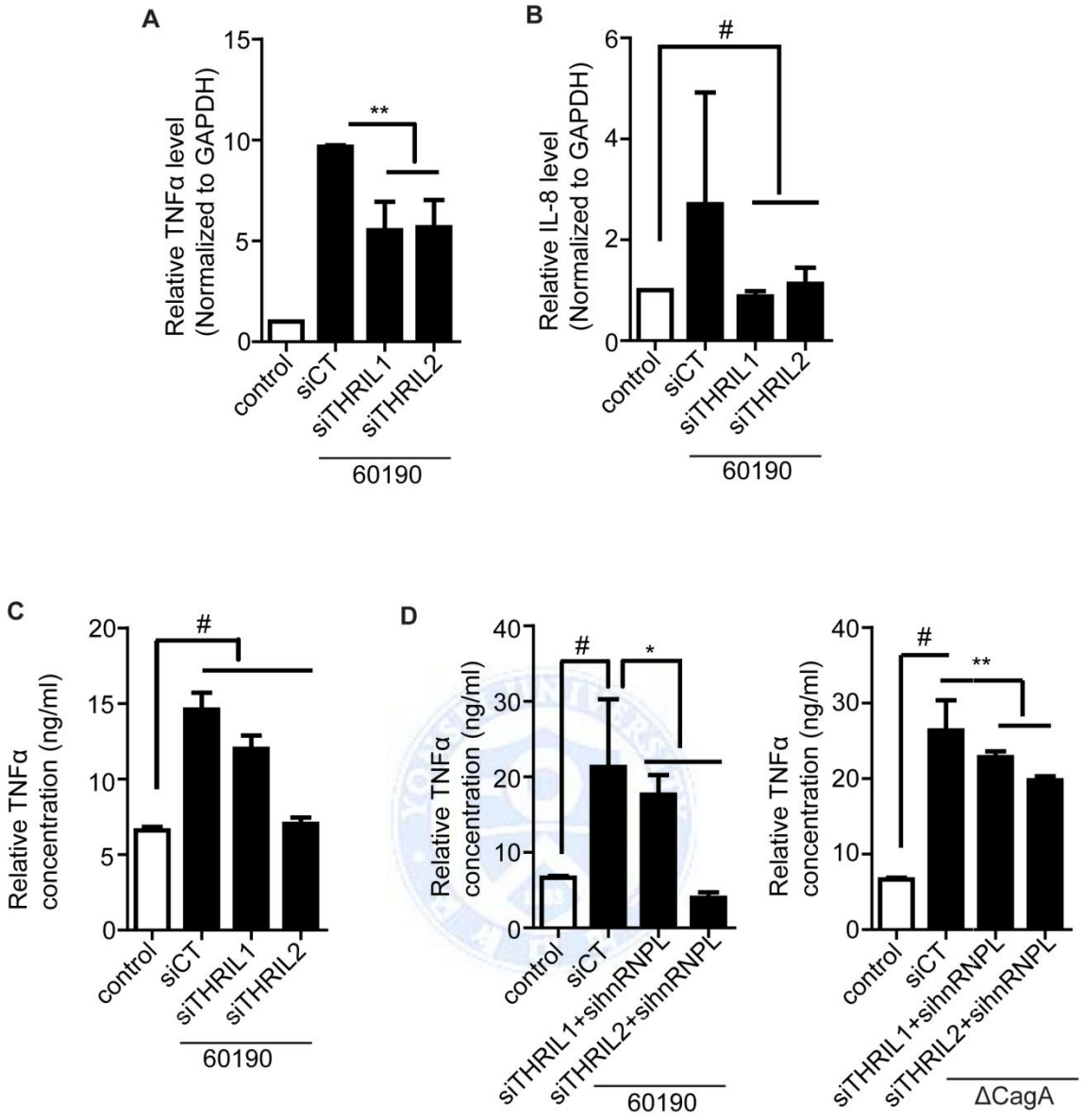


Figure 4. Knockdown of lincRNA THRIL inhibits TNFα mRNA and protein secretion by *H. pylori* infection. (A and B) TNFα and IL-8 mRNA level by CagA-positive *H. pylori* 60190 infection after knockdown of lincRNA THRIL. Results are mean ± SD of two independent experiments (#$P \leq 0.05$ compared with control or **$P \leq 0.01$compared with siCT) (C) TNFα protein secretion by CagA-positive *H. pylori* 60190 infection after knockdown of lincRNA THRIL (#$P \leq 0.05$ compared with control) (D) TNFα protein secretion by CagA-positive *H. pylori* 60190 and ΔCagA (an isogenic

