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Downregulation of tumor suppressor RACK1 by *Helicobacter pylori* infection promotes gastric carcinogenesis through the integrin β-1/NF-κB signaling pathway

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ABSTRACT

Receptor of activated protein kinase C 1 (RACK1) is downregulated in gastric cancer and is involved in modulating NF-κB signaling pathway activity. However, the underlying molecular mechanisms regulating RACK1 expression are unclear. In this study, we demonstrated that downregulated expression of RACK1 was observed in gastric cancer tissue compared to adjacent normal tissue and was correlated with poor prognosis in patients. *Helicobacter pylori* (*H. pylori*) infection downregulated RACK1 expression in concert with canonical NF-κB signaling pathway activation *in vivo* and *in vitro*. RACK1 overexpression suppressed NF-κB signaling pathway activation as well as the release of downstream proinflammatory cytokines. In addition, RACK1 downregulation increased integrin β-1 expression, while integrin β-1 silencing decreased NF-κB signaling activation. Moreover, *H. pylori* infection downregulated RACK1 but upregulated integrin β-1 expression at the precancerous lesion stages in human subjects. Our data indicate that *H. pylori* infection promotes the upregulation of integrin β-1 expression via downregulation of RACK1 expression, which subsequently leads to the elevated activation of the NF-κB signaling pathway, an essential step in *H. pylori*-induced carcinogenesis.

1. Introduction

Gastric cancer (GC) remains the fifth most frequently diagnosed cancer and the third leading cause of cancer-related death [1]. Receptor of activated protein kinase C 1 (RACK1), a 36-kDa cytosolic scaffold protein and member of the Trp-Asp (WD) repeat protein family, has been reported to have a suppressive effect on GC by negatively regulating the WNT and NF-κB signaling pathways [2,3]. Additionally, we analyzed The Cancer Genome Atlas (TCGA) and found that RACK1 expression was decreased in GC tissues in comparison with the corresponding normal tissues [4]. However, the mechanism underlying RACK1 expression regulation remains unclear.

Helicobacter pylori (*H. pylori*) is defined as a type-1 carcinogen and the major etiological factor for GC [5,6]. *H. pylori* infection causes chronic active gastritis in all infected subjects, which can progress to gastric atrophy, intestinal metaplasia, dysplasia and ultimately GC in a subset of individuals [7]. *H. pylori* infection-initiated gastric inflammation is believed to play an important role in *H. pylori*-induced carcinogenesis. Various virulence factors of *H. pylori* interact with the receptors or targets of the host, leading to the activation of inflammatory signaling pathways (e.g., the NF-κB signaling pathway) and the subsequent release of proinflammatory cytokines, which is a major step in the initiation and development of GC [8,9]. We also found that the NF-κB signaling pathway is positively associated with *H. pylori* infection through Gene Set Enrichment Analysis of differentially expressed genes between *H. pylori*-positive and *H. pylori*-negative GC from

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the TCGA database [4]. It has been documented that numerous regulators are involved in modulating the NF-κB signaling pathway [10]. Fan et al. [11] demonstrated that RACK1 acted as a novel negative regulator of the NF-κB signaling pathway, and subsequently inhibited cytokine induction and inflammatory reactions. Moreover, Zimmermann et al. [12] identified RACK1 as a negative regulator involved in *H. pylori*-induced NF-κB pathway activation by analyzing the nuclear translocation of P65 in AGS cells stimulated with *H. pylori*, TNF-α and IL-1β, although the underlying mechanisms were unclear.

Integrins are transmembrane receptors consisting of eighteen α-subunits and eight β-subunits that play essential roles in physiological functions and pathological progression (e.g., immune response, cell cycle progression, cell death, invasion, metastasis and angiogenesis) [13,14]. Moreover, interactions of integrins with *H. pylori* virulence factors activate downstream signaling pathways with oncogenic activity, which contributes to *H. pylori* pathogenesis [15,16]. RACK1 interacts with different beta-integrins and participates in regulating integrin functions [17]. However, the mechanistic linkage among *H. pylori* infection, RACK1 expression, beta-integrins and the NF-κB signaling pathway has not been reported.

This study aimed to investigate the role of RACK1, integrins and the NF-κB signaling pathway in *H. pylori*-induced carcinogenesis. Our results indicate that *H. pylori* downregulates the tumor suppressor RACK1 and subsequently upregulates integrin β-1. This process leads to the activation of the NF-κB signaling pathway and the release of proinflammatory cytokines, which can act as an essential step in promoting *H. pylori* carcinogenesis.

2. Materials and methods

2.1. Patients and tissue specimens

A tissue array (No. HStm-Ade180 Sur-02) containing 90 GC and paired normal tissues was obtained from Shanghai Outdo Biotech Co., LTD. Fresh primary GC specimens with adjacent normal tissues were collected during surgery from 23 patients who were not receiving any adjuvant therapy between December 2015 and April 2016 at The First Affiliated Hospital of Nanchang University. An additional 79 gastric tissue samples were collected from patients who underwent gastroduodenoscopy at the same hospital between July 2016 and February 2017, including 25 subjects with chronic gastritis (12 *H. pylori*-positive and 13 *H. pylori*-negative samples), 23 subjects with intestinal metaplasia (12 *H. pylori*-positive and 11 *H. pylori*-negative samples), and 31 subjects with dysplasia (17 *H. pylori*-positive and 14 *H. pylori*-negative samples). Pathologic scores were graded by pathologists using H&E staining according to the criteria of the World Health Organization [18] and the updated Sydney system [19]. *H. pylori* status was evaluated using a rapid urease test and immunohistochemistry. Sequence data from 49 GC cases (16 *H. pylori*-positive and 33 *H. pylori*-negative cases) and 35 cancer-adjacent normal cases were downloaded from the TCGA database. Informed consent was obtained from all patients, and all experiments were carried out according to the guidelines of the Ethics Committee of The First Affiliated Hospital of Nanchang University.

