Helicobacter pylori promotes epithelial-to-mesenchymal transition by downregulating CK2β in gastric cancer cells

So Dam Lee

Department of Medicine

The Graduate School, Yonsei University

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Directed by Professor Yong Chan Lee

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This certifies that the Doctoral Dissertation of
So Dam Lee is approved.

Thesis Supervisor: Yong Chan Lee

Thesis Committee Member#1: Ki Teak Nam

Thesis Committee Member#2: Jihyun Kim

Thesis Committee Member#3: Sung Kwan Shin

Thesis Committee Member#4: Jongki Hong

The Graduate School
Yonsei University

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<TABLE OF CONTENTS>

LIST OF FIGURES

ABSTRACT

***Helicobacter pylori* promotes epithelial-to-mesenchymal transition by downregulating CK2β in gastric cancer cells**

So Dam Lee

Department of Medicine Science
The Graduate School, Yonsei University

(Directed by Professor Yong Chan Lee)

Strains of *Helicobacter pylori* that are positive for the oncoprotein CagA (cytotoxin-associated gene A) are associated with gastric cancer and might be related to the epithelial-to-mesenchymal transition (EMT). Casein kinase 2 (CK2) is a serine/threonine protein kinase that plays a major role in tumorigenesis through signaling pathways related to the EMT. However, the role played by the interaction between CagA and CK2 in gastric carcinogenesis is poorly understood. Although CK2α protein expression remained unchanged during *H. pylori* infection, we found that CK2α kinase activity was increased in gastric epithelial cells. We also found that the CK2β protein level decreased in *H. pylori*-infected gastric cancer cells in CagA-dependent manner and demonstrated that CagA induced CK2β degradation via HDM2 (human double minute 2; its murine equivalent is MDM2). We observed that CagA induced HDM2 protein phosphorylation and that p53 levels were decreased in *H. pylori*-infected gastric cancer cells. In addition, downregulation of CK2β induced AKT Ser473 phosphorylation and decreased the AKT Ser129 phosphorylation level in gastric cancer cells. We also found that the downregulation of CK2β triggered the upregulation of Snail levels in gastric cancer cells. Furthermore, our *in vivo* experiments and functional assays of migration and colony formation suggest that CK2β downregulation is a major factor responsible for the EMT in gastric cancer. Therefore, CK2 could be a key mediator of the EMT in *H. pylori*-infected gastric cancer and could serve as a molecular target for gastric cancer treatment.

Key words : CK2; *helicobacter*. *pylori*; CagA; HDM2; EMT; cancer

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I. INTRODUCTION

Helicobacter pylori, a gram-negative bacterium that colonizes the gastric mucosa of humans, plays an important role in the pathogenesis of gastric adenocarcinoma and lymphoma [1-3]. The pathogenesis of infection is based on long-term host and bacterial interactions and is affected by bacterial virulence factors, the environment, and host factors. *H. pylori* injects the bacterial oncoprotein CagA (cytotoxin-associated gene A) into host gastric epithelial cells via the type IV secretion system [4-7]. Recent studies have suggested that chronic infection with *H. pylori* is associated with an increase in the epithelial-mesenchymal transition (EMT), which is implicated in gastric cancer progression and metastasis [8-10].

The murine double minute 2 (MDM2) protein (for which there is a human homolog, HDM2) is a multi-functional oncoprotein with p53-dependent and -independent roles in oncogenesis. p53 is inactivated by the MDM2 ubiquitin (Ub) ligase [11-12]. HDM2 is upregulated in human cancers of the brain, breast, ovary, lung, colon, prostate, and kidney [13-15], and HDM2 induces the EMT by enhancing Snail expression in breast, lung, and glioma cancers [16-18]. In contrast, p53 is a tumor suppressor involved in cell cycle arrest and apoptosis [19-21]. The regulation of HDM2 and p53 in *H. pylori*-infected cells is not well understood. *H. pylori* activates AKT (also known as protein kinase B or PKB), which causes the phosphorylation of HDM2 and subsequent degradation of p53 in gastric epithelial cells and the gastric mucosa [22-27]. p53 regulates invasion in hepatocellular carcinoma via Snail degradation [28-29]. However, the mechanisms underlying activation

of the EMT by HDM2 and p53 are poorly characterized. The vast majority of EMT-related signaling pathways converge to induce E-cadherin repressors, particularly Snail family proteins. Recent studies have demonstrated the roles of various post-translational modifications in regulating Snail and shown that they are controlled by protein kinases such as GSK-3β, FOXC2, TWIST1, and CK2 [30-33].

Casein kinase 2 (CK2) is a tetrameric enzyme with two catalytic (α and/or α') subunits and two regulatory CKβ subunits. CK2α/CK2α' and CK2β expression is unbalanced in different human cancer cells [34-36]. CK2 is a remarkably multi-functional protein kinase with more than 300 substrates, many of which are critical for cell growth, proliferation, and differentiation. However, a wealth of evidence indicates that CK2 plays a major role in tumorigenesis by enhancing the transforming potential of oncogenes and acting as an anti-apoptotic molecule [37-39]. Elevated CK2 expression and activity are associated with aggressive tumor behavior in a variety of human cancers, including mammary gland, head and neck, and kidney [34, 40]. In this study, we analyzed the functional role of CK2 in the EMT of gastric cancer. We have previously shown that the CagA protein of *H. pylori* induces cell migration and invasion by increasing CK2α activity in gastric epithelial cells such as AGS and MKN28 [41]. In contrast, CK2-specific siRNA and the CK2 inhibitor TBB suppressed cell migration and invasion in *H. pylori*-infected gastric epithelial cells. Although a few studies have shown a relationship between *H. pylori* and CK2β, other research has reported a correlation between *H. pylori* and CK2α [41-42]. The role of the CK2α catalytic subunits is largely uncharacterized, but several studies have already considered the role of the CKβ regulatory subunits in the EMT. Herein, we demonstrate that CK2 contributes to *H. pylori*-induced gastric carcinogenesis, and the downregulation of the CKβ regulatory subunit in *H. pylori*-infected gastric cancer could play a critical role in regulating the transcription factors involved in the EMT in gastric cancer.

II. MATERIALS AND METHODS

1. Cell culture and bacteria

AGS (CRL-1739, ATCC, USA), MKN28 (80102, KCLB, Korea), and MKN74 (80104, KCLB, Korea) gastric cancer cells and HEK293T (CRL-11268, ATCC, USA) cells were maintained in RPMI-1640 medium (Thermo, USA) or Dulbecco's modified Eagle's medium (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS, Thermo, USA) and 1% penicillin-streptomycin sulfate (Thermo, USA). All cultures were maintained in a 37°C incubator supplemented with 5% CO_2. The *H. pylori* strains, *H. pylori* 60190 (CagA+, 49503, ATCC, USA), *H. pylori* ΔVacA (CagA+), *H. pylori* ΔCagA (CagA-), and *H. pylori* 8822 (Cag PAI-), were cultured on agar plates containing 10% horse serum at 37℃ in a microaerobic atmosphere using a CampyContainer system (BBL, USA).

2. Tissue microarray construction from gastric cancer patients

To determine the relationship between the clinicopathological characteristics of gastric cancers and the expression of CK2 *in vivo*, we performed immunohistochemical (IHC) staining of CK2 using tissue microarrays (TMAs) constructed from 54 pairs of gastric cancer tissue samples and corresponding normal tissues that were obtained from patients who underwent curative surgery for gastric cancer at Yonsei University, Severance Hospital, between October 2018 and June 2020. This study was approved by the Institutional Review Board of Severance Hospital (IRB no. 4-2010-0265). The TMAs were constructed as described previously [43]. On hematoxylin and eosin (H&E)-stained slides of tumors, a representative area was selected, and the corresponding spot was marked on the surface of the paraffin block. With a biopsy needle, the selected area was punched out, and a 2-mm tissue core was placed into an 8 × 6 recipient block. Gastric cancer tissues and corresponding normal tissues were then extracted. Each tissue core was assigned a unique TMA location number that was linked to a database containing other clinicopathological data.

3. Immunohistochemical assay

For IHC, xenograft tumors were embedded in paraffin and fixed in 4% paraformaldehyde.

Then, 4-μm sections were de-paraffinized in xylene and sequentially rehydrated through 100%, 95%, and 70% ethanol, with a final step in distilled water. Antigen retrieval (S1699, DAKO, USA) was performed using a high-pressure cooker for 15 minutes, and then the samples were cooled on ice for at least one hour. Next, the sections were incubated in 3% H_2O_2 for 30 minutes and washed twice with phosphate-buffered saline (PBS). To reduce non-specific signals, we incubated the samples with serum-free blocking medium (X0909, DAKO, USA) for 2 hours at room temperature in a humidity-controlled chamber. The samples were incubated with anti-CK2α (PA5-28602, Thermo, USA), anti-CK2β (PA5-27416, Thermo, USA), anti-MDM2 (NB100-2736, Novus, USA), and anti-p53 (MA5-12557, Thermo, USA) antibodies overnight at 4℃ in a humidity-controlled chamber. After three washes in PBS, the sections were incubated in horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (K4003, DAKO, USA) or HRP-conjugated anti-mouse secondary antibody (K4001, DAKO, USA) for 15 minutes at room temperature in a humidity-controlled chamber. For IHC, DAB (K3468, DAKO, USA) was used to develop the antibodies, and Mayer's hematoxylin (S3309, DAKO, USA) was used for counterstaining. Signal development was conducted for the same amount of time for each section to prevent over-staining, and positive cells were measured using QuPath software (Queen's University, Belfast). The TMA blocks were sectioned to a thickness of 3-μm for automated immunostaining using Ventana Benchmark XT (Ventana Medical System, USA) with antibody against CK2α (PA5-28602, Thermo, USA) or CK2β (PA5-27416, Thermo, USA). All slides were detected with an ultraView universal DAB kit and counterstained with hematoxylin. The IHC results for tumor cells were scored using QuPath software.

4. Small interfering RNA (siRNA) transfection

Control and CK2 siRNA were synthesized by Bioneer. Co. Ltd. The siRNA duplexes were as follows: CK2α siRNA #1 sense strand, CAUUUAGUUACUGGGCAUA (dTdT); CK2α siRNA #1 antisense strand, UAUGCCCAGUAACUAAAUG (dTdT); CK2β siRNA #1 sense strand, GUGUACACACCCAAGUCAU (dTdT); CK2β siRNA #1 antisense strand, AUGACUUGGGUGUGUACAC (dTdT). AGS, MKN28, and MKN74 cells were

transfected with 20 nM siRNA using Lipofectamine RNAiMax according to the manufacturer's instructions (13778-150, Invitrogen, USA). The siRNA targeting the HDM2 sense and antisense strands were GCUUCGGAACAAGAGACCC and GGGUCUCUUGUUCCGAAGC (Santa Cruz, USA), respectively. CHIP siRNA, WWP1 siRNA, and p53 siRNA were purchased from Santa Cruz Biotechnology.

5. Plasmid transfection

AGS, MKN28, MKN74, and HEK293T cells were transfected with each construct using Lipofectamine 2000 according to the manufacturer's instructions (11668-019, Invitrogen, USA). pZw6-HA (CK2α, #27086), pZw12-Myc (CK2β, #27088), pCMV-Myc3-HDM2 (#20935), and pcDNA3 Flag p53 (#10838) were purchased from Addgene. Full-length constructs for CagA (AF202923) and the PR mutant on EPIYA were kindly provided by Professor M. Hatakeyama (Tokyo University). Six hours after transfection, the medium was replaced with fresh medium. For each analysis, cells were used 48 hours after transfection.

6. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated using TRIzol reagent (15596-026, Invitrogen, USA), and cDNA was synthesized using PrimeScript™ reverse transcriptase (2680A, Takara Bio Inc., Japan). The synthesized cDNA was amplified, and the PCR product was visualized on 1% agarose gel. The sequences of each forward (F) and reverse (R) primer used for PCR were as follows: CK2β-F, CAGAAGATACAAGTAGCCTC; CK2β-R, CTGCTGGAATACTGTAACTG; β-actin-F, GCTCGTCGTCGACAACGGCT; β-actin-R, CAAACATGATCTGGGTCATCTTCTC.