mutant of 60190 lacking CagA) infection after double knockdown of lincRNA THRIL and hnRNPL. Data are the mean of three independent experiments ± s.e.m. The asterisk represents a statistically significant difference compared with scrambled control (*$P \leq 0.05$; **$P \leq 0.01$; #$P \leq 0.05$ compared with control)

5. Down regulation of THRIL inhibits NF-kB translocation by CagA-dependent activation through canonical NF-kB pathway

NF-kB plays a critical role to regulate many proinflammatory cytokines including TNFα and IL-8, and is required for Akt-mediated phosphorylation of p65.[1,15] Based on our results, we investigated how lincRNA THRIL by CagA-positive *H. pylori* 60190 infection regulates TNFα and IL-8, and analyzed NF-kB activation as the upstream signaling pathway.

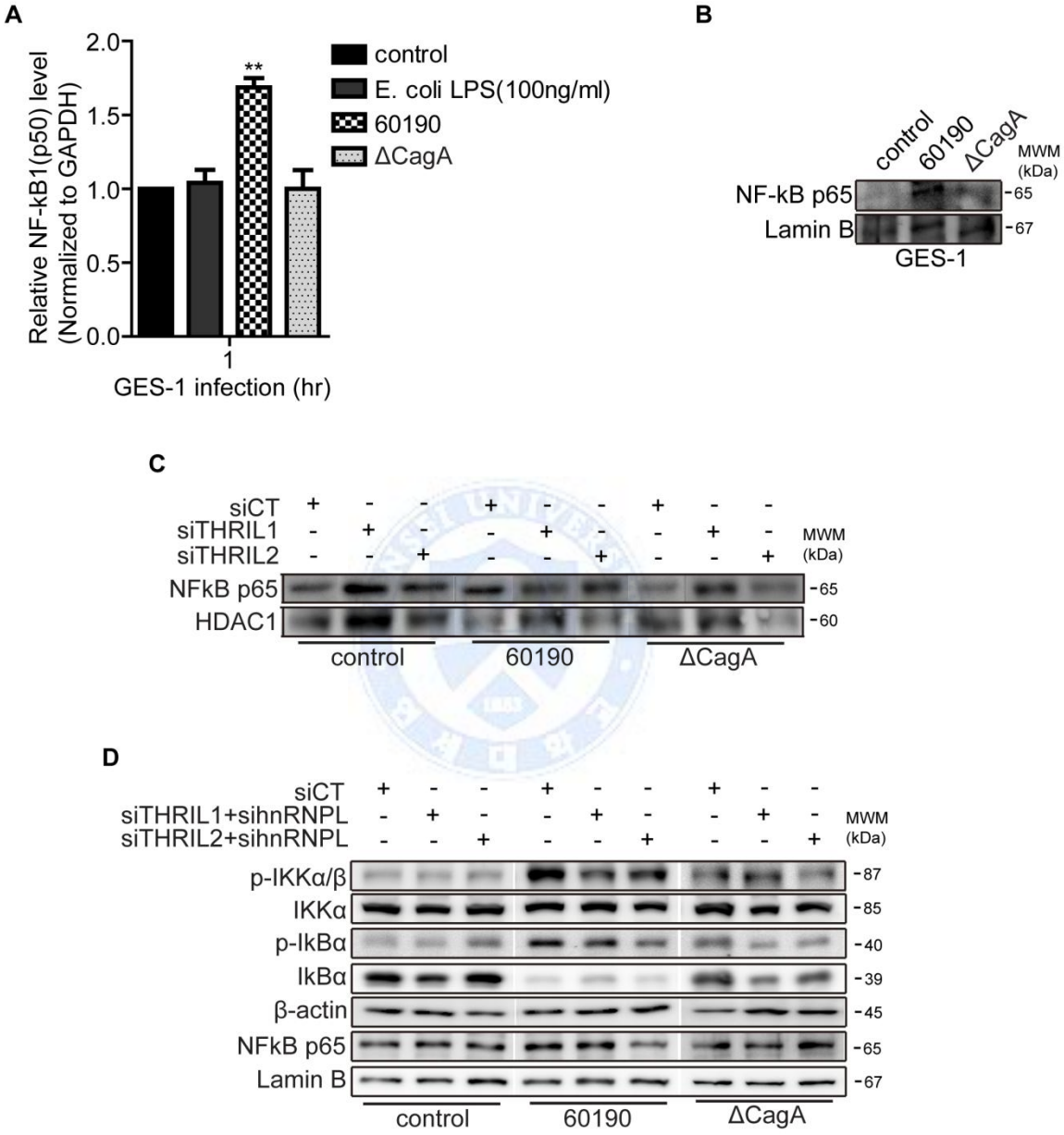
Prior to this, we checked whether NF-kB in GES-1 cell is activated by *H. pylori* infection. As a results, we confirmed that NF-kB1 (p50) mRNA level in GES-1 cell was up regulated in CagA-positive *H. pylori* 60190 infection compared to control (Figure 5A). To identify NF-kB translocation in protein level by *H. pylori* infection, GES-1 cells were fractionated with cytosol/nuclear. When compared to control and ΔCagA (an isogenic mutant of 60190 lacking CagA), the nuclear translocation of NF-kB was increased in CagA-positive *H. pylori* 60190 infection compared to control on western blot (Figure 5B). Immunofluorescence results were shown similar increased expression of NF-kB translocation in CagA-positive *H. pylori* 60190 infection (data not shown).