2.2. Cell lines and H. pylori

The immortalized human gastric cell line GES-1 was cultured in DMEM (Thermo Scientific HyClone, Beijing, China), while the AGS (Boster Biological Technology, Wuhan, China) and HGC-27 cell lines (Beijing Institute for Cancer Research, Beijing, China) were cultured in RPMI-1640 (Thermo Scientific HyClone, Beijing, China). All cell lines were cultured in medium supplemented with 10% fetal bovine serum (FBS), 100 U penicillin, and 100 μg/ml streptomycin (Gibco of Thermo Fisher ScientificInc., Waltham, USA) at 37 °C in an atmosphere of 5% CO_2. The wild-type strain *H. pylori* ATCC43504 (*cagA*$^+$) was obtained from the National Institute for Communicable Disease Control and Prevention, Chinese Centers for Disease Control and Prevention (Beijing, China). *cagA*$^+$ and *cagA*$^-$ *H. pylori* strains 7.13 were kindly provided by Dr. Richard Peek Jr. from the Vanderbilt Digestive Disease Research Center (Nashville, USA). *vacA*$^+$ and *vacA*$^-$ *H. pylori* strains 26695 were kindly provided by Dr. Chun-Hui Lan from Daping Hospital of the Army Medical University (Chongqing, China). *cagA*$^+$ *H. pylori* ATCC43504 and 7.13 strains and *vacA*$^+$ and *vacA*$^-$ *H. pylori* strains 26695 were cultured on Campylobacter agar plates containing 10% sheep serum at 37 °C under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2) for 24 h and then subcultured in Brucella broth supplemented with 10% FBS at 37 °C under a microaerophilic atmosphere for 16–18 h, as previously described [20]. *cagA*$^-$ *H. pylori* strain 7.13 was also cultured under the same conditions with 20 μg/ml kanamycin (Sigma-Aldrich). The bacterial densities of the *H. pylori* ATCC43504 and 7.13 strains were measured as previously described [20,21]. The bacterial density of *H. pylori* strain 26695 was estimated spectrophotometrically at an absorbance of 600 nm (OD_{600}), and viable counts were determined as colony-forming units (CFU)/ml (1 $OD_{600} = 10^9$ CFU/ml).

2.3. Infection of Mongolian gerbils with H. pylori

Specific pathogen-free male Mongolian gerbils (6–8 weeks, 30–50 g) were obtained from the Zhejiang Academy of Medical Sciences (Hangzhou, China) and maintained in an isolated clean room with a regulated temperature (20–22 °C), humidity (approximately 55%), and 12/12-h light/dark cycle with ad libitum rodent diet and water. Animal care and experimental protocols were in accordance with guidelines established by the Institutional Animal Care and Use Committee of Nanchang University. After one week of observation, the Mongolian gerbils were fasted for 12 h prior to the challenge. Subsequently, the gerbils were challenged using orogastric infusions of 500 μl of sterile Brucella broth (n = 8), 2×10^9 CFU/ml *cagA*$^+$ *H. pylori* strain 7.13 (n = 8) or 2×10^9 CFU/ml *cagA*$^-$ *H. pylori* strain 7.13 mutant (n = 8) once every 2 days for a total of 5 infusions. After the challenge, the Mongolian gerbils were fasted for 4 h. The animals were euthanized at 3 months post-challenge, and the gastric tissues were harvested for the following analyses.

2.4. Reagents and lentivirus

Cells were treated with different concentrations of the NF-κB signaling pathway activator recombinant human TNF-α (Sino Biological Inc, 10602-HNAE). GES-1 cells stably expressing RACK1 or empty vector were obtained from Novibio Biotechnology Inc. (Shanghai, China). Flag- and His-tagged intergrin β-1 plasmids and intergrin β-1 and control shRNA were purchased from Vigene Bioscience (Shandong, China). RACK1 shRNA and control shRNA were obtained from GENECHEM (Shanghai, China). GC cells in the exponential growth phase were plated into 6-well plates for 24 h and transfected with plasmids using FuGENE 6 (Promega, E2691) according to the manufacturer's protocol.

2.5. Immunoblotting

Western blotting (WB) was performed according to a standard method described previously [22] and using the following antibodies: anti-NF-κB P65 (Abcam, #32536, 1:10000), anti-phospho-NF-κB P65 (Ser 536) (CST, #3033, 1:1000), anti-IκBα (CST, #9242, 1:1000), anti-RACK1 (CST, #5432, 1:1000), anti-integrin β-1 (CST, #9699, 1:1000), anti-β-actin (TRANS, L30704, 1:1000) and anti-GAPDH (Boster Biological Technology, BA2913, 1:1000).