7. Western blot analysis

After being washed with PBS, cells were harvested and resuspended in NETN lysis buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1mM EDTA, 0.5% Nonidet P-40, 50 mM β-glycerophosphate, 10 mM sodium fluoride, 1 mM sodium orthovanadate, and protease inhibitor cocktail [Roche Molecular Biochemicals, USA]). After the protein in each lysate was quantified, sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris-Cl [pH 6.8],

2% SDS, 10% glycerol, β-mercaptoethanol, and 0.002% bromophenol blue) was added to equal amounts of proteins, which were then heated at 95°C for 5 minutes. The samples were loaded onto 6% or 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels. We used antibodies against CK2α (sc-12738, Santa Cruz, USA), CK2β (sc-12739, Santa Cruz, USA), CagA (sc-28368, Santa Cruz, USA), anti-Ub antibody (sc-8017, Santa Cruz, USA), p-HDM2 Ser166 (3521, Cell Signaling, USA), HDM2 (556353, BD Biosciences, USA), p53 (AHO0152, Invitrogen, USA), c-Myc (sc-40, Santa Cruz, USA), Snail (3895, Cell Signaling, USA), HA (3724, Cell Signaling, USA), Flag (F3165, Sigma, USA), p-AKT Ser473 (9271, Cell Signaling, USA), p-AKT Ser129 (13461, Cell Signaling, USA), CHIP (sc-133083, Santa Cruz, USA), WWP1 (ab43791, Abcam, USA), E-cadherin (3195, Cell Signaling, USA), N-cadherin (13116, Cell Signaling, USA), and Zo-1 (sc-10804, Santa Cruz, USA), with β-actin (sc-47778, Santa Cruz, USA) used as the loading control.

8. *In vivo* ubiquitination assay

HEK293T cells were transfected with His-Ub. Forty-eight hours after transfection, the cells were infected with *H. pylori* 60190 under CagA expression conditions in the presence of MG132 (25 μM) for 6 hours before harvesting. CK2β was immunoprecipitated using Protein A/G PLUS agarose beads (sc-2003, Santa Cruz, USA), and the immunoprecipitates were run on 6% SDS-PAGE gels. Ubiquitinated CK2β was detected using an anti-Ub antibody (sc-8017, Santa Cruz, USA).

9. Cell fractionation

AGS cells were infected with CagA-positive (60190) and CagA-negative (ΔCagA) strains of *H. pylori*. Six hours after infection, nuclear-cytoplasmic fractionation was conducted using a NE-PER nuclear and cytoplasmic extraction reagent kit (78833, Thermo, USA) according to the manufacturer's protocol.

10. Immunofluorescence assay

Cells were seeded on glass coverslips in 6-well plates. After overnight incubation, the cells were washed with PBS three times, fixed in 4% formaldehyde for 10 minutes, and permeabilized in 0.1% Triton X-100 in PBS for 3 minutes. Then the cells were washed

three times with PBS, blocked with 1% BSA in PBS, and incubated with primary antibodies (CagA, sc-28368, Santa Cruz, USA; CK2α, ab-76025, Abcam, USA; CK2β, ab-76025, Abcam, USA) overnight at 4°C. After being washed with PBS, the cells were incubated with FITC- or Texas Red-conjugated secondary antibodies for one hour at room temperature. The slides were mounted with DAPI, and all samples were photographed using a Zeiss LSM 700 confocal microscope (Carl Zeiss, Germany).

11. Stable CK2 knockdown using lentiviral shRNA

Four premade lentiviral CK2 short hairpin RNA (shRNA) constructs and a negative control construct in the same vector system (pLKO.1) were purchased from Sigma (shCK2α: TRCN0000320928, TRCN0000350294; shCK2β: TRCN000001081, TRCN0000199450; shCV: SHC001). Lentiviral helper plasmids (pCMV-Δ8.91R and pMDG) were obtained from Addgene. Transient lentivirus stocks were prepared in HEK293T cells according to the manufacturer's protocol. AGS and MKN74 cells that stably expressed the shRNA constructs were selected with puromycin (2 μg/ml) for 48 hours after lentivirus infection. The positive clones were identified and verified by western blotting.

12. CK2 kinase assay

CK2 activity in cell lysates was determined using a CK2 kinase assay kit (CycLex Co. Ltd., Japan) according to the manufacturer's instructions. The samples were assayed in triplicate, and statistical significance was determined.

13. Wound healing assay

AGS and MKN74 cells were cultured in 6-well plates until they reached 80-90% confluence. A sterile 1000 μl pipette tip was used to create a linear wound in the cell monolayer. After being washed, the cells were incubated in a medium containing 5% FBS for 24 hours. The width of the wound area was photographed 24 hours after scratching.

14. Clonogenic assay

AGS and MKN74 cells were seeded in 6-cm dishes at a density of 300, 600, or 900 cells per dish. After a 14-day incubation, the colonies were fixed with methanol and stained with

crystal violet. Then, the number of colonies containing more than 50 cells was counted.

15. Xenograft mouse model

For the *in vivo* xenograft assay, 5×10^6 AGS-shRNA CK2α#1, CK2α #2, shRNA CK2β#1, CK2β #2, or AGS-shRNA control cells and 8×10^6 MKN74-shRNA CK2α#1, CK2α #2, shRNA CK2β#1, CK2β #2, or MKN74-shRNA control cells were injected subcutaneously into the right hind lateral legs of NON-SCID mice. The mice were bred and housed in a specific pathogen free animal facility at Yonsei University College of Medicine. All the experiments were conducted using a protocol approved by the Institutional Animal Care and Use Committee (IACUC no. 2018-0330). Tumor sizes were measured with calipers every 5-10 days, and the volumes were calculated with the formula (length × width^2)/2. The mice were sacrificed at 60-110 days after injection, and the tumors were excised for photography. The tumor tissue of each group was stored in liquid nitrogen. Part of the tissue was fixed with 10% formaldehyde for H&E staining, IHC, and western blot experiments.

16. Statistical analysis

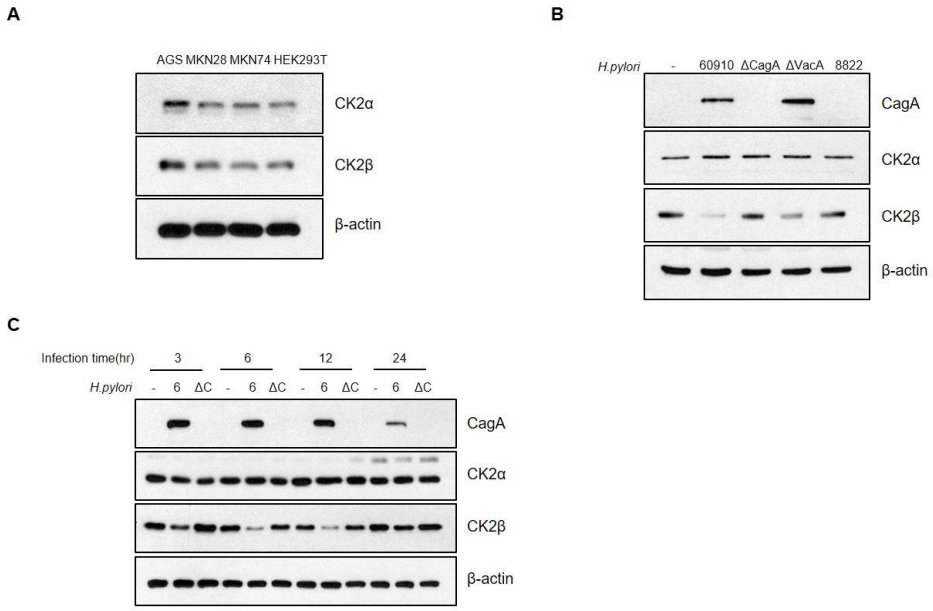
All experiments were done more than three times. All values are expressed as means ± standard deviations. Comparisons between two groups were analyzed using t-tests, and one-way ANOVA with a post hoc Bonferroni test was used for multiple comparisons. All analyses were performed in Graphpad Prism. Statistically significant differences were defined as those with a p value < 0.05. The significance level is indicated as $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

III. RESULTS

1. *H. pylori* regulates CK2 expression in human gastric cancer cells

We checked the basal CK2 protein expression levels in three gastric cancer cell lines (AGS, MKN28, and MKN74) and embryonic kidney epithelial cells (HEK293T) using western blotting. The expression levels of CK2α and CK2β were slightly higher in AGS cells than in the other cancer cell lines (Fig. 1A). To determine whether *H. pylori* regulates CK2 expression, we infected gastric cancer cells with CagA-positive and CagA-negative

H. pylori strains. *H. pylori* infection did not change the expression of CK2α; however, CK2β expression decreased in gastric cancer cells infected with CagA-positive strains (60190, ΔVacA), compared with the control. This suggests that *H. pylori* infection did not affect CK2α expression but induced CK2β downregulation in CagA-dependent manner (Fig. 1B). When AGS cells were infected with CagA-positive strain of *H. pylori* (60190) at different times, CK2β expression was reduced in a time-dependent manner. The level of CK2β expression decreased dramatically in AGS cells infected with *H. pylori* 60190 after 6 hours. The effect of CagA on CK2β expression was not observed after long-term *H. pylori* 60190 infection (Fig. 1C). The CagA-induced downregulation of CK2β was confirmed by immunofluorescence staining in 2D cell culture experiments (Fig. 1D). We overexpressed CagA using two different CagA vectors (WT, full-length constructs of CagA; PR, phosphorylation-resistant mutant) to examine the effect of the CagA oncoprotein on cellular CK2β expression. Although the overexpression of CagA did not change the level of CK2α, CK2β expression was significantly lowered by CagA WT vector transfection, compared with transfection with the empty vector or CagA PR vector. Therefore, intracellular CagA expression induced CK2β downregulation (Fig. 1E).



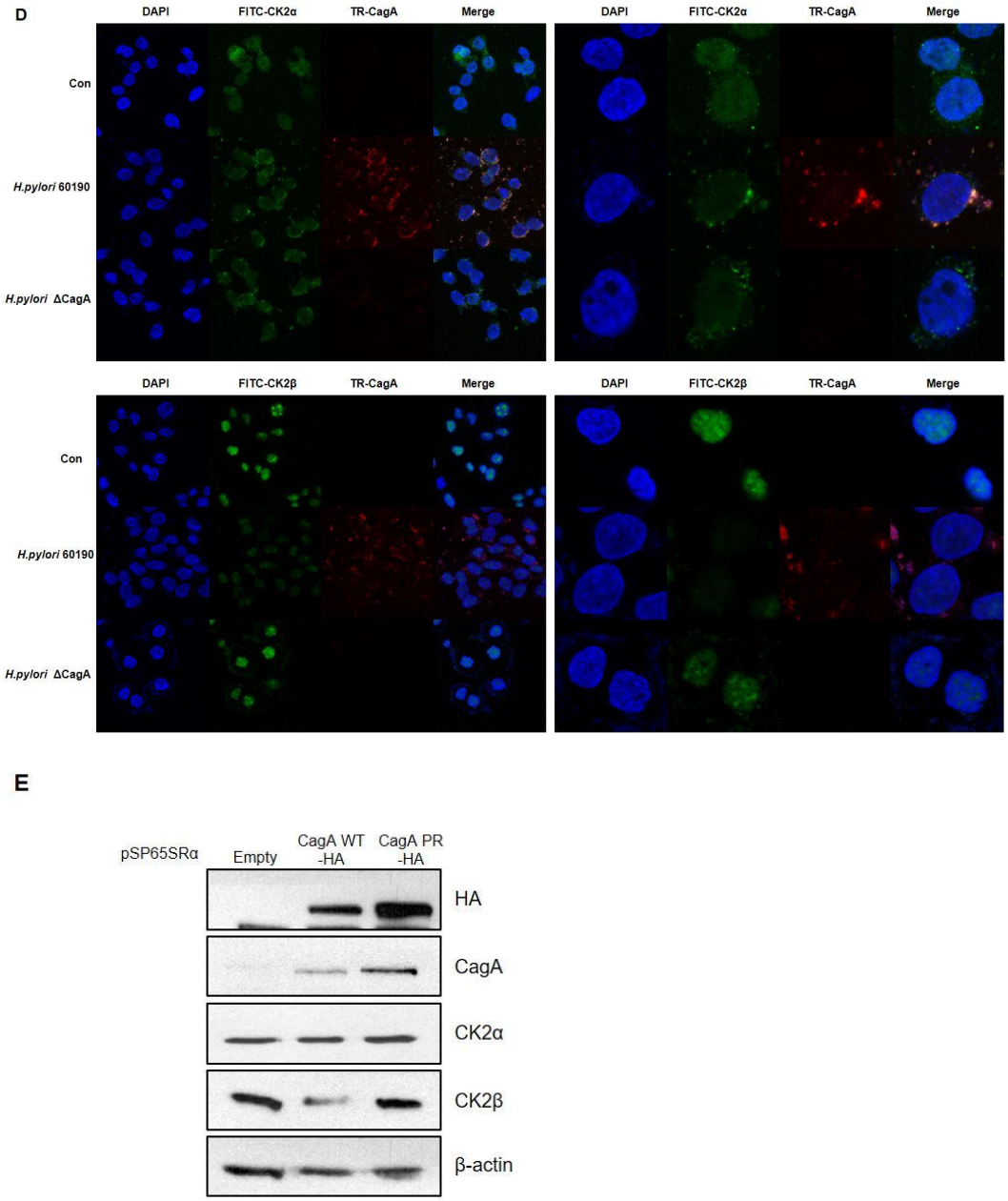


Fig. 1. *H. pylori* downregulates CK2β expression in human gastric cancer cells in CagA-dependent manner. (A-E) Expression of the CK2β protein was determined by western blotting and immunofluorescence staining. (A) Expression of the CK2 protein was

determined by western blotting in AGS, MKN28, MKN74, and HEK293T cells. β-actin was used as the loading control. (B) AGS cells were infected with several *H. pylori* strains for 6 hours at a multiplicity of infection of 100. (C) AGS cells were infected with CagA-positive *H. pylori* strain (60190) or CagA-negative strain (ΔCagA) for the indicated times prior to lysis. (D) AGS cells were infected with *H. pylori* 60190 or *H. pylori* ΔCagA for 6 hours. The cells were fixed for immunofluorescence staining of CK2α and CK2β. (E) AGS cells were transfected with HA-tagged CagA WT and CagA mutant expression vectors for 48 hours. CagA PR denotes a tyrosine phosphorylation mutant in the EPIYA motif.