Based on these results, siTHRILs-transfected GES-1 cells were infected with *H. pylori* and fractionated with cytosol/nuclear. siTHRILs decreased nuclear translocation of NF-kB p65 in CagA-positive *H. pylori* 60190 infection, but did not affect control and ΔCagA (Figure 5C). Also, double knockdown of lincRNA THRIL and hnRNPL attenuated NF-kB nuclear translocation in CagA-positive *H. pylori* 60190 infection, but did not affect control and ΔCagA (Figure 5D).

To elucidate NF-kB nuclear translocation signaling pathway, we examined activation of IKK and

IkB, which is mediated by phosphorylation and polyubiquitination in *H. pylori* infection after double knockdown of lincRNA THRIL and hnRNPL. In CagA-positive *H. pylori* 60190 infection, phosphorylation of IKKα/ß was reduced in siTHRIL and sihnRNPL compare to siCT, but did not affect control and ΔCagA (Figure 5D). In addition, IkBα, which is mediated by IKK, was degraded and its phosphorylation was also inhibited in siTHRIL and sihnRNPL compare to siCT (Figure 5D).

We indicated that translocation of NF-kB p65 within nucleus was suppressed by only CagA-positive *H. pylori* 60190 infection after double knockdown of lincRNA THRIL and hnRNPL on immunofluorescence (Figure 5E). Taken together, these results demonstrated that lincRNA THRIL regulates NF-kB translocation through CagA dependent activation by *H. pylori* infection.