2.6. Immunohistochemistry

Immunohistochemistry (IHC) was performed on the tissue arrays,

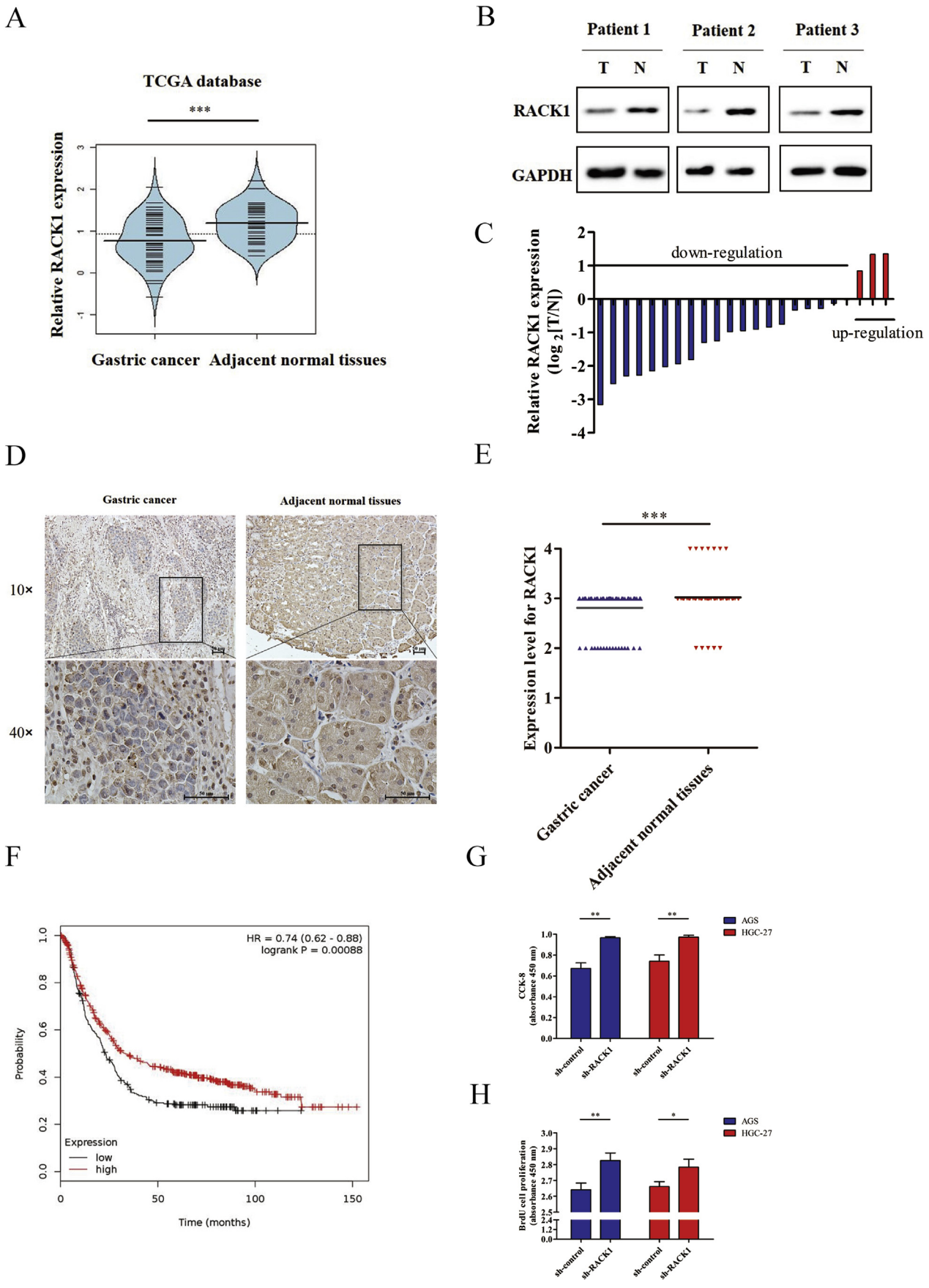

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Fig. 1. RACK1 is downregulated in GC tissues and is associated with poor prognosis in GC patients. (A) Normalized expression value of RACK1 (log 2 transformation and Z correction) in 49 GC tissues and 35 adjacent normal tissues from the TCGA database. (B) The expression level of RACK1 was measured in GC and the corresponding noncancerous tissues by WB. GAPDH was used as an internal control. (C) The graph summarizes the WB results from 23 GC and paired normal tissues. (D) IHC staining of RACK1 in GC and adjacent normal tissues from the tissue microarrays. Original magnification, 10 × and 40 × . (E) Tissue microarrays were stained with antibodies against RACK1, immunoreactive cells positive for RACK1 were semiquantitatively assessed, and the RACK1 protein expression levels are expressed as grades 1–4 in GC and adjacent normal tissues. Mean grades (−) for protein expression are shown. (F) Kaplan-Meier survival plots show the prognosis for 876 GC patients with high or low RACK expression. HR = 0.74, $P = 0.00088$. Cancer cell proliferation capacity was detected by CCK-8 (G) and BrdU (H) assays in GC cells transfected with the sh-RACK1 or sh-control plasmid. Scale bar = 50 μm $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

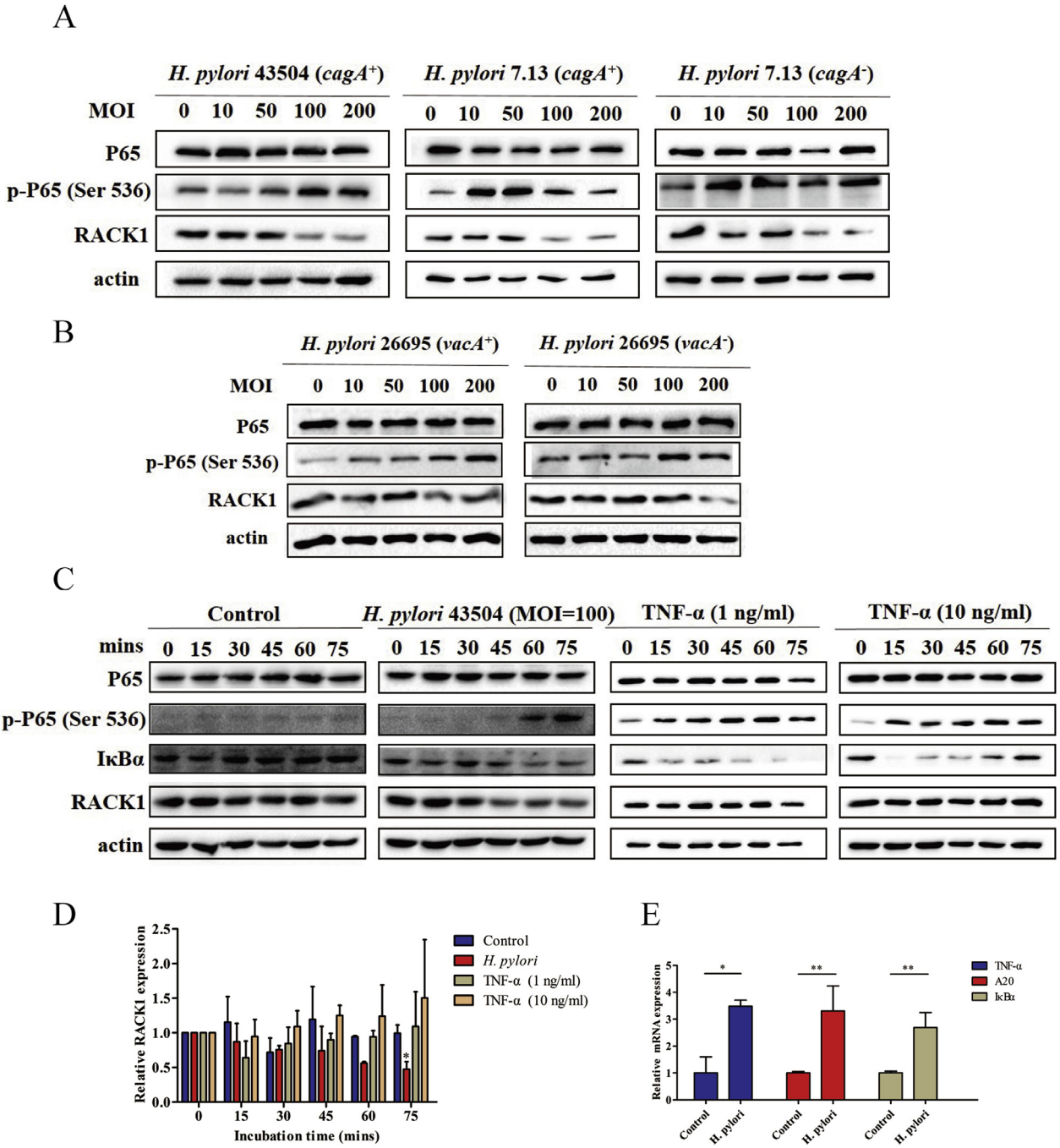

Fig. 2. *H. pylori* infection downregulated RACK1 in gastric epithelial cells independent of CagA and VacA and activated the NF-κB signaling pathway *in vitro*. (A–B) WB analysis of RACK1, P65 and p-P65 (Ser 536) in GES-1 cells cocultured with *H. pylori* strains at different MOIs (0, 10, 50, 100 and 200). (C) WB analysis of RACK1, P65, p-P65 (Ser 536) and IκBα in GES-1 cells with or without *H. pylori* strain 43504 and stimulated with TNF-α (1 ng/ml or 10 ng/ml) from 0 to 75 min. (D) The graph summarizes the relative RACK1 protein expression levels in GES-1 cells subjected to different stimulation. (E) mRNA expression of TNF-α, A20, and IκBα in GES-1 cells cocultured with *H. pylori* strain 43504 from 0 to 75 min $^{*}P < 0.05$, $^{**}P < 0.01$.