2. CK2 activity increases as CK2β decreases in human gastric cancer cells

Previously, we performed a CK2 kinase assay and found that *H. pylori* upregulates CK2 activity without affecting CK2α expression levels in gastric epithelial cells [41]. We also found that AGS, MKN28, and MKN74 cells infected with wild type *H. pylori* showed significantly increased CK2 kinase activity, whereas those infected with *H. pylori* ΔCagA did not. In addition, the CK2 inhibitor TBB completely abolished CK2 activation by *H. pylori* in gastric cancer cells (Fig. 2A; Supplementary Fig. S1A). To study the changes in CK2 activity that result from the downregulation of CK2β, we transfected gastric cancer cells with CK2β siRNA. At 24- and 48-hours post-transfection, CK2 activity was dramatically upregulated in the presence of CK2β downregulation (Fig. 2B; Supplementary Fig. S1B). We performed CK2 kinase assays in gastric cancer cells made CK2α- or CK2β-deficient using shRNA. We found that CK2 activity increased in the CK2β-deficient gastric cancer cells, whereas it was significantly reduced in CK2α-deficient gastric cancer cells (Fig. 2C; Supplementary Fig. S1C). We also transfected gastric cancer cells with CK2α and CK2β expression vectors. Whereas CK2 activity increased with CK2α overexpression, CK2β overexpression resulted in a marked reduction in CK2 activity compared with control cells (Fig. 2D; Supplementary Fig. S1D). This indicates that CK2β overexpression reduces the kinase activity of CK2. To confirm that *H. pylori*-mediated activation of CK2 is depended on the phosphorylation status of CagA protein, we transfected gastric cancer

cells with either CagA WT or PR vectors. We observed a higher CK2 activity with the CagA WT vector, indicating that CK2 activity is increased in CagA phosphorylation-dependent manner (Fig. 2E; Supplementary Fig. S1E).

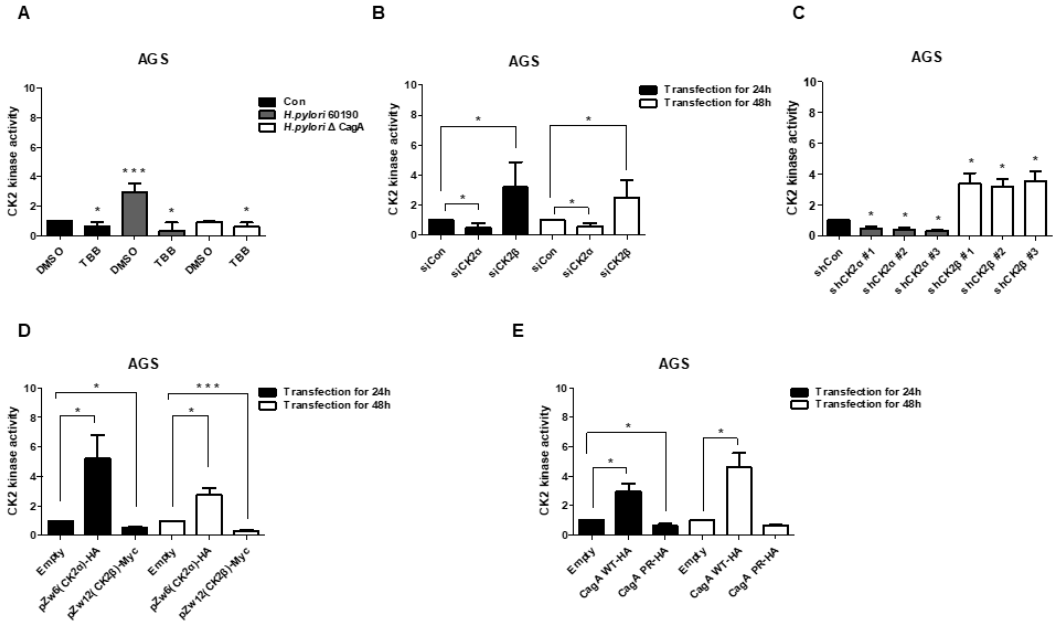


Fig. 2. *H. pylori* infection induces CK2 activation in CagA-dependent manner. (A-E) The activity of CK2 was determined using a kinase assay kit. (A) AGS cells were infected with *H. pylori* 60190 or *H. pylori* ΔCagA and co-treated with a CK2 inhibitor (100 μM) for 6 hours. (B) AGS cells were transfected with control siRNA, CK2α siRNA, or CK2β siRNA for 24 or 48 hours. (C) AGS-control (shCon) and CK2-silenced cells (shCK2α#1, #2, #3 and shCK2β#1, #2, #3) were incubated for 48 hours. (D) AGS cells were transfected with HA-tagged CK2α and Myc-tagged CK2β expression vectors for 24 or 48 hours. (E) AGS cells were transfected with HA-tagged CagA WT or CagA mutant expression vectors for 24 or 48 hours (ns, $P > 0.05$; *, $P < 0.05$; ***, $P < 0.001$).

3. CagA promotes the degradation of CK2β via a proteasomal pathway

We tried to identify the mechanism by which intracellular CagA downregulates CK2β

expression in gastric cancer cells. First, we used RT-PCR to determine the expression of *CK2β* mRNA in AGS cells infected with *H. pylori* 60190. Those results showed that *H. pylori* 60190 infection did not affect the expression of *CK2β* mRNA (Fig. 3A). Next, to determine whether the downregulation of the CK2β protein was due to proteasomal degradation, we blocked the proteolytic activity of the 26S proteasome using MG132. Although the expression of CK2β decreased in AGS cells infected with CagA-positive *H. pylori* 60190, that downregulation was recovered by MG132 treatment (Fig. 3B). Thus in gastric cancer cells, CK2β undergoes proteasomal degradation following infection with CagA-positive *H. pylori* strain. Overall, these results suggest that intracellular CagA downregulates CK2β through increased proteasomal degradation. We also examined the association between CagA and CK2β to evaluate the CagA-dependent ubiquitination of CK2β. As binding between CagA and CK2β increased in *H. pylori*-infected cells, the level of ubiquitinated CK2β in infected cells became higher than that in control cells (Fig. 3C). This suggests that CK2β ubiquitination increases in CagA-dependent manner.

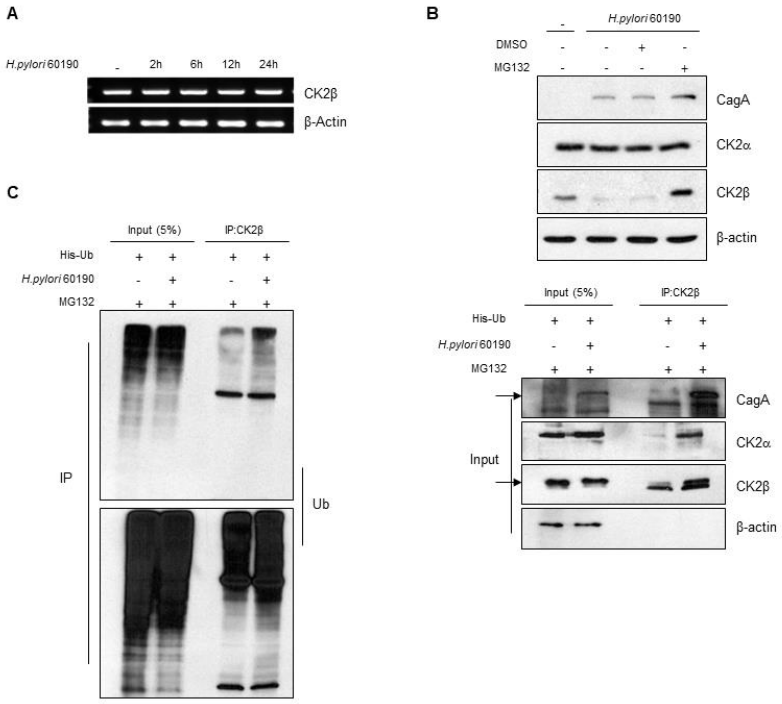


Figure 3. *H. pylori* promotes the degradation of CK2β via a proteasomal pathway. (A) RT-PCR analysis of CK2β expression in AGS cells infected with *H. pylori* 60190. β-actin was used as the loading control. (B) Western blotting of CK2β expression in AGS cells infected with *H. pylori* 60190 for 6 hours. Proteasomal degradation was blocked by treatment with MG132 (25 μM). (C) HEK293T cells were transiently transfected with a plasmid encoding ubiquitin (Ub), and 48 hours after transfection, the cells were infected with *H. pylori* 60190 and treated with MG132 (25 μM) for 6 hours. Cell lysates were immunoprecipitated with an anti-CK2β antibody and immunoblotted with an anti-Ub antibody. β-actin was used as the loading control.

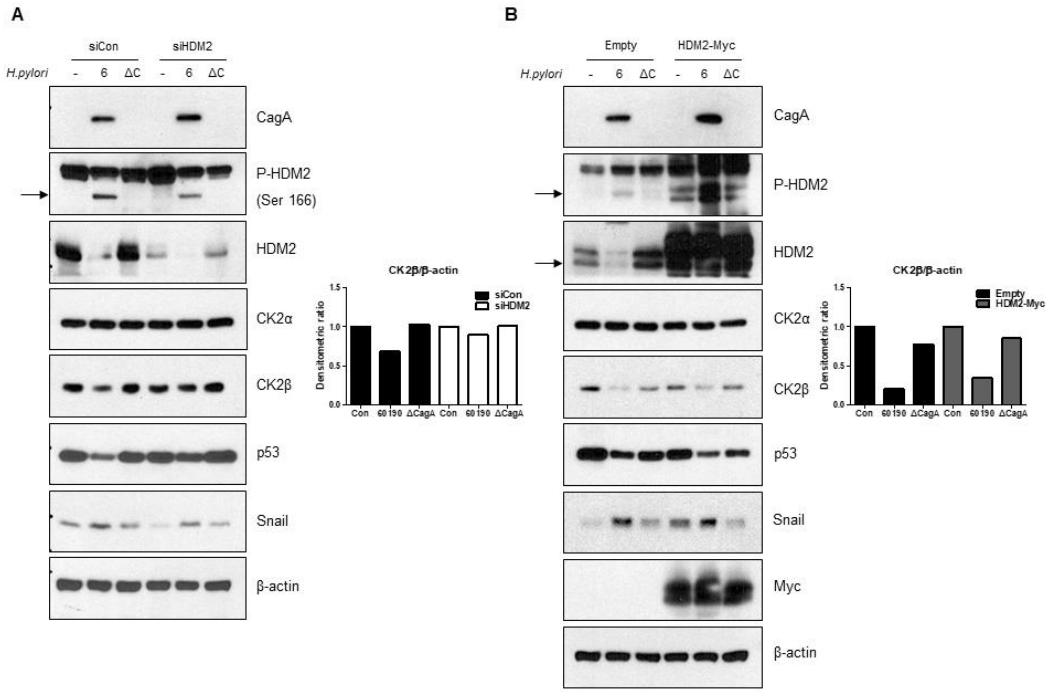
4. Proteasomal degradation of CK2β is mediated by CagA-associated E3 ligase complex

