Fork head box M1 is overexpressed in *Helicobacter pylori*-induced gastric carcinogenesis and is negatively regulated by hsa-miR-370

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LW, JZ, JJ and YF designed the study; YF, LW, JZ, LS, XL and HY performed the study; YF, SL, ZL, YS, WL HY and CC analyzed and interpreted data; JJ supervised the study; and YF, LW, JZ and JJ wrote the paper.

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Abstract

*Helicobacter pylori* (*H. pylori*) is implicated in human gastric mucosa. Fork head box M1 (FoxM1), the key positive regulator of cell proliferation is overexpressed in gastric cancer. MicroRNAs are important post-transcriptional regulators of gene expression. In this study, we explored the effect of *H. pylori* infection on FoxM1 expression and possible mechanisms. The expression of FoxM1 was gradually increased in human gastric specimens from inflammation to cancer. FoxM1 was time- and concentration-dependently upregulated in gastric epithelial-derived cell lines infected with *H. pylori*. CagA, the key virulence factor of *H. pylori*, was associated with increasing FoxM1 expression. *H. pylori* and CagA inhibited the expression of *P27Kip1* (cyclin-dependent kinase inhibitor 1B, CDKN1B) and promoted cell proliferation by upregulating FoxM1. The expression of hsa-miR-370 was decreased in human gastritis and gastric cancer. FoxM1 was directly downregulated by hsa-miR-370 in gastric cell lines. *H. pylori* and CagA inhibited hsa-miR-370 expression, which led to overexpression of FoxM1 and cell proliferation. Furthermore, the overexpression of FoxM1 and reduced expression of hsa-miR-370 were confirmed on August 30, 2021. © 2013 American Association for Cancer Research. mcr.aacrjournals.org Downloaded from Author Manuscript Published OnlineFirst on April 10, 2013; DOI: 10.1158/1541-7786.MCR-13-0007 Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
in *H. pylori*-infected C57BL/6J mice. *H. pylori* and its key virulence factor CagA can upregulate the expression of FoxM1 in a hsa-miR-370-dependent manner, which affects the expression of P27$^{Kip1}$ and promotes proliferation of gastric cells. These findings may provide further information for better understanding the mechanism of *H. pylori* carcinogenesis.

**Keywords**: FoxM1; *Helicobacter pylori*; Gastric cancer; P27$^{Kip1}$; hsa-miR-370

**Introduction**

Gastric cancer (GC) is the fourth diagnosed cancer and the second most common cause of cancer-related death worldwide, especially in developing countries[1, 2]. Most patients are diagnosed at an advantaged stage because of lack of early specific symptoms, so prognosis remains poor even after extensive surgery and adjuvant therapy.

Gastric carcinogenesis is a complex, multistep and multifactorial event. *H. pylori* infection is involved in the early stage of GC pathogenesis by inducing chronic gastritis [3-6]. This chronic gastritis can persist for decades and may result in nonresolving inflammation, which is a major driver of GC[6], and the interaction of host–pathogen contributes to this carcinogenesis[7]. The protein CagA of *H. pylori* is closely associated with GC and can intervene in signal pathways in cells[8]. To date, however, the underlying molecular mechanisms of carcinogenesis induced by *H. pylori* remain to be elucidated.

Fork head Box M1 (FoxM1), a member of the Fox transcription factor family[9],
is a key positive cell-cycle regulator in cell proliferation. Aberrant expression of FoxM1 is involved in several tumor types, including hepatocellular carcinoma, basal cell carcinoma, breast cancer, lung cancer, prostate cancer, glioblastomas and GC[10-18], which implies an oncogene role in carcinogenesis. We previously reported that FoxM1 is upregulated in GC, and its inhibition leads to cellular senescence[18], but the relevance of *H. pylori* infection and FoxM1 expression associated with the pathogenesis of GC remains undefined.

MicroRNAs (miRNAs), a family of small noncoding RNAs, are important negative regulators of post-transcriptional gene expression by directly targeting the 3’ untranslated regions (3’UTRs) of target mRNAs, eventually promoting the degradation or translation suppression of target mRNAs[19, 20]. MiRNAs are frequently deregulated in many types of human cancers and play critical roles in tumorigenesis, serving as tumor suppressors or oncogenes[21, 22]. Recent studies have demonstrated that several miRNAs, such as miR-146a, miR-155 and miR-21, are involved in *H. pylori*-induced infection [23-25]. MiR-370 has been verified as a tumor suppressor in bladder cancer and cholangiocarcinoma cell lines [26, 27]. Furthermore, FoxM1 is predicted as a putative target of miR-370 by bioinformatics.

Here, we aimed to identify the role of FoxM1 in *H. pylori* infection and the pathogenesis of GC and regulation by miR-370 *in vitro* and *in vivo*.

**Materials and methods**

**Patients and tissue specimens**
Resected tissues from 25 patients with GC were harvested at surgery. Gastritis species were collected from 34 patients undergoing gastroscopy. All these patients underwent surgery at Qilu Hospital, Shandong University, from 2009 through 2011. None of the patients had received adjuvant chemotherapy before surgery. The diagnosis of all GC and gastritis was histopathologically confirmed. General information about patients is in Supplementary Table 1. The study was approved by the Ethics Committee of Shandong University School of Medicine.

**Experimental animals**

C57BL/6J mice were purchased from the Experimental Animal Center of Shandong University and maintained in the Animal Center in Shandong University School of Medicine. Male mice aged 6 weeks were used in all experiments. We randomly divided 40 mice into 2 groups for treatment: infection (n=24) and control (n=16). The infection group received by intragastric gavage $1 \times 10^9$ colony-forming units (CFUs) of *H. pylori* (standard SS1 strain) after 12-h fasting every other day for 3 times. The control group received 1ml phosphate buffered saline (PBS) each time in the same way. At 8 months, mice were killed and stomach tissue samples underwent hematoxylin and eosin staining and immunohistochemistry (IHC) assay. All animal care and experimental protocols were approved by the Ethics Committee of Shandong University School of Medicine.