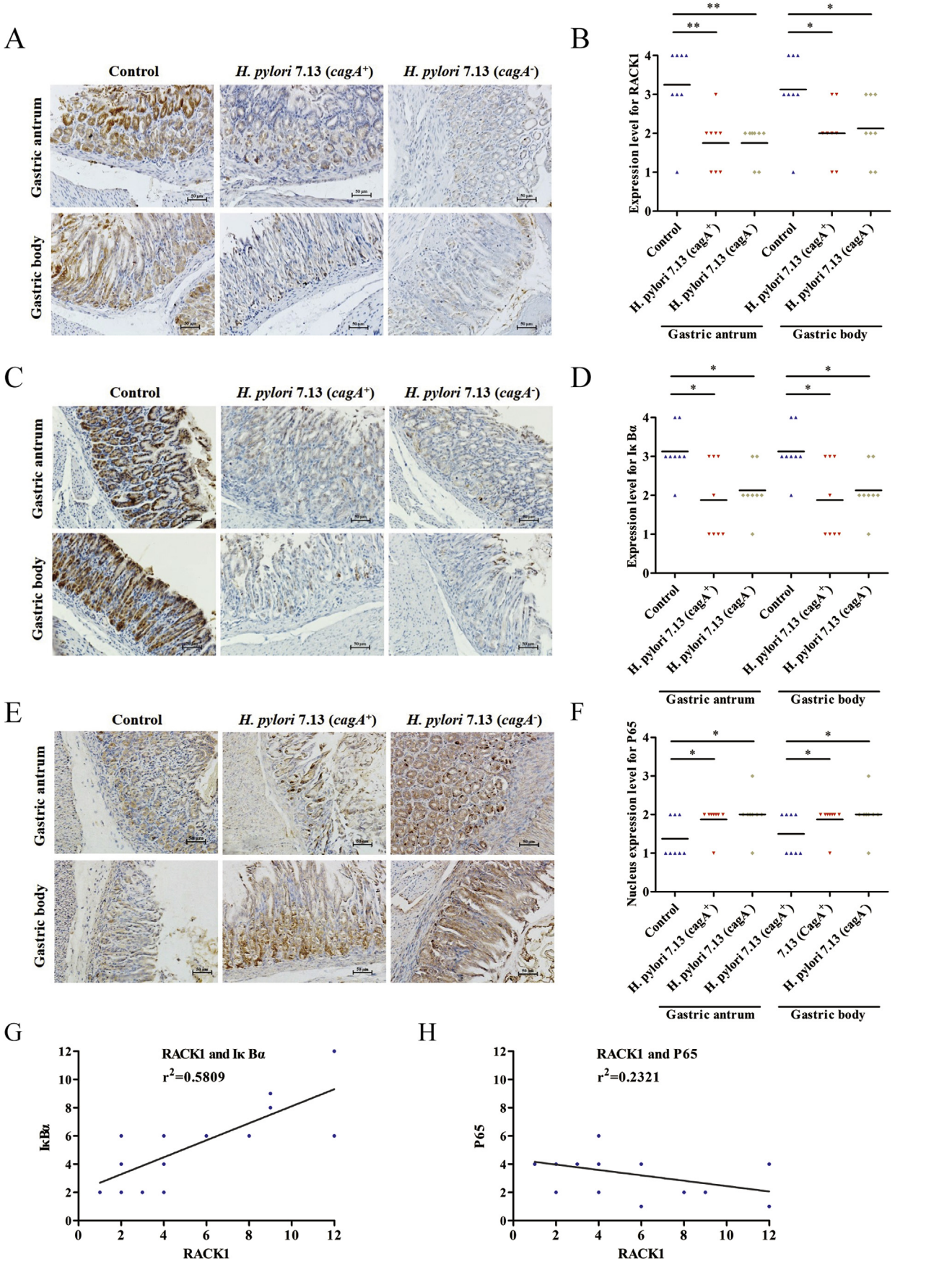

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Fig. 3. *H. pylori* infection downregulated RACK1 in gastric epithelial cells independent of CagA and activated the NF-κB signaling pathway *in vivo*. IHC staining of RACK1 (A), IκBα (C) and nuclear P65 (E) in gastric tissues collected from Mongolian gerbils that were orogastrically challenged with Brucella broth, *cagA*$^+$ *H. pylori* 7.13 strain or cagA$^-$ *H. pylori* 7.13 strain. Graphs B, D and F summarize the IHC staining results for RACK1, IκBα and nuclear P65, respectively. (G–H) Positive associations between RACK1 and IκBα and negative associations between RACK1 and nuclear P65 were detected. Original magnification, 20 × , Scale bar = 50 μm *$P < 0.05$, **$P < 0.01$.

paraffin sections of human biopsy specimens and Mongolian gerbil gastric tissues according to a standard method described previously [22] and using the following antibodies: anti-NF-κB P65 (Abcam, #32536, 1:200), anti-NF-κB P65 (phospho S536) (Abcam, # 131109, 1:200), anti-IκBα (Abcam, #32518, 1:100), anti-RACK1 (Abcam, #129084, 1:100), and anti-integrin β-1 (Abcam, #179471, 1:1400). A total of 100 cells were counted in five random fields, and the scores were evaluated based on the ratio and intensity of stained cells as described previously [20].

2.7. Quantitative real-time PCR analysis

Total RNA from gastric cell lines was isolated using an RNAprep Pure Cell Kit (TIANGEN, DP430) and reverse-transcribed into cDNA using a FastKing RT Kit (TIANGEN, KR116). Quantitative real-time PCR (qRT-PCR) was performed using a SYBR® Premix Ex Taq™ II Kit (Takara, RR820A) and StepOnePlus™ Real-Time PCR System (Applied Biosystems). The primers for *TNF-α* were 5′-CCTCTTCTTCCTTCCTGA TCG-3' (forward) and 5′-ATCACTCCAAAGTGCAGCAG-3' (reverse). The primers for *IL-8* were 5′-TCTGGCAACCCTAGTCTGCT-3' (forward) and 5′-AAACCAAGGCACAGTGGAAC-3' (reverse). The primers for *A20* were 5′-ATGCACCGATACACACTGGA-3' (forward) and 5′-GGATGATCTCCC GAAACTGA-3' (reverse). The primers for *IκBα* were 5′-GCTGATGTCA ATGCTCAGGA-3' (forward) and 5′-CCCCACACTTCAACAGGAGT-3' (reverse). The primers for *GAPDH* were 5′-ACAGTCAGCCGCATCTT CTT-3' (forward) and 5′-ACGACCAAATCCGTTGACTC-3' (reverse). The primers for *Urease A* were 5′- TGTTGGCGACAGACCGGTTCAAATC-3' (forward) and 5′- GCTGTCCCGCTCGCAATGTCTAAGC-3' (reverse).

2.8. Cell counting kit-8 (CCK-8) and bromodeoxyuridine (BrdU) cell proliferation assay

AGS and HGC-27 GC cells (100 μl, 1×10^5 cells/ml) in the exponential growth phase were seeded into 96-well plates and incubated at 37 °C and 5% CO_2 for 24 h and then transfected with plasmids. After culture for another 48 h, 10 μl of CCK-8 solution (TransGen Biotech, China) was added to each well, followed by incubation under the above conditions for 2 h. Optical density values were measured at a wavelength of 450 nm using a Molecular Devices SpectraMax M2^e.