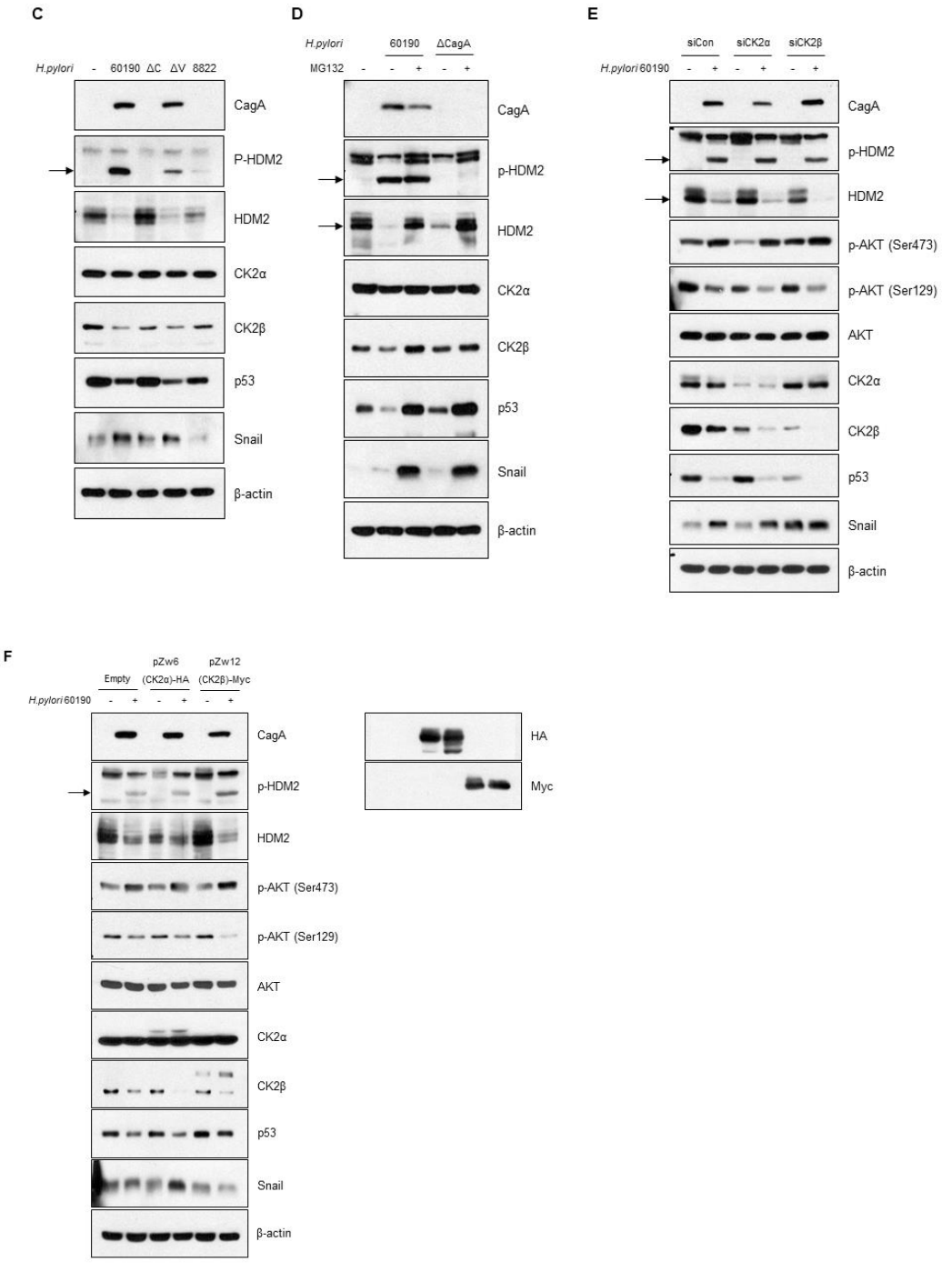
To determine whether CagA induces the ubiquitination of CK2β after *H. pylori* infection, we examined the CK2β level after siRNA-transfection of *H. pylori* 60190-infected AGS cells with proteasome-related E3 ligases (CHIP, WWP, and HDM2). CK2β downregulation was blocked in AGS cells with HDM2 knockdown (Supplementary Fig. S2A, lane 7 *vs*. lane 8). Thus, HDM2 knockdown inhibited CagA-dependent CK2β degradation. To confirm that CK2β degradation was mediated by HDM2, we compared HDM2 knockdown with HDM2 overexpression in gastric cancer cells. The level of CK2β remained unchanged in *H. pylori* 60190-infected gastric cancer cells with HDM2 knockdown (Fig. 4A). Furthermore, *H. pylori* 60190-infected cells stably transfected with an HDM2 expression vector showed lower expression of CK2β (Fig. 4B), indicating that CK2β is degraded in an HDM2-dependent manner. Previously, we observed that CagA increased the Snail-mediated EMT by reducing GSK-3 activity in gastric cancer cells [8]. Also, several studies have shown that CK2β depletion in breast cancer induces the production of Snail protein, which is one of the major intracellular CK2 targets. The increases in GSK3β (S9) phosphorylation observed in CK2β-downregulated HK-2 cells led us to re-evaluate the need for CK2β to control Snail stability in cancer [44-45]. However, how CagA protein

contributes to the EMT through CK2β dysregulation has not yet been explored. Therefore, we investigated the regulatory factors of HDM2, such as phosphorylated HDM2 (p-HDM2) and p53. We found that AGS cells infected with *H. pylori* 60190 showed increased phosphorylation of HDM2 at Ser166, which is known to increase the activity of HDM2. Although the level of Snail increased, HDM2 and p53 decreased in *H. pylori* 60190-infected gastric cancer cells, compared with the control; however, the levels of p-HDM2 and Snail decreased and p53 levels increased in *H. pylori* 60190-infected HDM2-knockdown cells (Fig. 4A). In contrast, levels of p-HDM2 and Snail were higher and p53 levels were lower than the control in *H. pylori* 60190-infected gastric cancer cells overexpressing HDM2 (Fig. 4B).

Interestingly, total HDM2 protein levels decreased in *H. pylori*-infected cells, possibly through proteasomal degradation. When AGS cells were infected with various *H. pylori* strains, the CagA-positive strains (60190, ΔVacA) reduced CK2β, HDM2, and p53 expression levels. Increases in expression of p-HDM2 and Snail were also found following infection with the CagA positive strains (Fig. 4C). Furthermore, the low expression of HDM2 and p53 was reversed by MG132 treatment, suggesting that the downregulation of HDM2 and p53 was caused by proteasomal degradation (Fig. 4D). To further confirm that CagA induces CK2β degradation, we transfected AGS cells with CK2 siRNA and then infected them with *H. pylori* 60190. Similar to the results in Fig 4C, p-HDM2 and Snail increased and CK2β and p53 decreased in the *H. pylori* 60190-infected cells (Fig. 4E). We also found that Snail increased and CK2β and p53 decreased in CK2β-deficient cells. Interestingly, the levels of CK2-regulated factors changed dramatically in *H. pylori* 60190-infected CK2β knockdown cells. As previously reported in other cell lines, the p-AKT (Ser473) level was increased by CK2β knockdown, but the p-AKT (Ser129) level was decreased [45]. We also found that CagA-induced CK2β degradation altered the phosphorylation ratio between the two canonical AKT activation sites (pSer473 increased slightly, and pSer129 decreased strongly) in AGS cells (Fig. 4E, lane 1 *vs*. lane 5). Furthermore, p-AKT (Ser473) increased, and p-AKT (Ser129) decreased, but Snail levels

did not change in *H. pylori* 60190-infected CK2β-overexpressing cells (Fig. 4F). To determine whether p53 regulates Snail expression in *H. pylori*-infected gastric cancer cells, we transfected AGS cells with p53 siRNA and p53 overexpression vector. The Snail level increased with p53 knockdown (Fig. 4G, left panel, lane 1 *vs*. lane 4); however, the Snail level decreased in p53 overexpressing cells (Fig. 4G, right panel, lane 1 *vs*. lane 4). Thus, the expression of Snail in gastric cancer cells increased with p53 knockdown or *H. pylori*-mediated CK2β degradation (Fig. 4G).





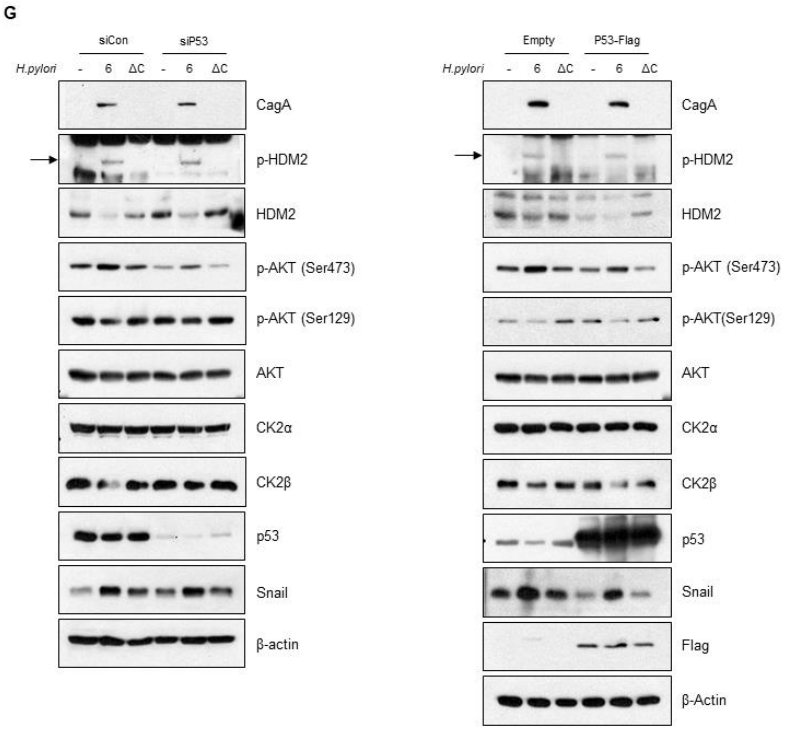


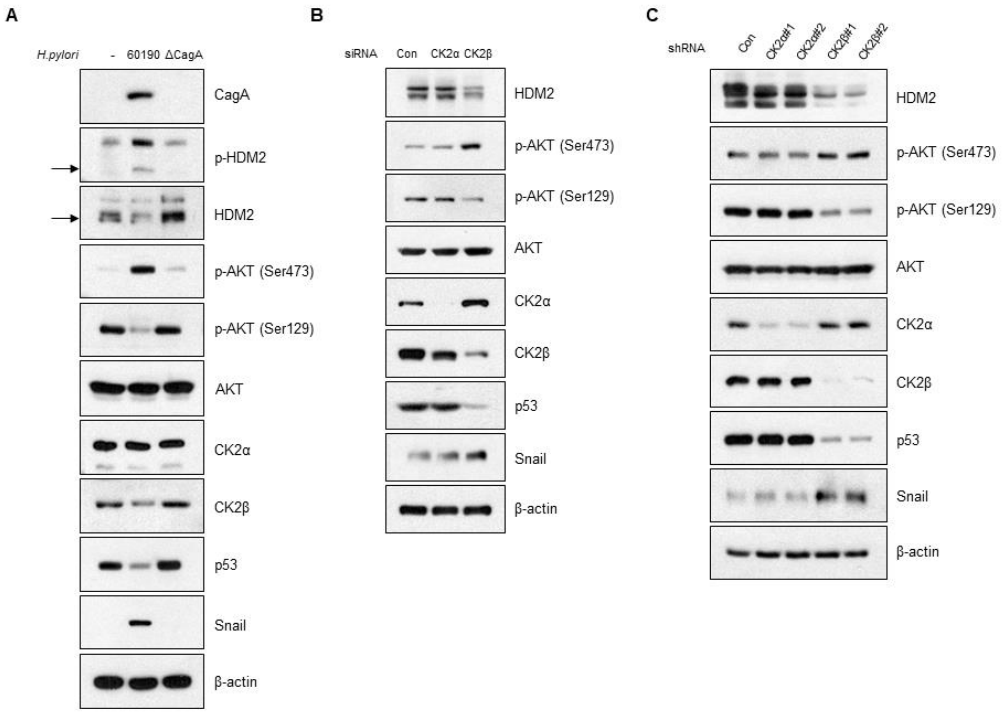
Figure 4. *H. pylori* mediates CK2β ubiquitination in an HDM2-dependent manner. (A-F) The expression of each protein was determined by western blotting. (A, B) AGS cells were transfected with HDM2 siRNA or Myc-tagged HDM2 vector for 48 hours. Subsequently, the cells were exposed to *H. pylori* 60190 or *H. pylori* ΔCagA for 6 hours. β-actin was used as the loading control. (C) AGS cells were infected with several *H. pylori* strains for 6 hours. (D) AGS cells were infected with *H. pylori* 60190 or *H. pylori* ΔCagA for 6 hours in the presence or absence of MG132 (25 μM). (E, F) AGS cells were transfected with CK2α siRNA or CK2β siRNA or with HA-tagged CK2α or Myc-tagged CK2β for 48 hours. Subsequently, the cells were infected with *H. pylori* 60190 for 6 hours. (G) AGS cells were transfected with p53 siRNA or Flag-tagged p53 for 48 hours and then infected with *H. pylori* 60190 or *H. pylori* ΔCagA for 6 hours.