A
Relative NF-kB1(p50) level
(Normalized to GAPDH)
2.0
1.5
1.0
0.5
0.0
**
control
E. coli LPS(100ng/ml)
60190
ΔCagA
1
GES-1 infection (hr)
B
control
60190
ΔCagA
MWM
(kDa)
NF-kB p65
Lamin B
65
67
GES-1
C
siCT
siTHRIL1
siTHRIL2
MWM
(kDa)
NFkB p65
HDAC1
65
60
control
60190
ΔCagA
D
siCT
siTHRIL1+sihnRNPL
siTHRIL2+sihnRNPL
MWM
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IKKα
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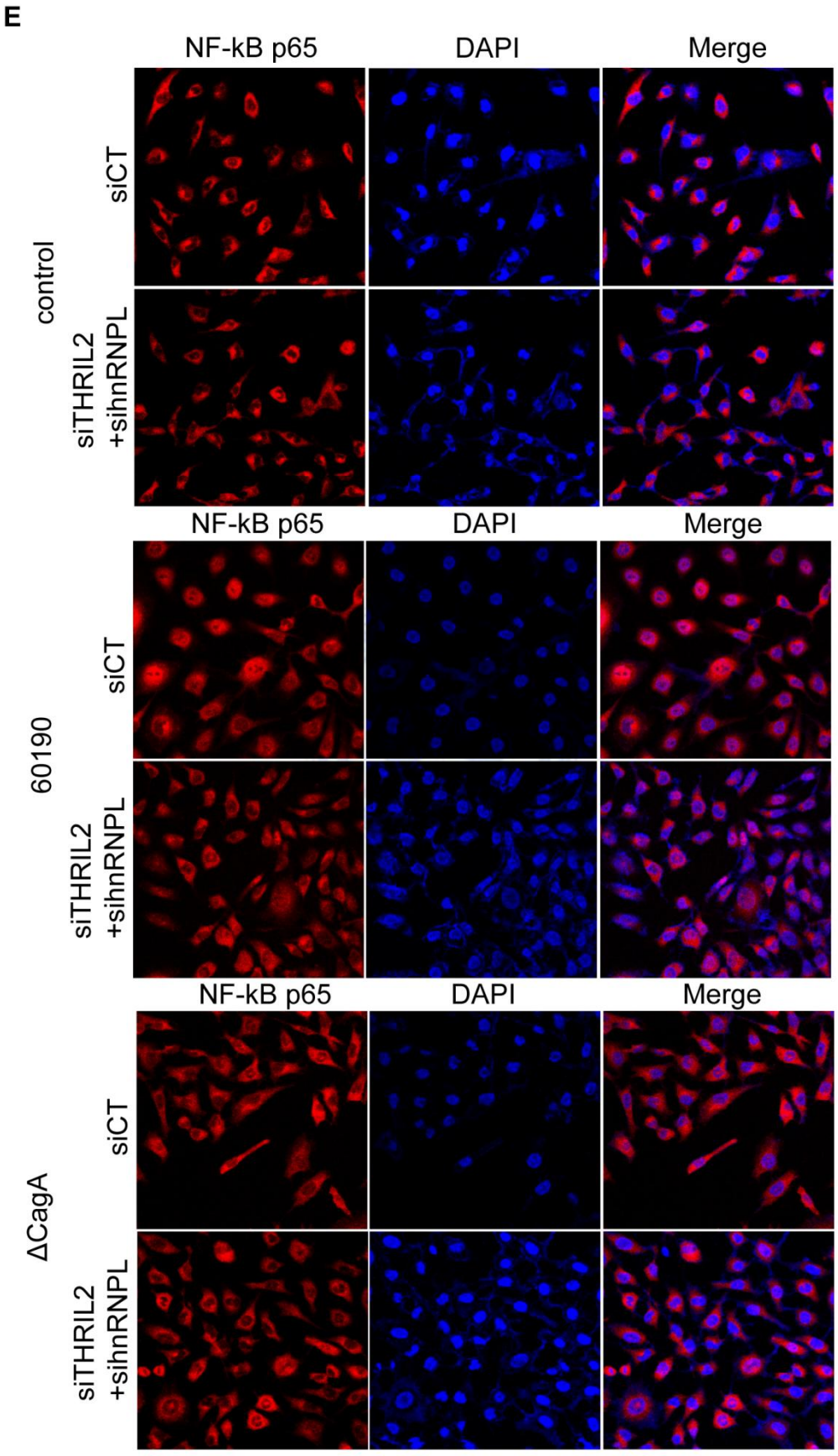
60190
ΔCagA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NF-kB p65
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control
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siTHRIL2 +sihnRNPL
60190
siCT
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siCT
siTHRIL2 +sihnRNPL