Following transfection with RACK1 shRNA or control shRNA for 48 h, the GC cells were incubated with BrdU solution for 12 h and processed according to the instructions of the BrdU assay kit (CST, #6873). Absorbance was measured at 450 nm using a Molecular Devices SpectraMax M2^e.

2.9. Luciferase activity assays

Cells were transfected with the NF-κB-RE vector (Promega, N1111) and the pGL 4.13 vector (Promega, E6681) using FuGENE 6 and co-cultured with *H. pylori* under different conditions. Luciferase activity assays were performed using a Nano-Glo® Dual-Luciferase® Reporter Assay System (Promega, N1610). Each transfection was performed in triplicate.

2.10. Statistical analysis

The data are summarized as the mean ± SD of three independent experiments. Chi-square tests were performed to evaluate differences in categorical variables. One-way analysis of variance (ANOVA) was used to determine the differences in numerical variables. Mann-Whitney tests were used to determine the differences in numerical variables between differently defined groups. Kaplan-Meier survival curves and log-rank (Mantel-Cox) tests were used for survival analysis. The results were considered statistically significant at $P < 0.05$.

3. Results

3.1. RACK1 is downregulated in GC tissues and is associated with poor prognosis in GC patients

To explore the role of RACK1 in gastric carcinogenesis, we analyzed the TCGA database and found that RACK1 expression was markedly lower in GC tissues than in the adjacent normal tissues ($P < 0.001$, Fig. 1A). We further examined RACK1 expression in 23 pairs of human gastric cancer samples using WB and in 90 pairs of human GC samples in a tissue array using IHC. WB data showed that RACK1 was downregulated in 87.0% (20/23) of GC patients compared with that in matched normal tissues ($P < 0.001$, Fig. 1B–C). Consistent with this result, the microarray data showed that GC tissues had significantly lower levels of RACK1 expression than the adjacent normal tissues ($P < 0.001$, Fig. 1D–E). The Kaplan-Meier plotter database (www.kmplot.com) was utilized to analyze the association between RACK1 expression and overall survival in 876 GC patients. We observed that lower expression levels of RACK1 (n = 256 vs. n = 620) were significantly associated with poor prognosis (log-rank $P = 0.00088$, Fig. 1F). Moreover, CCK-8 and BrdU proliferation assays showed that RACK1 downregulation significantly promoted GC cell growth ($P < 0.05$, Supplementary Figs. 1A–B, Fig. 1G–H).

3.2. H. pylori infection downregulated RACK1 in gastric epithelial cells independent of CagA and VacA and activated the NF-κB signaling pathway

Because of the decreased RACK1 expression noted in GC tissues, we sought to investigate how *H. pylori* infection modulates RACK1 expression and the interaction between RACK1 expression and activation of the canonical NF-κB signaling pathway. Canonical NF-κB activation involves the phosphorylation and degradation of IκBα, which leads to the translocation of the P65 and P50 dimer from the cytoplasm to the nucleus. Subsequently, the transcription of a number of NF-κB target genes was initiated, a pivotal step in inflammation-associated carcinogenesis [9,10]. GES-1 cells were cocultured with different MOIs of wild-type *H. pylori* strains 43504, 7.13 and 26695 or isogenic *cagA*$^-$ *H. pylori* strain 7.13 or isogenic *vacA*$^-$ *H. pylori* strain 26695. WB data revealed that *H. pylori* infection (MOI = 100 or 200) downregulated RACK1 independent of CagA and VacA and promoted the induction of p-P65 (Ser 536), thus indicating canonical NF-κB signaling pathway activation (Fig. 2A–B). To further validate this result, GES-1 cells were cocultured with wild-type *H. pylori* strain 43504 (MOI = 100) or treated with TNF-α (1 ng/ml or 10 ng/ml) for 0, 15, 30, 45, 60 and 75 min. Both *H. pylori* and TNF-α stimulation promoted the production of p-P65 (Ser 536) and the degradation of IκBα, while decreased RACK1 expression was observed at 75 min in only the *H. pylori* group (Fig. 2C–D). In addition, expression of NF-κB signaling pathway target genes (*TNF-α*, *A20* and *IκBα*) was increased by *H. pylori* infection (Fig. 2E), further indicating the activation of the canonical NF-κB signaling pathway.

To further validate our *in vitro* results, Mongolian gerbils were challenged with Brucella broth, wild-type *H. pylori* strain 7.13 or isogenic *cagA*$^-$ *H. pylori* strain 7.13 for 3 months. Giemsa staining and PCR