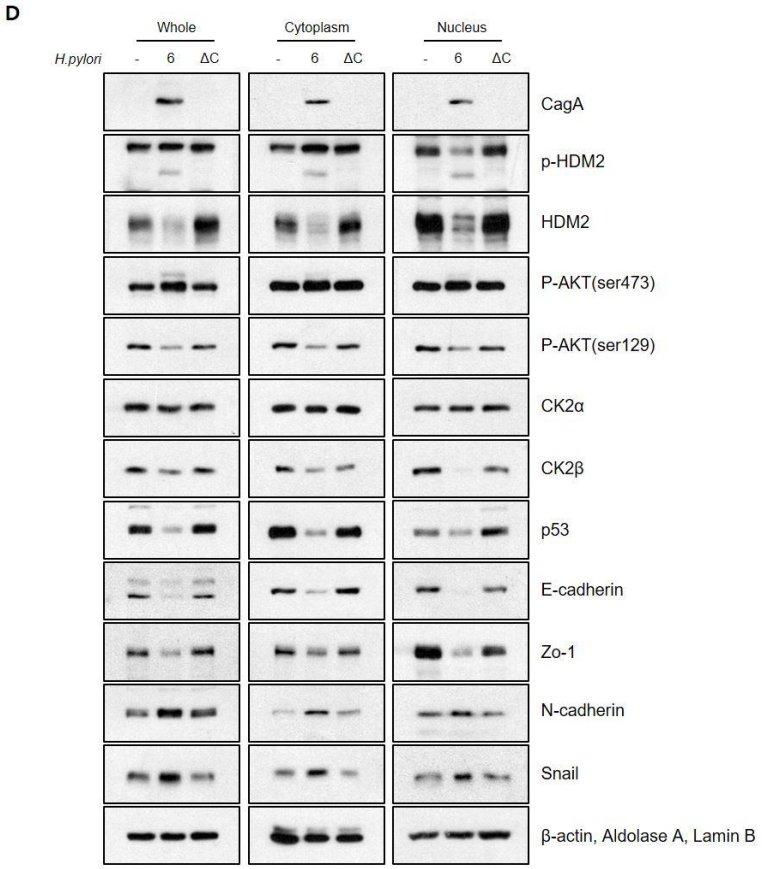
5. CK2β-downregulation promotes the expression of EMT markers and tumor growth

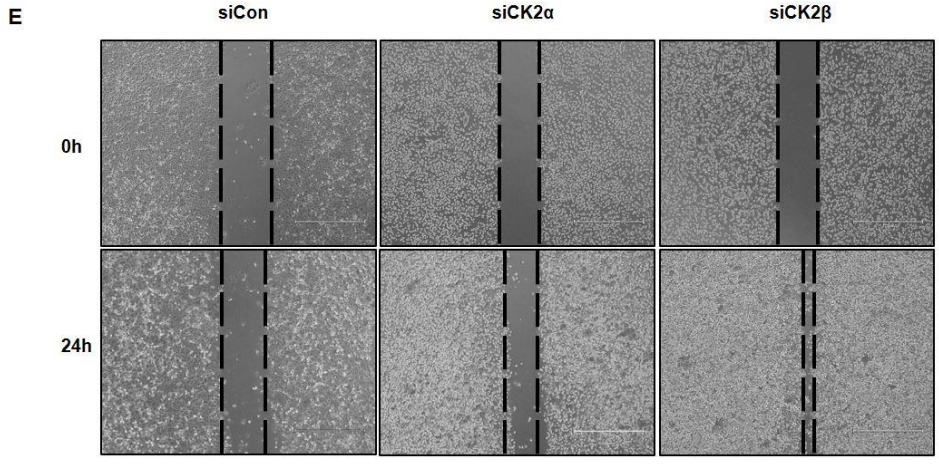
in vitro

We searched for the mechanism by which *H. pylori* CagA regulates CK2β-related protein expression *in vitro*. We infected AGS cells with the *H. pylori* CagA-positive strain (60190) or CagA-negative (ΔCagA) strain. *H. pylori* 60190 upregulated the expression of p-HDM2, p-AKT (Ser473), and Snail and downregulated p-AKT (Ser129) and p53 expression (Fig. 5A). Similarly, when we knocked down CK2α or CK2β in AGS cells using siRNA or shRNA, we found that p-AKT (Ser473) and Snail increased and p-AKT (Ser129) and p53 decreased in CK2β-deficient cells (Fig. 5B and 5C). To identify the CK2-related changes in different intracellular locations, we performed a nuclear-cytoplasmic fractionation assay. CK2β, HDM2, and p53 levels decreased in both the nucleus and cytoplasm of AGS cells infected with the *H. pylori* CagA-positive strain (60190) (Fig. 5D). We also found that p-HDM2 and p-AKT (Ser473) increased; however, the p-AKT (Ser129) level decreased in both the nucleus and cytoplasm of *H. pylori* 60190-infected AGS cells. Next, we analyzed the effect of CK2β downregulation on the expression of EMT markers, E-cadherin and Zo-1 (epithelial markers) and N-cadherin and Snail (mesenchymal markers) in *H. pylori* 60190-infected AGS cells. E-cadherin and Zo-1 decreased and N-cadherin and Snail increased in the nucleus and cytoplasm, as well as in whole cells of *H. pylori* 60190-infected AGS cells (Fig. 5D). This suggests that the downregulation of CK2β leads to EMT changes in CagA-dependent manner. Our previous studies showed that *H. pylori* increases cellular invasiveness and motility through CK2 activation [9-10, 48]. To better define the phenotypic changes induced by CK2β downregulation, we performed a wound healing assay in CK2-deficient gastric cancer cells. At 24 hours after wound stimulation, the migration of AGS and MKN74 cells increased dramatically when CK2β was inhibited (Fig. 5E and 5F, Supplementary Fig. S3A and S3B). In addition, we performed a colony formation assay in gastric cancer cells deficient in CK2α or CK2β. The rate of cell survival increased in CK2β-knockdown cells (Fig. 5G). Also, the cell survival rate increased in CagA WT-overexpressing gastric cancer cells (Supplementary Fig. S3C). Therefore, knockdown of the CK2β subunits markedly increased the proliferation and migratory

potential of gastric cancer cells *in vitro*.







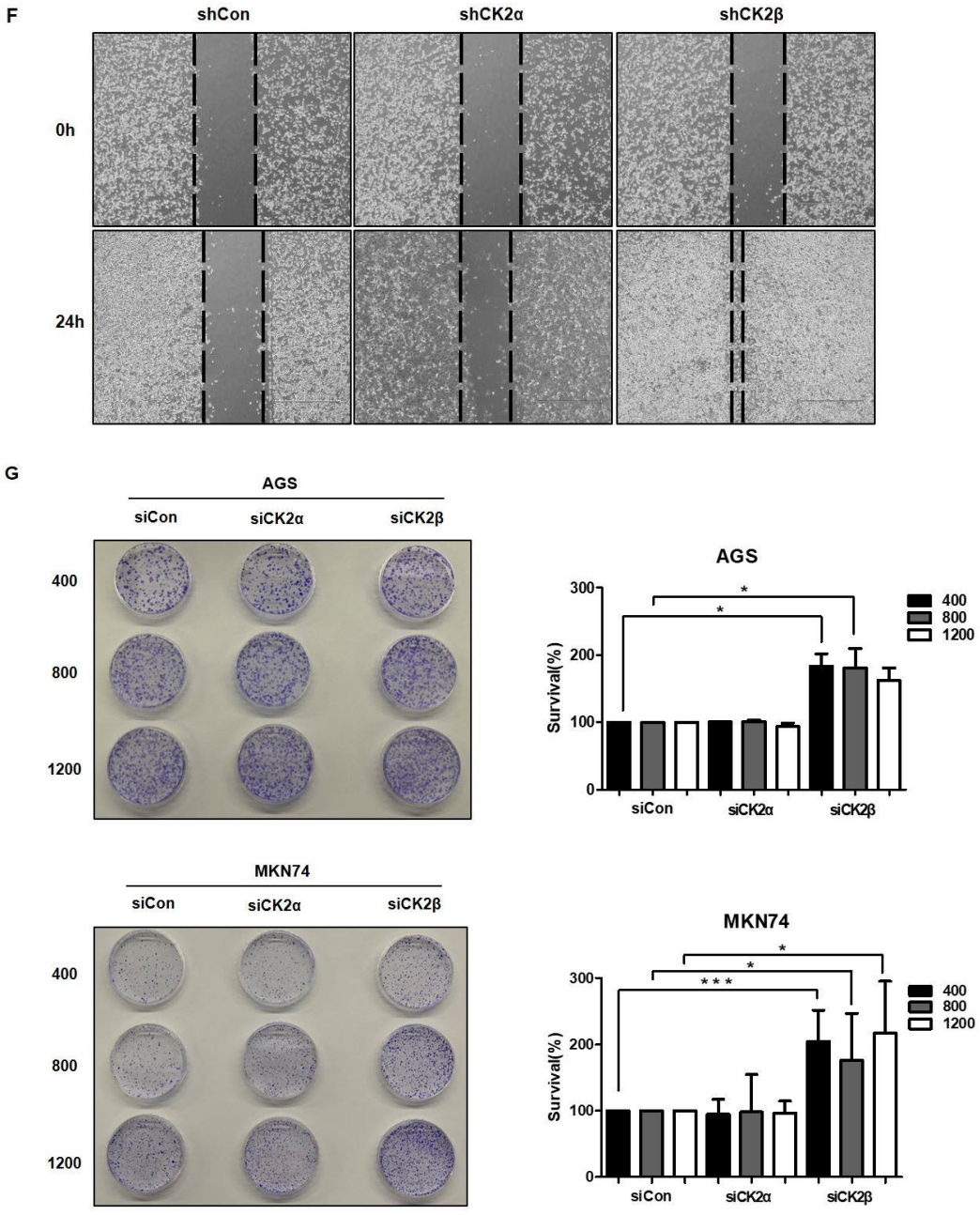


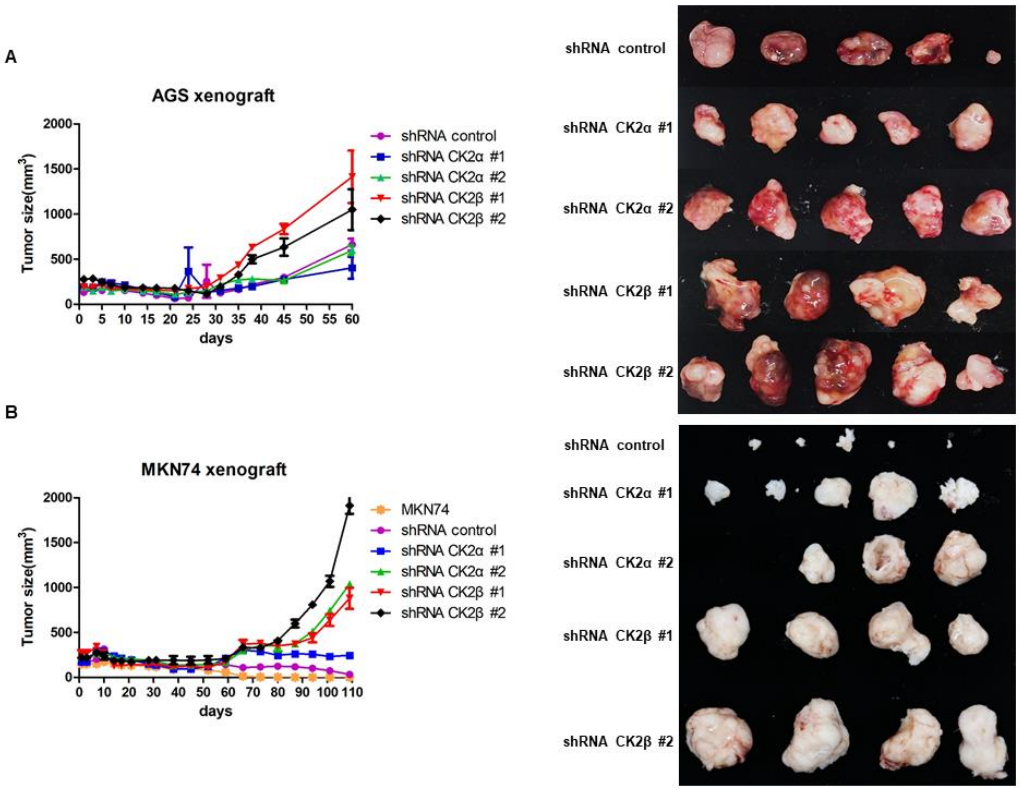
Figure 5. ***H. pylori*** **regulates the HDM2-AKT-p53-Snail axis, to induce the epithelial-mesenchymal transition.** (A-G) The expression of each protein was analyzed by western