Figure 5. CagA-positive *H. pylori* 60190 infection after double knockdown of THRIL and hnRNPL suppressed NF-kB nuclear translocation. (A) The expression of NF-kB1 (p50) was determined by qRT-PCR in *H. pylori* infection for 1 hr. Data are the mean of three independent experiments ± s.e.m. The asterisk represents a statistically significant difference compared with control (**$P \leq 0.01$) (B) Protein expression of NF-kB p65 by *H. pylori* infection on western blot (C) NF-kB p65 expression by *H. pylori* infection after siTHRILs on western blot (D) NF-kB activation-related protein expression by *H. pylori* infection after siTHRIL and sihnRNPL in GES-1 cells was detected on western blot (E) NF-kB nuclear translocation was confirmed using immunofluorescence by *H. pylori* infection after knockdown of THRIL and sihnRNPL in GES-1 cell.

6. hnRNPL as an RNA binding protein interacts with cagA

We showed that NF-kB nuclear translocation was inhibited in CagA positive *H. pylori* 60190 infection after siTHRIL and sihnRNPL. Also, Li z et al. reported lincRNA THRIL interacts with hnRNPL.[11] Based on these results, we tried to elucidate whether lincRNA THRIL interacts with CagA or not. We conducted immuoprecipitation using Flag tagged-empty or CagA wt (wild type) plasmid vector to analyze relation between hnRNPL and CagA after siTHRIL and sihnRNPL in GES-1 cells. When total lysate was immunoprecipitated with hnRNPL, the expression of hnRNPL was decreased in siTHRIL and sihnRNPL compared to siCT in both p3x-Flag-empty and CagA wt. On the other hand, Flag was expressed in only p3x-Flag-CagA wt in total lysate. When compared to p3x-Flag-empty, the Flag expression was increased in p3x-Flag-CagA wt in siCT after IP. In contrast to, the Flag expression was decreased in siTHRIL and sihnRNPL compared with siCT (Figure 6A). In the same manner, the total lysate was co-immunoprecipitated with Flag. The expression of hnRNPL and Flag was presented in only p3x-Flag-CagA wt (Figure 6B). The IP results suggest that hnRNPL which binds to lincRNA THRIL interacts with CagA to regulate NF-kB signaling pathway by CagA positive-*H. pylori* infection.

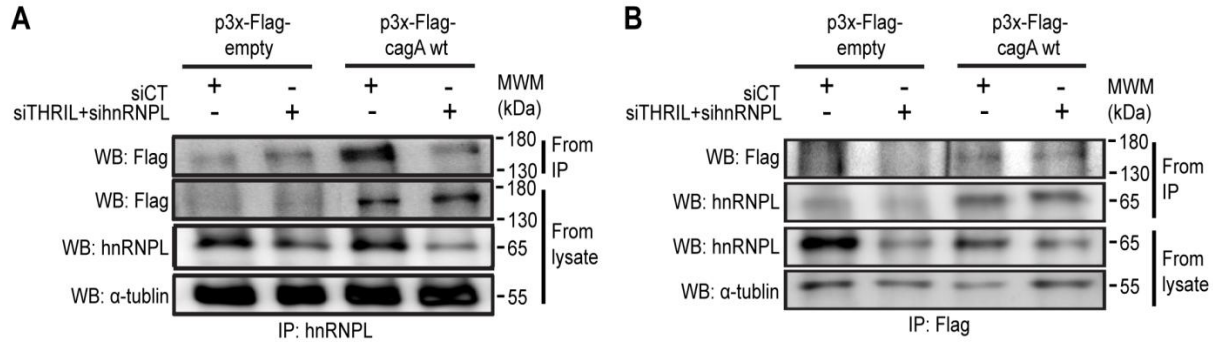

Figure 6. hnRNPL interacts with CagA. GES-1 cells were transfected with p3x-Flag-empty or CagA wt after siTHRIL and sihnRNPL. Western blot analysis (A) Flag and hnRNPL expression by IP of hnRNPL (B) Flag and hnRNPL expression by IP of Flag.