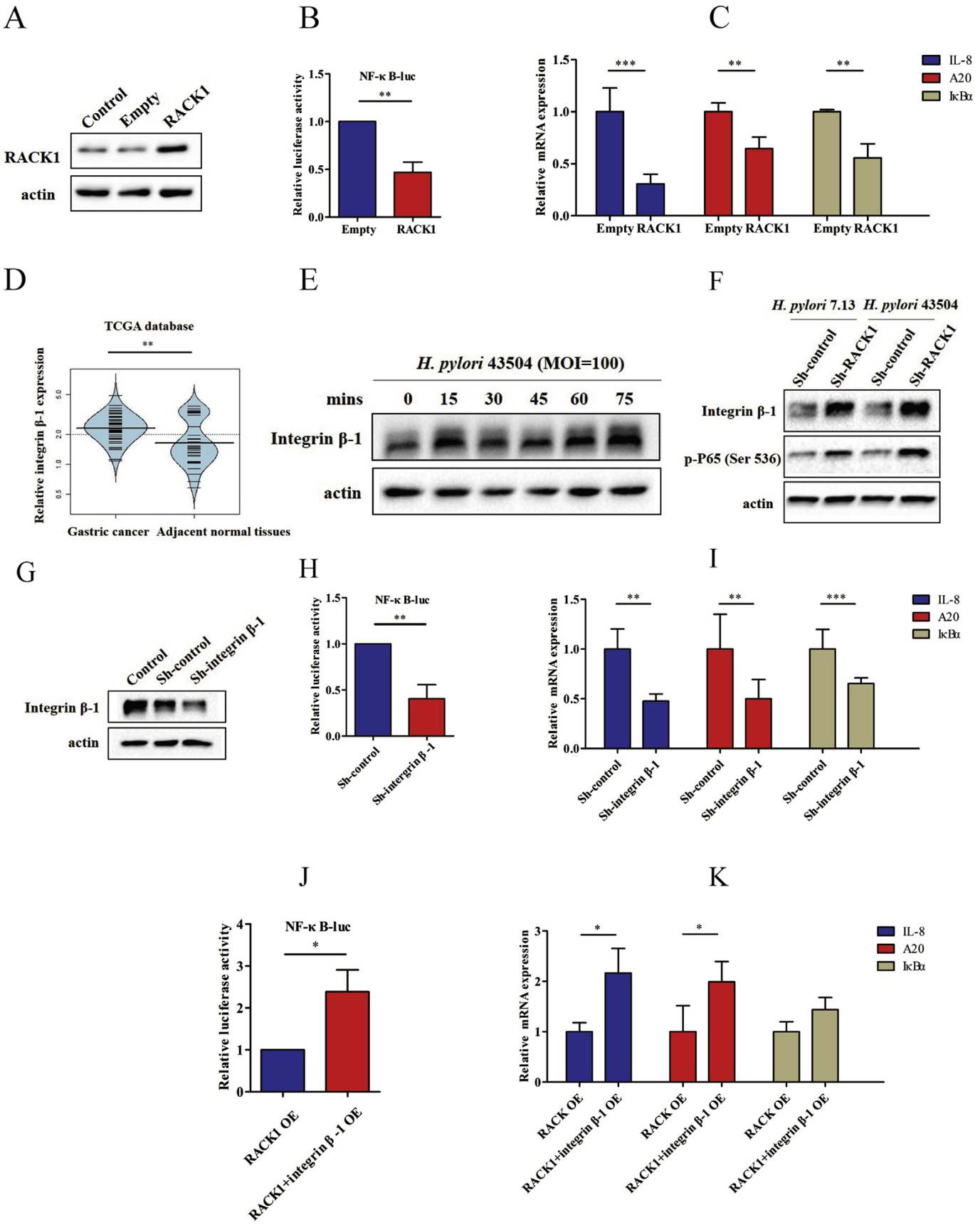

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Fig. 4. RACK1 negatively regulated *H. pylori* infection-induced NF-κB activation by modulating integrin β-1 (A) WB analysis of GES-1 cells stably overexpressing RACK1 or empty vector control. (B) Luciferase reporter assay for NF-κB-Luc in GES-1 cells overexpressing RACK1 or empty vector control stimulated with *cagA*$^+$ *H. pylori* strain 43504 (MOI = 100) for 75 min. (C) qRT-PCR analysis of IL-8, A20 and IκBα in GES-1 cells overexpressing RACK1 or empty vector control stimulated with *cagA*$^+$ *H. pylori* strain 43504 (MOI = 100) for 75 min. (D) Normalized expression value of integrin β-1 (log 2 transformation and Z correction) in GC and adjacent normal tissues from the TCGA database. (E) WB analysis of integrin β-1 in GES-1 cells cocultured with *cagA*$^+$ *H. pylori* strain 43504 (MOI = 100) from 0 to 75 min. (F) WB analysis of integrin β-1 and p-P65 (Ser 536) in AGS-1 cells with RACK1 shRNA or control shRNA stimulated with *cagA*$^+$ *H. pylori* strain 7.13 or 43504 (MOI = 100) for 75 min. (G) WB analysis of integrin β-1 in AGS cells with integrin β-1 shRNA or control shRNA. (H) Luciferase reporter assay for NF-κB-Luc in AGS cells with integrin β-1 shRNA or control shRNA stimulated with *cagA*$^+$ *H. pylori* strain 43504 (MOI = 100) for 75 min. (I) qRT-PCR analysis of IL-8, A20 and IκBα in AGS cells with integrin β-1 shRNA or control shRNA stimulated with *cagA*$^+$ *H. pylori* strain 43504 (MOI = 100) for 75 min. Luciferase reporter assay for NF-κB-Luc (J) and qRT-PCR analysis of IL-8, A20 and IκBα (K) in the following two groups: (1) AGS cells with RACK1 overexpression (OE) and (2) AGS cells with RACK1 and integrin β-1 overexpression. The cells were cocultured with *cagA*$^+$ *H. pylori* strain 43504 (MOI = 100) for 75 min $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

confirmed that *H. pylori* colonized the gerbils challenged with strain 7.13 and its *cagA*$^-$ mutant (Supplementary Figs. 2A–B). H&E staining results showed sparse neutrophils in the squamous junctions and squamous epithelial hyperplasia in a subset of animals infected with *cagA*$^+$ or *cagA*$^-$ *H. pylori* strain 7.13 (Supplementary Fig. 2C). IHC staining was performed to evaluate the expression of RACK1, IκBα and nuclear P65. The data showed that *H. pylori* infection downregulated the expression of RACK1 (Fig. 3A–B) and IκBα (Fig. 3C–D) and subsequently promoted the translocation of P65 from the cytoplasm to the nucleus (Fig. 3E–F). Levels of RACK1 were positively correlated with levels of IκBα ($P < 0.0001$, Fig. 3G) but negatively correlated with levels of nuclear P65 ($P < 0.05$, Fig. 3H).

3.3. RACK1 negatively regulated H. pylori infection-induced NF-κB activation by modulating integrin β-1

To determine whether RACK1 plays a role in regulating the activity of the NF-κB signaling pathway, GES-1 cells with stable RACK1 overexpression (Fig. 4A, Supplementary Figs. 3A–B) were established and then cocultured with *H. pylori* strain 43504 for 75 min. Compared to the GES-1 control group, the RACK1 overexpression group had significantly inhibited NF-κB-mediated transcription activity as detected using an NF-κB luciferase reporter (Fig. 4B) and decreased expression of selected NF-κB target genes (Fig. 4C). In our previous study, we identified that the integrin-mediated signaling pathway was positively associated with *H. pylori* using TCGA data, and integrin β-1 was involved in multiple biological processes [4]. We showed that the expression of integrin β-1 was higher in GC tissue than in adjacent normal tissue (Fig. 4D). Additionally, *H. pylori* increased integrin β-1 protein levels *in vitro* (Fig. 4E). Knocking down endogenous RACK1 upregulated the expression of integrin β-1 and increased the formation of p-P65 (Ser 536) (Fig. 4F). In contrast, knocking down endogenous integrin β-1 significantly suppressed NF-κB-mediated transcriptional activity and target gene expression (Fig. 4G–I). To further establish the role of integrin β-1 in the RACK1-mediated suppression of NF-κB signaling activation, AGS cells overexpressing RACK1 were transfected with an integrin β-1 plasmid and subsequently cocultured with *H. pylori* strain 43504. The suppressive effect of RACK1 on the NF-κB signaling pathway was rescued by integrin β-1 overexpression according to NF-κB reporter luciferase assay and qRT-PCR results (Fig. 4J–K).