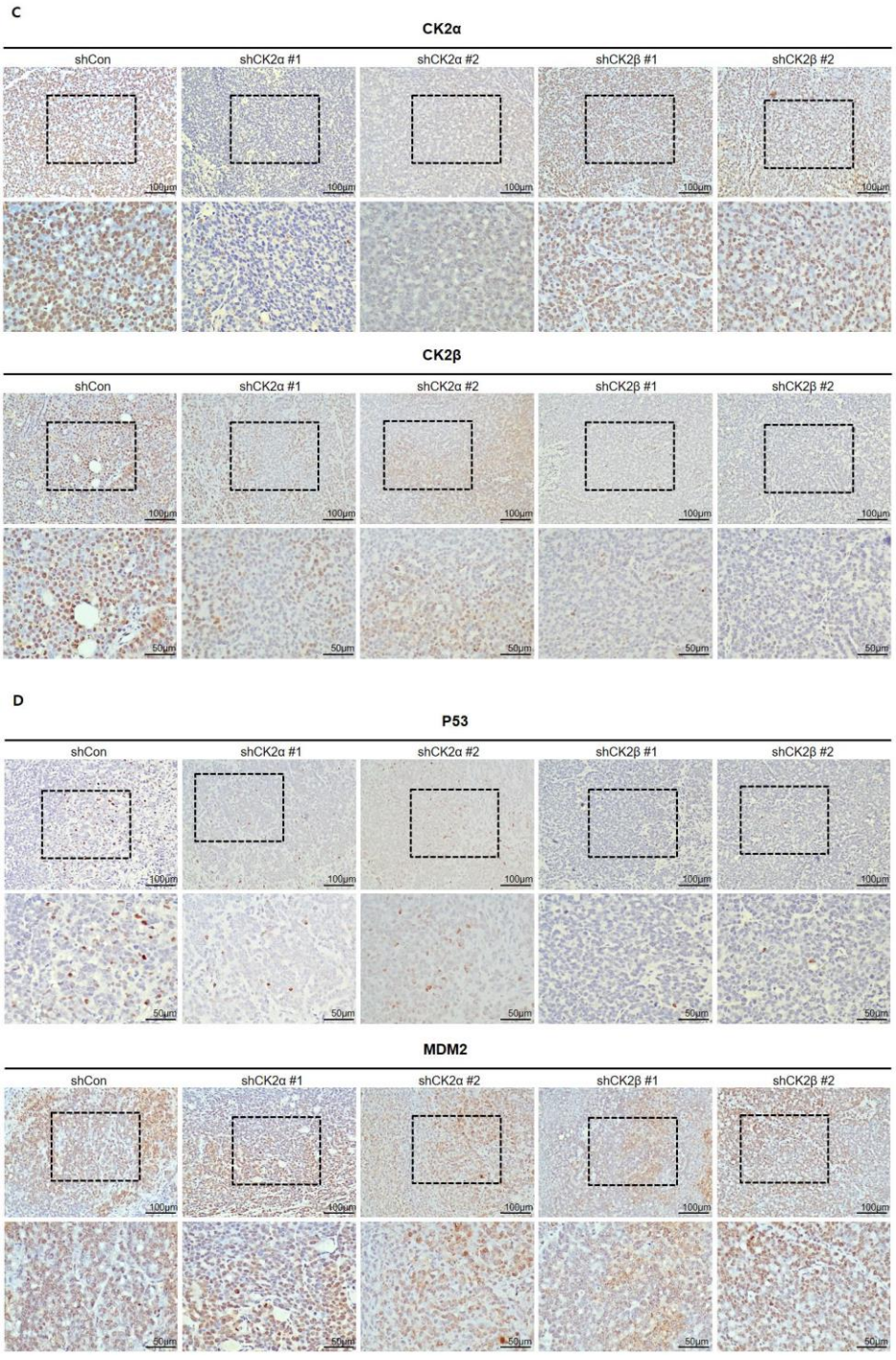
blotting and functional assays. (A) AGS cells were infected with *H. pylori* 60190 or *H. pylori* ΔCagA for 6 hours. β-actin was used as the loading control. (B) AGS cells were transfected with control siRNA, CK2α siRNA, or CK2β siRNA for 48 hours. (C) AGS-control (shCon) and CK2-silenced cells (shCK2α#1, #2 and shCK2β#1, #2) were incubated for 48 hours. (D) AGS cells were infected with *H. pylori* 60190 for 6 hours and then analyzed using a nuclear-cytoplasmic fractionation reagent kit. A sample of each cytoplasmic and nuclear extract was analyzed by western blotting. (E-F) The wound healing assay was used to determine the cell migration ability of gastric cancer cells. The images were captured 24 hours after scratching. (E) AGS cells were transfected with CK2α siRNA or CK2β siRNA for 48 hours. (F) AGS-control (shCon), and CK2 silenced cells (shCK2α#1, #2 and shCK2β#1, #2) were incubated for 48 hours. (G) The colony formation ability of AGS and MKN74 cells transfected with CK2α siRNA or CK2β siRNA for 48 hours. The survival rate was measured 10 days after transfection. Each experiment was performed in triplicate (right panel, ns, $P > 0.05$; *, $P < 0.05$; ***, $P < 0.001$).

6. CK2β downregulation correlates with tumorigenesis *in vivo*

Our *in vivo* animal study used AGS and MKN74 xenograft mouse models to evaluate the effect of CK2β downregulation on gastric tumorigenesis. Tumor growth and weight were significantly increased in the CK2β-deficient xenograft mouse models (Fig. 6A and 6B, Supplementary Fig. S4A). We then used western blotting to measure the levels of CK2, MDM2, and p53 protein in those tumors. In agreement with our *in vitro* data, p53 expression significantly decreased in tumors formed from CK2β-deficient cells, compared with those from control or CK2α-deficient cells (Supplementary Fig. S4B). We performed IHC staining of CK2α, CK2β, p53, and MDM2 in CK2-deficient xenograft mouse tumor samples (Fig. 6C and 6D). As a result, we found that the level of p53 decreased, but MDM2 increased in the CK2β-deficient mouse groups (Fig. 6E). To dissect the potential relationship between CK2α or CK2β expression and *H. pylori* infection in human gastric cancer, 54 paired human gastric cancer tissues and adjacent normal tissues were analyzed

using IHC. The number of CK2β-positive cells was higher in the *H. pylori*-positive cancer tissues than the *H. pylori*-positive normal tissues (Fig. 6F). Interestingly, the CK2α- and CK2β-positive cells are increased in the *H. pylori*-infected gastric cancer tissues compare to non-infected cancer tissues. This result is not consistent with the *in vitro* studies. We postulated that *H. pylori*-mediated CK2β downregulation may participate in the neoplastic progression. Once gastric cancer is established, the CK2 regulation might be deranged and this is why both CK2α and CK2β expression is increased in *H. pylori*-infected gastric cancer tissues. Indeed, the percentage of CK2β-positive cells decreased significantly in *H. pylori*-positive normal gastric tissues than in *H. pylori*-negative normal tissues; though the level of CK2α-positive cells did not show any changes in *H. pylori*-negative normal tissues compared with *H. pylori*-negative normal tissues. Taken together, our results suggest that CK2β downregulation mediates EMT activation and tumorigenesis in 54 paired gastric cancer cells.





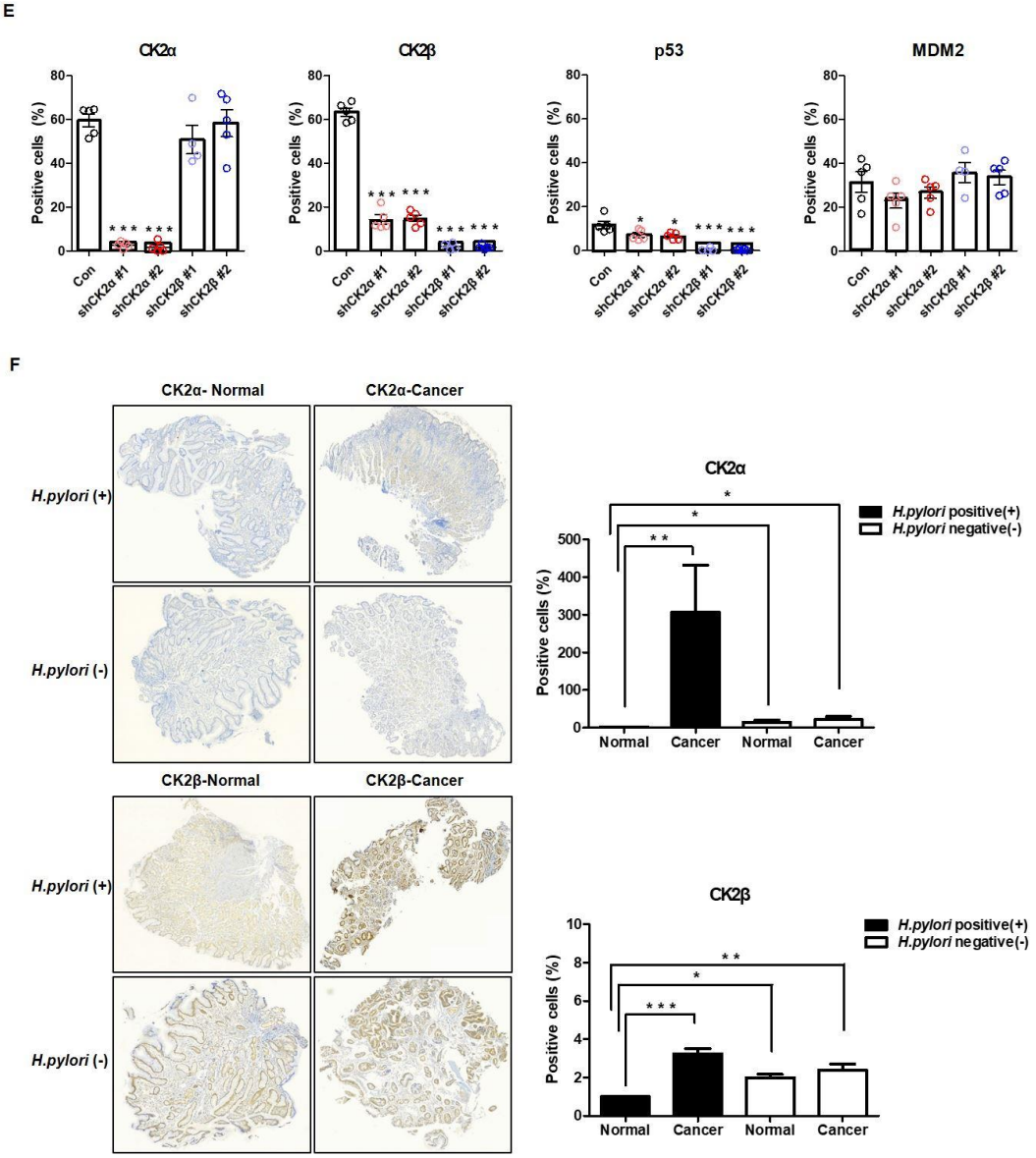


Figure 6. Tumor growth increase in xenograft mouse model upon CK2β downregulation and *H. pylori* infection regulates CK2α and CK2β expression in human gastric cancer. (A, B) Tumor size after subcutaneous injection of mice with AGS-control (shCon) or CK2-silenced cells (shCK2α#1, #2 and shCK2β#1, #2) and MKN74-

control (shCon) or CK2-silenced cells (shCK2α#1, #2 and shCK2β#1, #2). The tumor size was measured every 1-2 weeks for 60-110 days. Mice were sacrificed 2-4 months after the injection. (C, D) Immunohistochemical detection of CK2α, CK2β, p53, and MDM2 expression in AGS xenograft mouse tissue. (E) The percentage of DAB-positive cells in AGS xenograft tissue was measured in a 20× high-power field. Data are presented as the mean ± SEM (n = 4-5 per group). (F) Immunohistochemical detection of CK2α (upper) and CK2β (lower) expression in non-infected and *H. pylori*-infected gastric cancer tissues. We measured the positive cells, and each experiment was performed in triplicate (right panel, ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

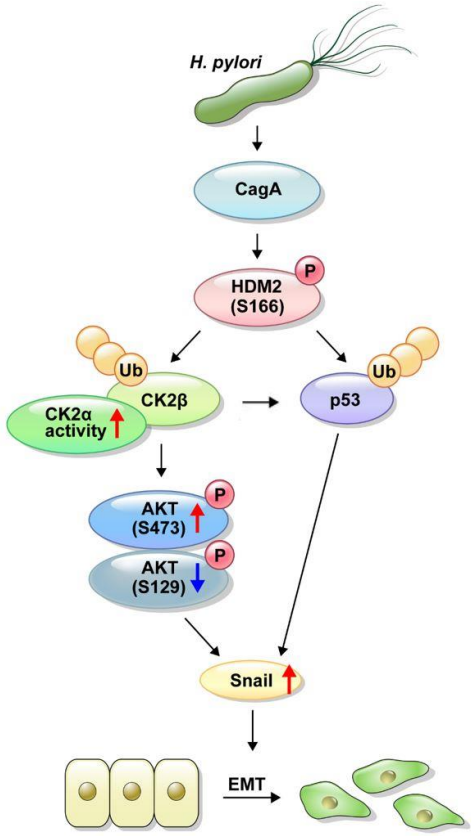


Figure 7. Schematic diagram of *H. pylori* CagA-mediated CK2β downregulation and EMT activation.