7. LincRNA THRIL modulates NF-kB pathway by targeting CagA

We investigated CagA positive *H. pylori* 60190 after siTHRIL and sihnRNPL inhibited NF-kB translocation, but did not know how THRIL regulates NF-kB pathway by CagA positive *H. pylori* 60190 infection. However, our results were shown hnRNPL, RNA binding protein playing a dual role in nuclear/cytosol, interacts with CagA through co-immunoprecipitation. This finding suggests that lincRNA THRIL regulates TNFα and IL-8 through canonical NF-kB translocation by interacting with CagA (Figure 7).

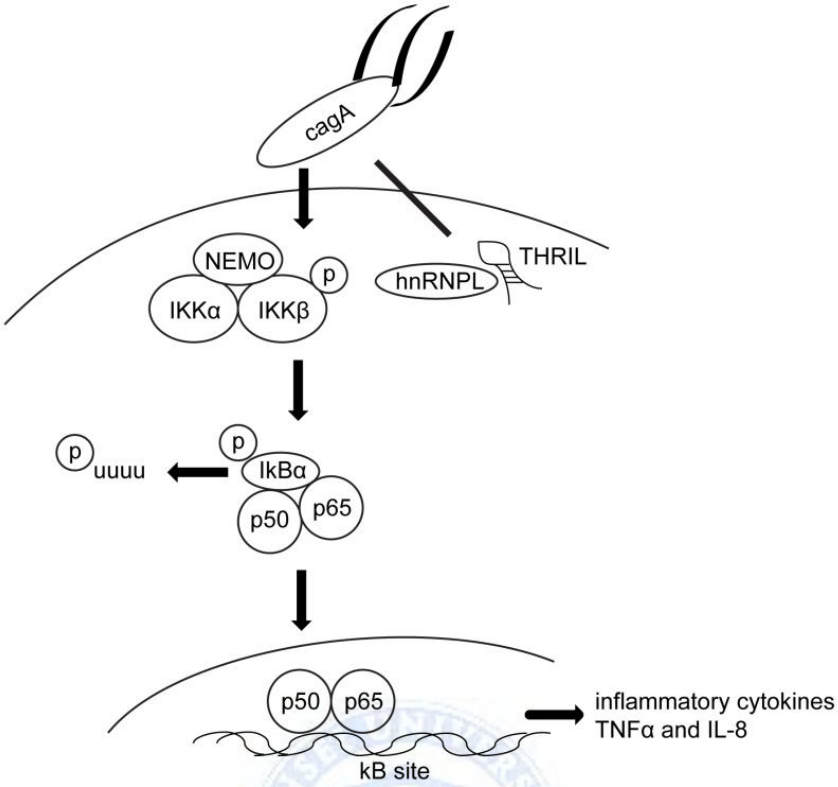

Figure 7. A schematic diagram of canonical NF-kB translocation pathway by *H. pylori* infection. Long non-coding RNA THRIL regulates TNFα and IL-8 through canonical NF-kB translocation by targeting CagA.

Ⅳ. DISCUSSION

Helicobacter pylori (H. pylori) is Gram negative bacterium that leads to chronic gastric gastritis, peptic ulceration and gastric cancer by colonizing the gastric epithelium. *H. pylori*-induced gastric inflammation and cancer are closely associated with cag pathogenicity island (PAI) that is composed of about 30 genes encoding through a type Ⅳ secretion system. *H. pylori* CagA protein, which is injected on the Cag PAI, initiates tyrosine phosphorylation at EPIYA motifs and induces the change of the morphology.[16] Also, CagA stimulates large amounts of pro-inflammatory cytokines and chemotactic activating cytokine such as tumor necrosis factor alpha (TNFα), interleukin1, 6 and 8 (IL-1, IL-6 and IL-8) in gastric epithelial cells. These cytokines induce the infiltration at inflammatory sites, and are far likely to be associated with occurrence *H. pylori*-induced gastric diseases.[17-19]