3.4. RACK1 expression was significantly lower, but integrin β-1 expression and NF-κB activation were higher in H. pylori-positive subjects than in H. pylori-negative subjects

By analyzing the different gene expression levels in *H. pylori*-positive and *H. pylori*-negative GC from the TCGA database, we found that there were no significant differences in RACK1 and integrin β-1 expression levels between the two groups (Fig. 5A–B). Subsequently, IHC was performed to evaluate the expression of RACK1, integrin β-1 and p-P65 (Ser536) in gastric tissues from various histopathologic stages of gastric carcinogenesis (chronic gastritis, intestinal metaplasia and dysplasia). RACK1 expression was lower in the *H. pylori*-positive groups than in the negative groups with intestinal metaplasia ($P = 0.167$) and dysplasia ($P < 0.05$) (Fig. 5C–D), whereas integrin β-1 was increased (intestinal metaplasia and dysplasia: $P < 0.05$, Fig. 5E–F). Additionally, an increased level of p-P65 (Ser536), indicating NF-κB activation, was observed in the *H. pylori*-positive group with chronic gastritis but not in the negative group (Fig. 5G–H). Taken together, our data indicated that *H. pylori* downregulated the expression of RACK1 and upregulated the expression of integrin β-1 and p-P65 (Ser 536) *in vivo*.

4. Discussion

RACK1 exerts either promoting or suppressive effects on infections and cancers in cell type-, tissue- and pathogen-specific manners due to its opposite roles in cell growth, dual functions in apoptosis, and different effects on metastasis [23,24]. Several studies have reported that the expression of RACK1 is upregulated in various cancers (e.g., breast cancer, pulmonary adenocarcinoma and hepatocellular carcinoma) and correlated with poor clinical outcomes in patients [25–29]. However, downregulated RACK1 expression has been observed in GC and was correlated with poor tumor differentiation and infiltration depth; RACK1 plays a suppressor role in the WNT and NF-κB signaling pathway in GC [2,3]. Consistent with these findings, our results analyzing data from the TCGA database and GC samples paired with normal tissues showed that RACK1 expression was downregulated in GC and negatively associated with disease severity in patients. Moreover, knocking down RACK1 expression promoted the growth of GC cells, which indicated that RACK1 acts as a tumor suppressor in GC.

What is the underlying molecular mechanism leading to the downregulated expression of RACK1 in GC? *H. pylori* is a major risk factor for the development of GC, and it has been estimated that at least 90% of GC is attributable to *H. pylori* infection [6]. CagA and VacA are thought to be the major virulence factors of *H. pylori* [30], and a previous study confirmed that the RACK1 protein interacts with *H. pylori* VacA [31]. Our *in vitro* results showed that *H. pylori* infection led to decreased RACK1 expression in a time- and MOI-dependent manner, however, this effect was independent of the virulence factors CagA and VacA. In addition, infection with *H. pylori* strain 7.13 or its *cagA*-deficient mutant in Mongolian gerbils decreased the levels of gastric RACK1 expression, further indicating that *H. pylori*-induced suppression of gastric RACK1 is independent of CagA. Recent studies proposed a new regulatory system in which RACK1 is controlled by phosphorylation and subsequent protein degradation in plants [32], and RACK1 phosphorylation has been reported in mammals [33,34]. Further investigations of how *H. pylori* affect the protein stability of RACK1 and the role of virulence factors in this process are needed. *H. pylori*-infected gerbils in our study developed more severe inflammation and epithelial hyperplasia than the uninfected control gerbils. However, there was no dysplasia or GC development in these infected gerbils, which is inconsistent with previously reported results [21,35]. The histopathological discrepancy between our study and previous studies is likely explained by the difference in the virulence of *H. pylori* strain 7.13 used in each study [36].

It has been proposed that *H. pylori* act as an initiation factor in the histopathological cascade of gastric carcinogenesis due to promotion of