IV. DISCUSSION

In this work, we found that CagA from *H. pylori* infection significantly reduced CK2β expression by modifying HDM2 in gastric cancer cells, and that reduction was associated with gastric tumorigenesis and the EMT. In our previous study, CK2 activity increased in *H. pylori*-infected gastric cancer cells, but CK2α expression did not change [41]. Indeed, as Casein kinase showed the display an array of various activity including proliferation, migration, metabolism and so on. It is postulated that CK2 related to various activities are variable and dynamic in nature depending on the substrate specificity, conformational changes, cell types and intracellular location as well. We also found that p53, HDM2, E-cadherin, and Zo-1 were downregulated, and Snail and N-cadherin were upregulated when CK2β was silenced (Fig. 5D). A schematic diagram of the signaling pathway is depicted in Fig. 7. CK2β plays a global role in the control of cell growth, proliferation, and cell death. Notably, nuclear CK2β expression was higher in gastric cancer tissues than in normal tissues [38, 46-48]. Moreover, CK2 is known to cooperate with GSK3β in the phosphorylation of Snail, a transcription factor that plays a key role in initializing and maintaining the EMT process [31]. The EMT, which is part of normal cellular plasticity, is defined by a loss of epithelial cell morphology, reduced expression of proteins mediating cell-to-cell contact, remodeling of the actin cytoskeleton, and acquisition of a mesenchymal cell shape. It involves multiple signaling pathways in which several protein kinases act as key regulators [30]. Phosphorylation operated by the CK2 holoenzyme and GSK3β targets the transcription factor Snail for degradation; downregulation of CK2β is sufficient to stabilize Snail and induce an EMT-like phenotype in normal human breast epithelial cells [38]. We believe that CK2 activation in *H. pylori* mediated gastric cancer cell is mainly due to increased cellular level of free form CK2α unbound to CK2β. And this free form CK2α mediated kinase activity may be an important factor mediating EMT activation by *H. pylori* in gastric cancer**.** We also observed that EMT-related markers (Snail) increased in CK2β-deficient gastric cancer cells (Fig. 4E).

Numerous studies have shown that HDM2 phosphorylation increases along with the total

protein levels of HDM2, inducing the proteasomal degradation of p53 [14, 19]. In contrast, other studies have shown that increased HDM2 phosphorylation is not accompanied by an increase in the total protein level of HDM2 or decreased p53 [13, 24]. Our data show that CagA increased the phosphorylation of HDM2, which induced the downregulation of p53 and HDM2 (Fig. 4A). Recently, the CK2-induced phosphorylation of HDM2 has been shown to influence the p53-HDM2 interaction. Although HDM2 regulates both the stability of p53 and its transactivation functions, those processes are not regulated by the CK2 phosphorylation of HDM2 [48].

We employed *in vivo* xenograft mouse models and immunohistochemical analysis of human gastric cancer tissue microarray to further explore our findings in human gastric cancer. As expected, tumor growth and weight were significantly increased in the xenograft mouse models using CK2β-deficient AGS and MKN 74 cell lines (Fig. 6A and 6B). Also, we confirmed that p53 level were decreased while MDM2 expression increased in the CK2β-deficient tumor tissue which were consistent with our hypothesis (Figure 6E). Next, we analyzed 54 paired human gastric cancer and normal tissues for the confirmation of CK2α and CK2β expression in relation to *H. pylori* infection in human (Fig. 6F). Interestingly, the CK2α- and CK2β-positive cells are increased in the *H. pylori*-infected gastric cancer tissues compare to non-infected cancer tissues. Although this result is somewhat inconsistent with our *in vitro* data, we believe that *H. pylori*-mediated CK2β downregulation may play a role in preneoplastic process. Therefore, in established gastric cancer tissues, the CK2 regulation might be deranged causing simultaneous CK2α and CK2β overexpression in *H. pylori*-infected gastric cancer tissues. Nevertheless, the CK2β-positive cells decreased significantly in *H. pylori*-positive normal tissues compared to *H. pylori*-negative normal tissues while CK2α-positive cells did not show any changes. Therefore, our results suggest that CK2β downregulation mediates EMT activation and tumorigenesis in *H. pylori*-infected gastric cancer cells.

Our study is the first to evaluate the mechanism of CK2 activation by *H. pylori* in gastric cancer cells. CK2 activation is directly related to CK2β degradation by CagA-induced

HDM2 phosphorylation in *H. pylori*-infected gastric cancer cells (Fig. 4A). *H. pylori* infection is reported to be associated with increased levels of p53 and HDM2 proteins in tissue from gastric cancer patients [49]; however, our data provide solid evidence that *H. pylori* phosphorylates HDM2, thereby downregulating p53 expression in gastric cancer. Interestingly, the levels of MDM2 increased in CK2β-deficient mice (Fig. 6D and 6E). As already reported and similar to other cell lines [45], CK2β downregulation altered the phosphorylation ratio between the two canonical AKT activation sites (pSer473 slightly increased, pSer129 strongly reduced) in HK-2 cells. We also found that CK2β downregulation increased AKT Ser473 phosphorylation and decreased AKT Ser129 phosphorylation in AGS cells. By affecting the balance of AKT phospho-sites, the three-dimensional structure of CK2 present in the cells apparently has somewhat consequential effects on the phosphorylation of AKT substrates. That finding is particularly relevant because unbalanced CK2 subunit expression has been suggested to occur in cancer, which implies the existence of free catalytic subunits in tumor cells. In summary, we have shown that the downregulation of CK2β in *H. pylori*-infected gastric cancer cells promotes the EMT in CagA-dependent manner.

V. CONCLUSION

This study demonstrates that *H. pylori*-induced CagA is required to reduce the CK2β protein. In the presence of the *H. pylori* CagA-positive strains, CK2β underwent proteasomal degradation in HDM2-dependent manner. Furthermore, CK2 may be a key mediator of EMT in *H. pylori*-infected gastric cancer and may serve as a molecular target for CK2 inhibition in gastric cancer therapy.

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APPENDICES

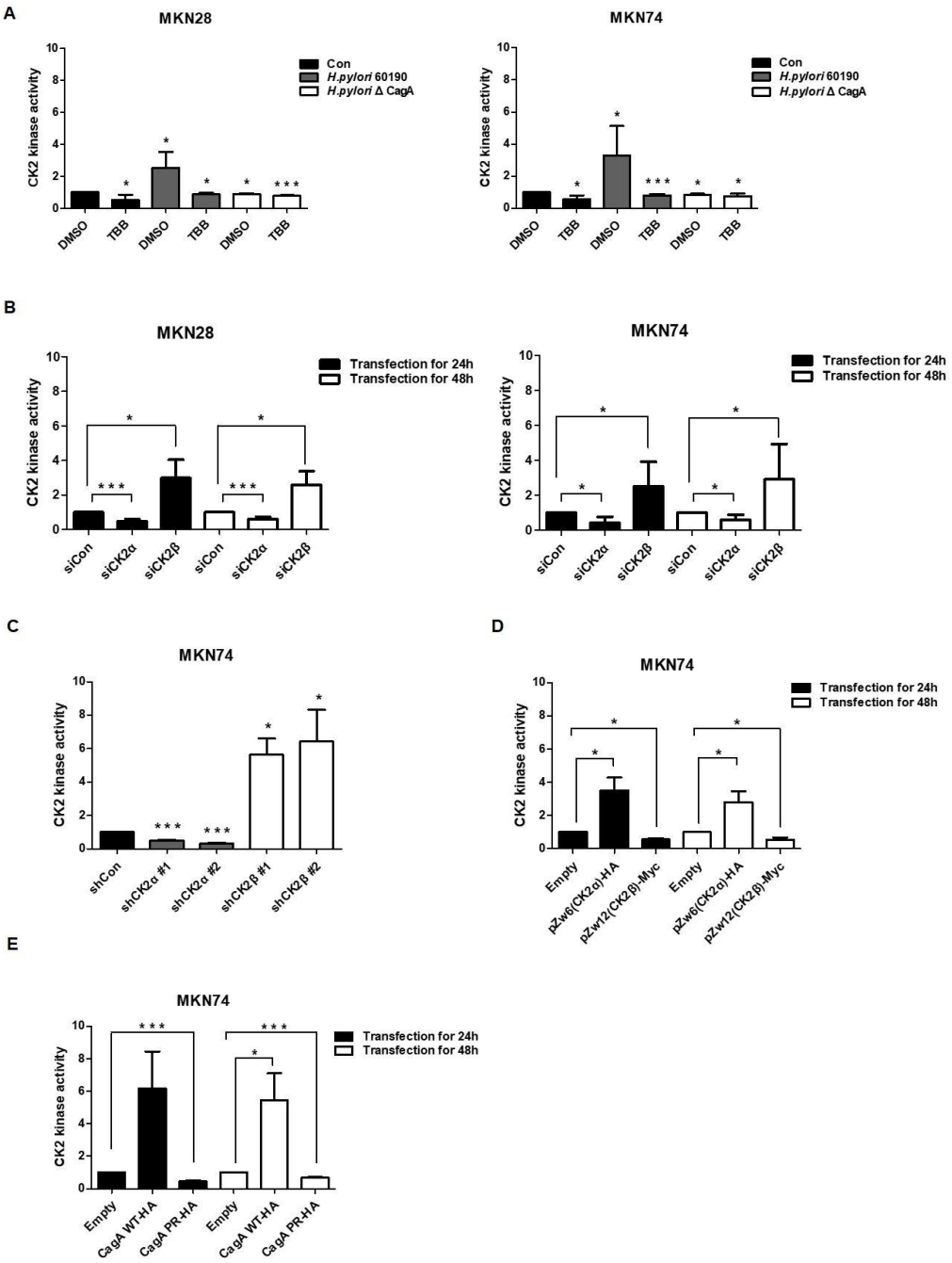


Figure S1. (A-E) CK2 activity was determined using a kinase assay kit. (A) MKN28 and MKN74 cells were infected with *H. pylori* 60190 or *H. pylori* ΔCagA and co-treated with a CK2 inhibitor (100 μM) for 6 hours. The kinase activity was quantified using GraphPad Prism software (ns, $P > 0.05$; *, $P < 0.05$; ***, $P < 0.001$). (B) MKN28 and MKN74 cells were transfected with control siRNA, CK2α siRNA, or CK2β siRNA for 24 or 48 hours. (C) MKN74-control (shCon) and CK2-silenced cells (shCK2α#1, #2 and shCK2β#1, #2) were incubated for 48 hours. (D) MKN74 cells were transfected with HA-tagged CK2α or Myc-tagged CK2β expression vectors for 24 or 48 hours. (E) MKN74 cells were transfected with HA-tagged CagA WT or CagA mutant expression vectors for 24 or 48 hours.