In recent year, long non-coding RNAs are interestingly demonstrating that it could regulate the lipopolysaccharide-induced inflammation and immune response.[9,10,20-22] The studies have usually implicated in LPS or Pam-stimulated innate immune response and adaptive immune response including T cell activation, development and differentiation.[5] *H. pylori* infection is the strongest virulence factor that causes innate immune response-related chronic gastric inflammation and adenocarcinoma. In gastric epithelial cells, *H. pylori* infection is known to induce increased expression of Toll-like receptor 4 (TLR4), and the TLR4 signaling is correlated with activation of NF-kB nuclear translocation.[23,24] Also, phosphatidylinositol 3-kinase (PI3K) and Akt phosphorylation play essential role in NF-kB activation by *H. pylori*-induced innate immune response.[15,25] However, lincRNAs by *H. pylori*-induced gastric inflammation have not been studied yet even if recent study is that immune response by *H. pylori* infection is regulated by microRNA.[4]

Recently, lincRNA THRIL is newly reported that it regulates gene expression of Pam3CSK4-stimulated TNFα by forming RNP complex through interaction with hnRNPL. Also, Li z et al. was

shown dysregulation of lincRNA THRIL reduced Pam-stimulated the expression of inflammation-related cytokines including IL-8, CXCL10, CCL and CSF1 in macrophages.[11]

In our study, lincRNA THRIL regulates in both mRNA level and protein secretion of TNFα and IL-8 by CagA-positive *H. pylori* 60190 infection. The regulation of lincRNA THRIL by CagA-positive *H. pylori* infection suppresses NF-kB translocation within nucleus through canonical NF-kB pathway in GES-1 cells.

In this study, we demonstrated that lincRNA THRIL regulates expression of TNFα and IL-8 by *H. pylori* infection as previously reported.[11] TNFα and IL-8 are well known proinflammatory cytokines as target genes for NF-kB. In our data, the expression of TNFα and IL-8 by CagA-positive *H. pylori* 60190 infection after knockdown of THRIL was decreased when compared with the expression in siCT. This finding considers that the regulation of lincRNA THRIL by CagA-positive *H. pylori* 60190 infection is thought to be associated with TNFα and IL-8 level by NF-kB activation. CagA-positive *H. pylori* infection activates the IKK complex in gastric epithelial cells and induces NF-kB activation, leading to the induction TNFα as proinflammatory cytokine and IL-8 as chemotactic activating cytokine.[26,27] Moreover, IKK activation requires phosphorylation of IkBα at conserved serine residues (serine 32 and 36) within NH_2-terminal domain and the phosphorylation initiates degradation of endogenous IkBα and nuclear translocation of NF-kB.[1,28,29]

Based on our results, we first tried to elucidate that NF-kB nuclear translocation could be regulated in CagA-positive *H. pylori* infection after knockdown of THRIL. Only CagA-positive *H. pylori* 60190 infection was resulted in suppressed phosphorylation of IkBα and IKKα/ß by siTHRIL and hnRNPL. NF-kB p65 nuclear translocation corresponding with its expression was also shown decreased expression in only CagA-positive *H. pylori* 60190 infection after dysregulation of lincRNA THRIL and hnRNPL.

This finding indicates that lincRNA THRIL regulates TNFα expression through NF-kB pathway by

targeting CagA in transcription level. We showed hnRNPL binds to CagA in endogenous RNP level. However, there is limit to elucidate posttranscriptional regulation between lincRNA THRIL and binding site of CagA. To figure out the mechanism how lincRNA THRIL targets CagA-dependent activation, we should clarify through additional experiments such as RNA immunoprecipitation for analysis of RNA-protein interaction.

In summary, our findings suggest that lincRNA THRIL by CagA-dependent activation suppressed the expression of TNFα and IL-8 through NF-kB pathway. lincRNA THRIL can be a new biomarker as therapeutic target for *H. pylori*-induced gastric inflammation and adenocarcinoma.