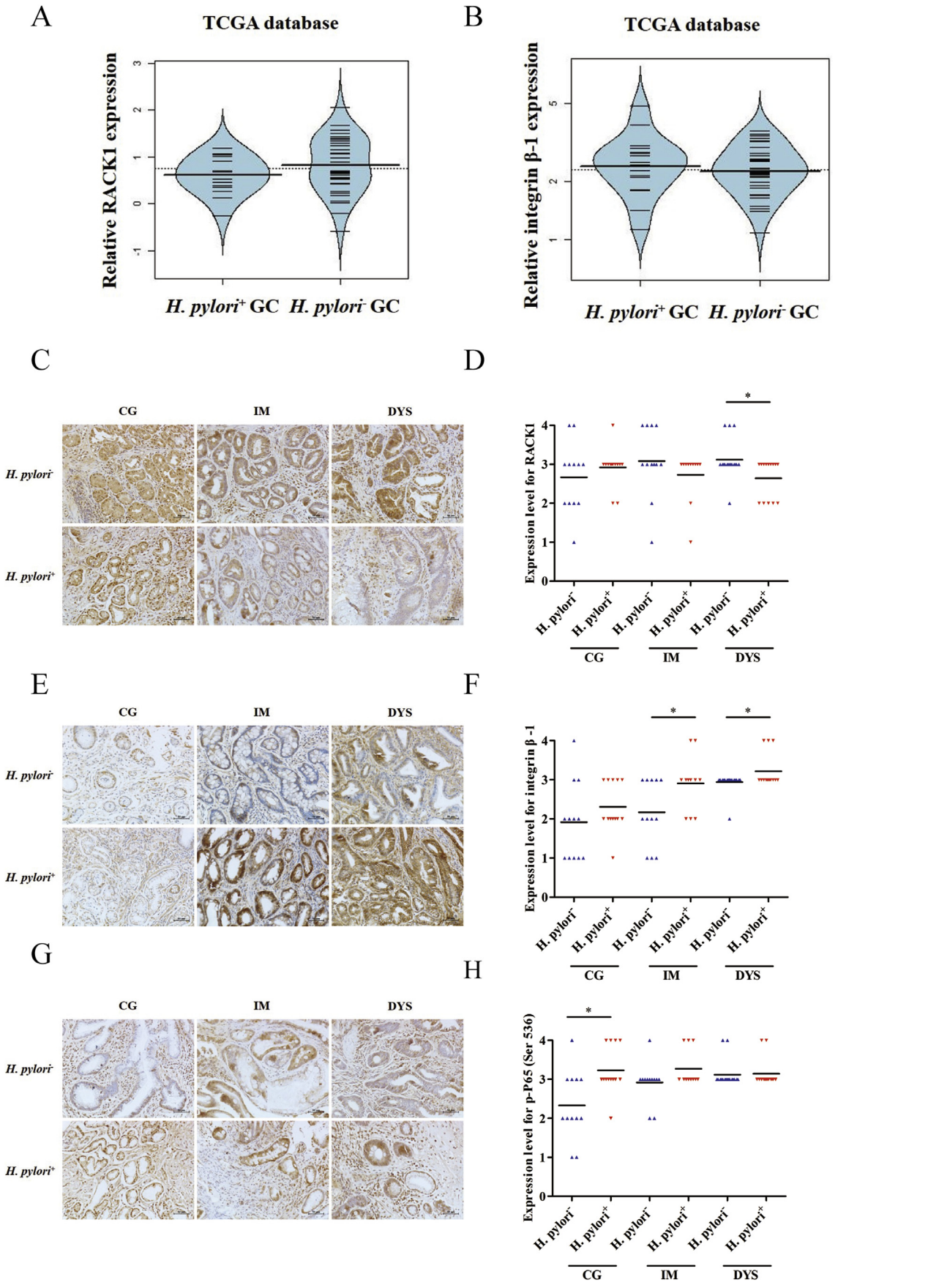

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Fig. 5. RACK1 expression was significantly lower, but integrin β-1 expression and NF-κB activation were higher in *H. pylori*-positive subjects than in *H. pylori*-negative subjects. Normalized expression values of RACK1 (A) and integrin β-1 (B) in *H. pylori*-positive and *H. pylori*-negative GC patients from the TCGA database. IHC staining showing the expression of RACK1 (C), integrin β-1 (E) and p-P65 (Ser 536) (G) in chronic gastritis (CG), intestinal metaplasia (IM) and dysplasia (DYS) with or without *H. pylori* infection. Graphs D, F, and H summarize the IHC staining results for RACK1, integrin β-1 and p-P65 (Ser 536) in the gastric samples. Immunoreactive cells were semiquantitatively assessed, and the protein expression levels are expressed as grades 1–4. The mean grades (−) for protein expression are shown. Original magnification, 20 × , Scale bar = 50 μm $^{*}P < 0.05$.

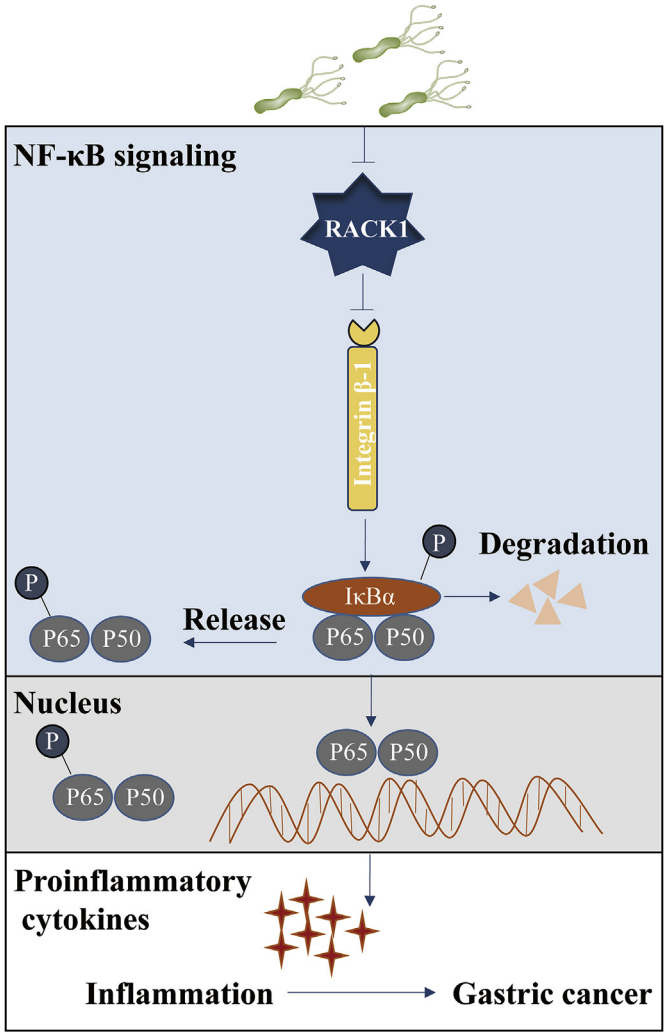

Fig. 6. Schematic model of *H. pylori*-induced GC development. Downregulation of the tumor suppressor RACK1 by *H. pylori* infection promotes gastric carcinogenesis through the integrin β-1/NF-κB signaling pathway.

the inflammatory response such as the release of various proinflammatory cytokines (e.g., IL-8, and TNF-α) [37,38]. NF-κB, an important regulator of central inflammatory and immune responses, can be activated by different virulence factors of *H. pylori* [8,9,39]. NF-κB activation fundamentally links *H. pylori*-induced inflammation and GC [40]. In this study, our data indicate that *H. pylori* infection leads to the activation of the canonical NF-κB signaling pathway *in vivo* and *in vitro* as well as the release of proinflammatory cytokines. Moreover, RACK1 can function as a regulator of the *H. pylori*-induced activation of the NF-κB signaling pathway based on the following evidence: (1) *H. pylori* infection led to a decreased protein level of RACK1 in concert with the increased activation of the NF-κB signaling pathway *in vivo* and *in vitro*; (2) levels of RACK1 were positively associated with levels of IκBα but negatively associated with levels of nuclear P65 in the *H. pylori*-infected Mongolian gerbil model; and (3) knocking down RACK1 promoted the activation of the NF-κB signaling pathway, whereas overexpressing RACK1 inhibited this process. These findings are further supported by a previous study reporting an association between RACK1 expression and the NF-κB signaling pathway [11].

Our previous study analyzed data from the TCGA database and revealed that integrin was involved in multiple pathological processes of *H. pylori* infection and that the integrin-mediated signaling pathway was closely associated with *H. pylori* [4]. In addition, RACK1 was reported to regulate beta-integrin function [17]. In this study, we found that integrin β-1 was higher in GC tissue than in adjacent normal tissue and that *H. pylori* could upregulate integrin β-1 expression. Moreover, knocking down integrin β-1 inhibited NF-κB signaling pathway activation, while decreased RACK1 expression negatively regulated integrin β-1 expression and subsequently activated the *H. pylori*-induced NF-κB signaling pathway and proinflammatory cytokine release. The typical pathological evolution of *H. pylori*-induced GC is atrophic gastritis, intestinal metaplasia, dysplasia and ultimately GC [7]. Although there were no differences in RACK1 and integrin β-1 expression among the *H. pylori*-positive and *H. pylori*-negative GC groups from the TCGA database, we found that *H. pylori* infection suppressed RACK1 expression and increased integrin β-1 and p-P65 (Ser 536) expression in the early stage of GC development. These expression changes might be one of the major molecular mechanisms that *H. pylori* utilize to promote the progression of gastric carcinogenesis.

In summary, we propose a working model illustrating the role of RACK1 in *H. pylori*-induced gastric carcinogenesis (Fig. 6). In this model, *H. pylori* infection suppresses RACK1 expression, which then upregulates integrin β-1 expression, followed by NF-κB signaling pathway activation. The subsequent release of proinflammatory cytokines promotes the progression of *H. pylori*-induced inflammation to GC. In conclusion, we demonstrate, for the first time, the role of RACK1 and integrin β-1 in *H. pylori*-induced NF-κB activation. The *H. pylori*-RACK1-integrin β-1-NF-κB axis could potentially be used to develop a new therapeutic paradigm for curing GC.

Conflicts of interest

The authors declare that there were no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.02.039.

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