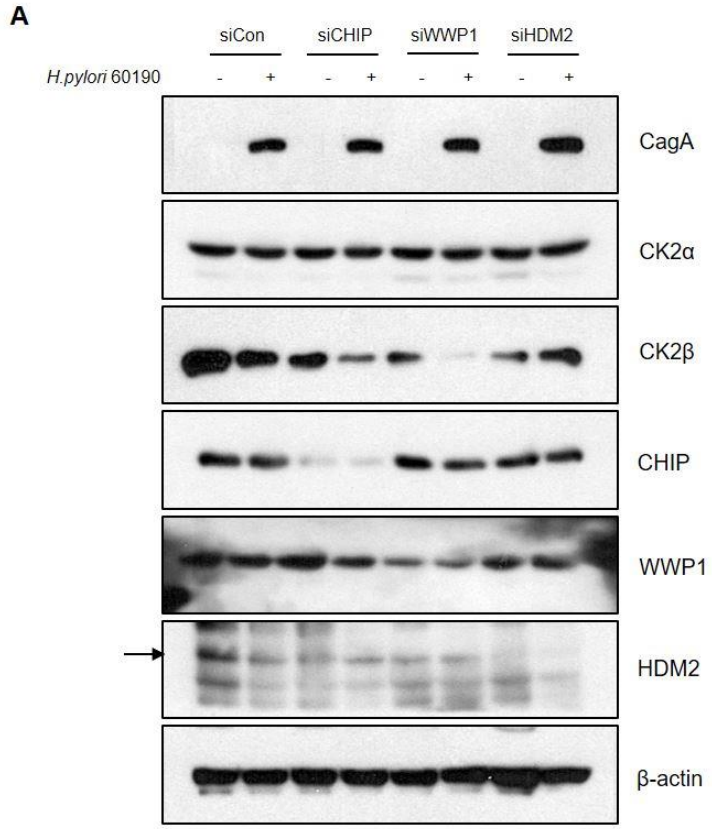
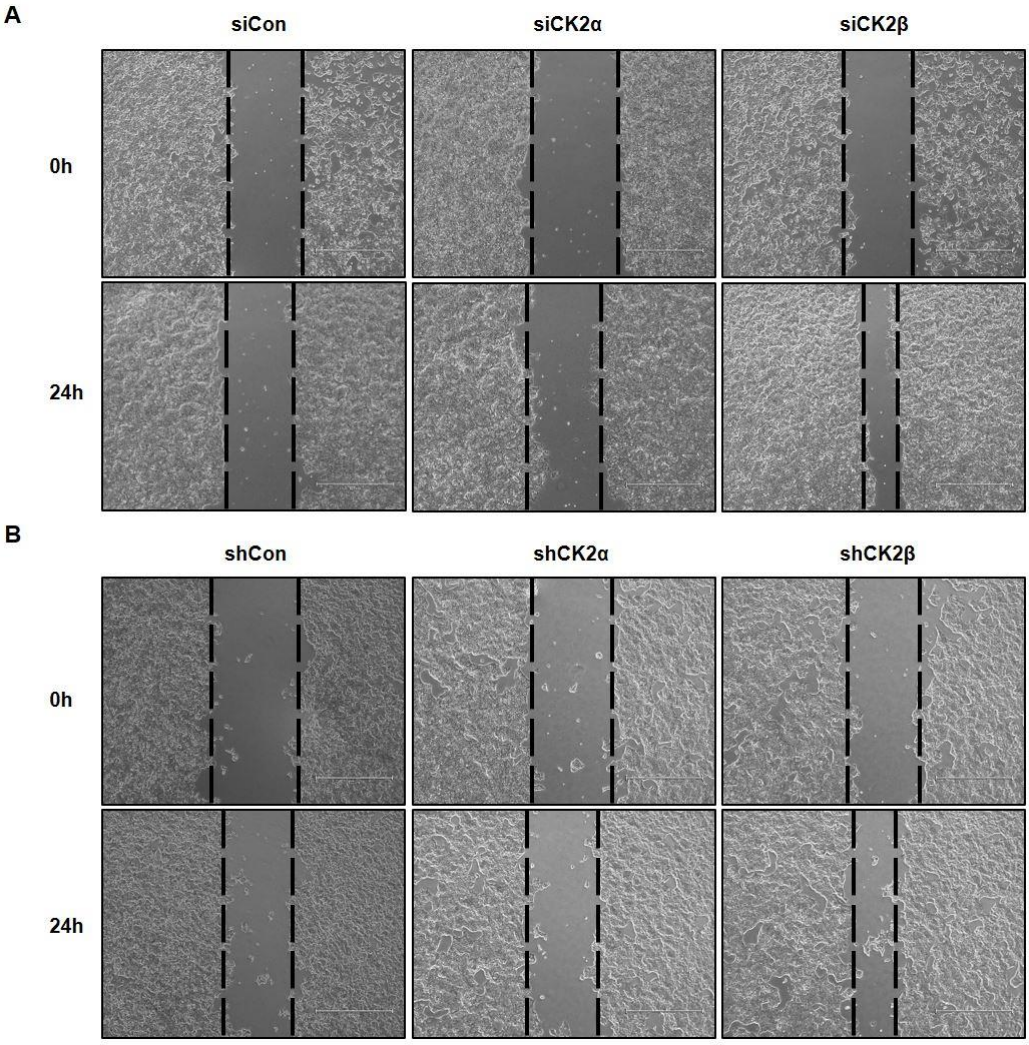


Figure S2. (A) AGS cells were transfected with control siRNA, CHIP siRNA, WWP1 siRNA, or HDM2 siRNA for 48 hours. β-actin was used as the loading control.



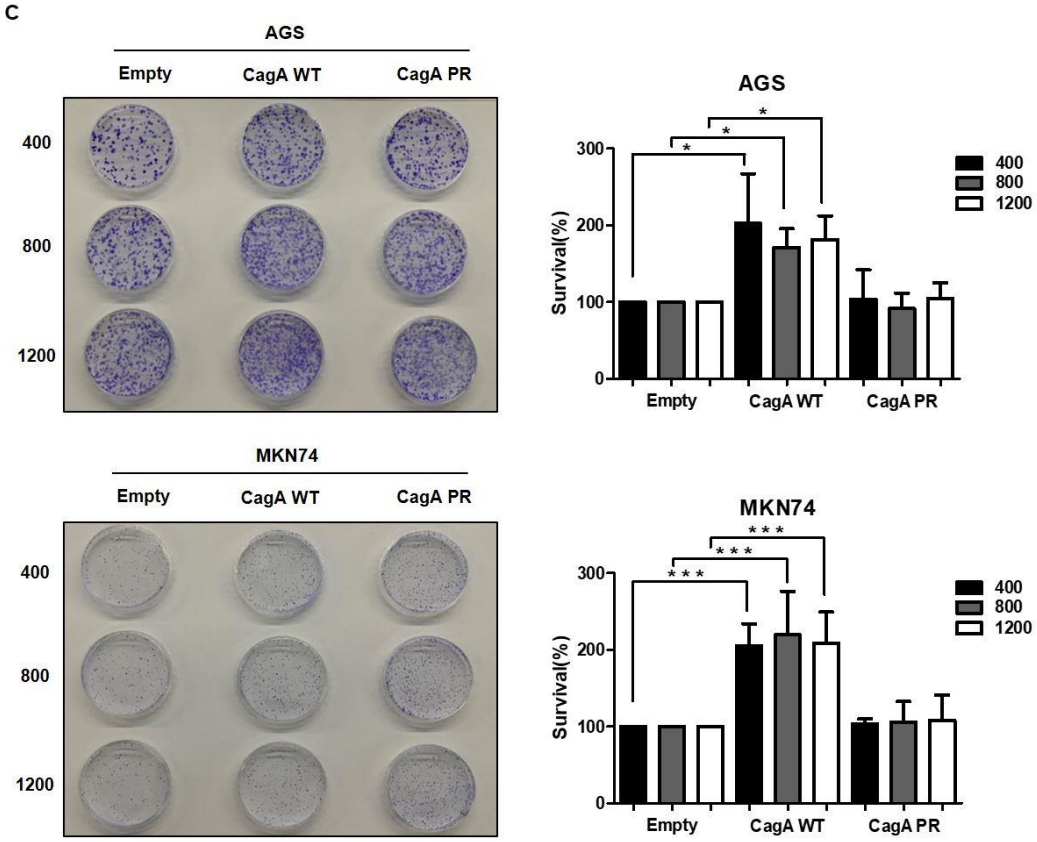


Figure S3. (A-B) The wound healing assay was used to determine the cell migration ability of gastric cancer cells. The images were captured 24 hours after scratching. (A) MKN74 cells were transfected with CK2α siRNA or CK2β siRNA for 48 hours. (B) MKN74-control (shCon), and CK2 silenced cells (shCK2α#1, #2 and shCK2β#1, #2) were incubated for 48 hours. (C) The survival rate of AGS and MKN74 cells after transfection with CagA WT and CagA mutant expression vectors was measured using a clonogenic assay. The survival rate was quantified using GraphPad Prism software (ns, $P > 0.05$; *, $P < 0.05$; ***, $P < 0.001$).

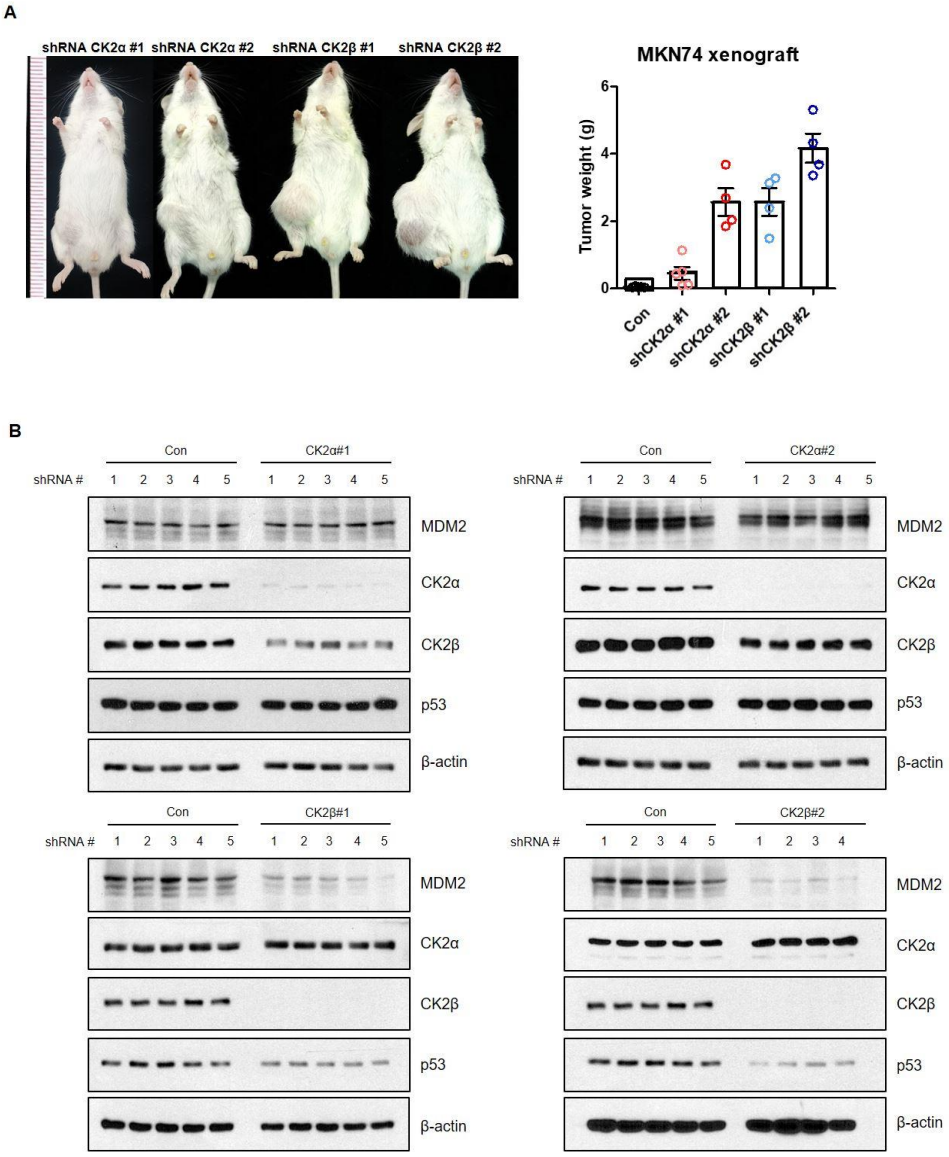


Figure S4. (A) Tumor size after subcutaneous injection of mice with MKN74-silenced cells (shCK2α#1, #2 and shCK2β#1, #2). The MKN74 xenograft mice were sacrificed 4 months after injection, and the tumor weight was measured. Data are presented as the mean ± SEM (n = 4-5 per group). (B) The expression of CK2α, CK2β, p53, and MDM2 in AGS xenograft mouse samples was analyzed by western blotting.

ABSTRACT(IN KOREAN)

위암세포주에서 헬리코박터 파일로리 매개 CK2β 조절에 의한 상피간엽전이 촉진의 기전 연구

<지도교수 이용찬>

연세대학교 대학원 의학과

이 소 담

내용

헬리코박터 파이로리는 대표적인 세포 독소 관련 유전자 A인 CagA를 지니고 있으며, GSK3를 불안정화시켜 상피간엽전이를 일으킨다. CK2는 카세인 인산화 효소로써 GSK3와 같은 역할을 하며 상피간엽전이와 관련된 신호 전달 경로를 통해 종양 형성에 중요한 역할을 한다. 따라서 CK2가 CagA에 의한 상피간엽전이를 조절할 수 있다고 사료된다. 본 학위논문 연구에서는 CagA가 CK2에 미치는 영향을 알아보고자 하였다. 그 결과, 위암세포주에서 헬리코박터 파이로리의 CagA 양성 균주 감염시에서만 유비퀴틴화 되어 프로테아좀에 의해 CK2β가 분해되었다. 이러한 CK2β의 감소는 CK2α 활성도를 증가시켜고, 상피간엽전이와 관련 있는 유전자인 Snail의 발현을 증가시켰다. 더 나아가 CagA에 의해 HDM2의 인산화가 증가하여 CK2β의 감소가 일어남으로써 대표적인 종양 억제 유전자인 p53의 발현을 감소시켰다. 또한 CK2β의 감소에 의해 AKT 473번 세린의 인산화 증가와 AKT 129번 세린의 인산화 감소가 일어났다. 종합적으로, 본 연구는 CagA가 위암세포주에서 존재하는 CK2β를 불안정화시킴으로써 CK2β가 암의 치료제로서 적용 가능함을 시사한다.

핵심되는 말: CK2; 헬리코박터 파일로리; CagA; 상피간엽전이; 암