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Ⅴ. CONCLUSION

Long non-coding RNAs are being newly magnified to regulate innate and adaptive immunity-related inflammatory diseases. *Helicobacter pylori* infection is one of the well known innate immune response-related gastric inflammation. In *H. pylori*-induced inflammation, lincRNAs have not been studied and its mechanism is complex and poorly understood. Here, we tried to demonstrate the effect of lincRNA in *H. pylori* infection. We found that lincRNA TRHIL regulates *H. pylori* induced-TNFα and IL-8 as proinflammatory cytokines by CagA-dependent activation through NF-kB pathway.

Consequently, lincRNA THRIL could be a new biomarker that being therapeutic target for *H. pylori* infection.

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ABSTRACT (IN KOREAN)

헬리코박터로 기인한 염증반응에서 NF-kB전좌의 억제에 의한 long non-coding RNA THRIL의 조절

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이 나 금

위염 과 위암 의 가장 큰 명백한 원인은 헬리코박터 파이로리 감염 및 세포 독소 관련 유전자 CagA로 알려져 있다. 그럼에도 불구하고, 헬리코박터 파이로리 선택적인 위암발생에 대해서는 거의 알려져 있지 않다. 최근에, 흥미롭게도 lncRNA가 병원균에 의한 면역 반응의 조절에 관여하는 것이 보고 되고 있다. 더불어, 선천적 면역반응과 관련된 lncRNA들은 RNA 결합 단백질과 결합함으로써 전염증성 사이토카인을 조절한다는 것이 보고 되어왔다. THRIL은 다양한 조직에서 발현 되고 특히 위 점막에서 과발현 되어 있는 것으로 알려져 있다. THRIL은 hnRNPL과 상호작용함으로써 지질다당류로 유도되는 종양괴사인자알파를 조절하는 것으로 처음 보고 되었다.

본 연구에서, 헬리코박터 감염에 의한 위 정상 상피세포의 발생에서 THRIL의 영향을 조사하였다. THRIL의 발현은 GES-1 세포와 다양한 암세포주에서 qRT-PCR에 의해 측정되었다. THRIL의 하향조절은 두 가지 siRNA에 의해 진행되었고 병원균 감염을 위해 헬리코박터균주 60190 (CagA+), 8822 (CagA-) 그리고 ΔCagA (CagA가 없는 60190의 돌연변이 유전자)가 사용되었다. 세포들은 헬리코박터 감염다중도 1:500으로 다양한 시간에서 감염되었다. 그 후, 헬리코박터 감염에 의한 종양괴사인자알파 분비는 ELISA 분석으로 측정되었고 THRIL을 표적으로 하는 siRNA 처리 후 헬리코박터 감염에 의한 NF-kB 경로의 신호를 분석하였다.

위 정상 상피 세포와 비교 하였을 때, THRIL의 발현은 다양한 암 세포주에서 과발현 되었다. 헬리코박터균주 8822 (CagA-)와 ΔCagA (CagA가 없는 60190의 돌연변이 유전자)는 종양괴사인자알파, 인터루킨8 그리고 THRIL의 발현에는 영향을 주지 않았다. 암 단백 CagA를 가진 60190 균주에서만 종양괴사인자알파와 인터루킨8이 유도되었고 이에 대해 THRIL의 발현은 역비례하여 하향조절 되었다. 또한, hnRNPL 유전자 억제가 암 단백 CagA를 가진 헬리코박터균주 감염에 의해 유도되는 종양괴사인자알파와 인터루킨8의 발현의 감소가 THRIL의 변질에는 영향없이 유도되는 것을 분석하였다. siRNA에 의한 THRIL의 하향조절은 암 단백 CagA를 가진 헬리코박터균주의 감염과 기저 상태 모두에서 종양괴사인자알파와 인터루킨8 mRNA의 발현을 감소시켰다. 또한, THRIL과 hnRNPL에 대한 이중 유전자억제는 암 단백 CagA를 가진 헬리코박터균주 감염에 의한 종양괴사인자알파의 유도를 약화시켰다.

결론적으로, THRIL과 hnRNPL은 암 단백 CagA로 유도된 위 염증반응을 NF-kB 의존적으로 조절한다고 여겨진다.

핵심되는 말: long non-coding RNA, 헬리코박터파일로리, 종양괴사인자알파, NF-kB