**H. pylori and bacterial culture**

The standard strains of *H. pylori* (NCTC 11637, 26695 and SS1) were kindly provided by Dr. Jianzhong Zhang (Chinese Disease Control and Prevention Center).
All strains were cultured in Brucella broth with 5% fetal bovine serum (FBS) under microaerobic conditions (5% O₂, 10% CO₂, and 85% N₂) at 37°C. The strains were harvested at exponential amplification phases by centrifugation and added to gastric cell lines at different bacteria-to-cell ratios and times.

Cell lines and cell culture

The gastric epithelial-derived cell lines AGS and BGC-823 were obtained from China Academia Sinica Cell Repository (Shanghai). AGS cells were cultured in F12 medium supplemented with 10% FBS. BGC-823 cells were cultured in RPMI 1640 medium supplemented with 10% FBS. Both cell lines were incubated in a humidified atmosphere containing 5% CO₂ at 37°C without antibiotics.

Plasmid construction and transfection

The sequence fragment of 212 bp incorporating the whole length of pre-miR-370 was amplified from normal human genomic DNA with the primers sense, 5'-AAGGGATCCTACTTGAGGGATGGGCGATA-3', and antisense, 5'-TCAAAGCTTCCCGAGCTCTGGTGTTAGAC-3'. The amplified fragment was cloned into the specific vector pSilencer3.1-H1 (Ambion, USA) to construct the pSilencer-miR-370 expression plasmid. Likewise, for constructing the anti-miR-370 interfering plasmid, the miR-370 complementary sequence was synthesized as DNA oligonucleotides and cloned into the pSuper vector (Oligoengine, USA) after annealing. Firefly luciferase reporter vectors with the intact putative miR-370 recognition sequence of the wild type (pGL3-FoxM1-wild-3'UTR) and mutant (pGL3-FoxM1-mutant-3'UTR) were cloned from the FoxM1 3'UTR. The authenticity
of DNA sequences was confirmed by sequencing. All plasmids were transfected into cell lines by the Lipofectamine 2000 method (Invitrogen, USA). The si-FoxM1 oligonucleotides were also transfected with Lipofectamine 2000, with final concentration 100 nM.

**RNA extraction and qRT-PCR**

Total RNA was extracted by use of Trizol reagent (Invitrogen). For detection of miR-370, RNA was converted into cDNA with use of the TaqMan MicroRNA Reverse Transcription kit, then real-time PCR was performed with specific primers (TaqMan MicroRNA Assay) and TaqMan Universal PCR Master Mix (Applied Biosystems, USA). For detecting FoxM1 and P27<sup>Kip1</sup>, 1 μg RNA was converted into cDNA by the MMLV Reverse Transcription System (Promega, USA) and real-time PCR involved use of the SYBR Premix Ex Taq system (TaKaRa, Japan). All PCR reactions were run on an ABI 7500 sequence detector (Applied Biosystems, USA). U6 snRNA or β2-microglobulin (β2-M) was used as an endogenous control. All real-time PCR reactions were performed in triplicate, and relative quantification was calculated by the ΔΔCt method (95% CI) with calibration to the corresponding endogenous control. Primers for real-time PCR were for FoxM1, sense, 5'-TGCAGCTAGGGATGTGAATCTTC-3', and anti-sense, 5'-GGAGCCCAGTCCATCAGAACT-3'; P27<sup>Kip1</sup>, sense, 5'-ATGTCAAACGTGCGAGTGTCTAA-3', and anti-sense, 5'-TTACGTTTGACGTCTTCTGAGG-3'.

**Luciferase assay**
The P27Kip1 reporter plasmid, constructed to harbour the P27Kip1 promoter sequence (-1358/+132), and control TK plasmid were added into cells by use of Lipofectamine 2000. Cells were infected with H. pylori by the ratio 1:100 for 6 h. Luciferase activity was determined with the Dual Luciferase Reporter Assay system (Promega, USA), and the P27Kip1 promoter-driven firefly luciferase activity was normalized to that of TK Renilla. For luciferase assays of miRNA target validation, pSilencer-miR-370, wild-type or mutant 3’UTR of FoxM1 and TK plasmids were co-transfected into AGS cells. After 48 h, cells were lysed in passive lysis buffer and luciferase activity was determined.

**Western blot and IHC assay**

According to standard western blot procedures, proteins were separated by 10% SDS-PAGE and then transferred to PVDF membrane (Bio-Rad, USA), which were blocked in 5% nonfat milk, then incubated with the primary antibodies FoxM1 (1:500), P27Kip1 (1:200) and β-actin (1:500) for 1-2 h under room temperature, then horseradish peroxidase-labeled goat-anti-mouse or goat-anti-rabbit IgG antibody (1:5000, all Santa Cruz Biotechnology). Protein levels were detected by use of enhanced chemiluminescence (Pierce) and visualized on X-ray film. FoxM1 levels in tissue specimens were determined by IHC staining with primary antibody (FoxM1, 1:250) incubation at 4°C overnight. Negative controls were treated without the primary antibody.

**Colony-formation assay**

AGS cells were infected with H. pylori standard strain 26695 or transfected with
FoxM1 siRNA and pSilencer-miR-370 for corresponding times, then seeded into 6-well plates (300 cells/well) for 10–14 days. Plates were stained with Giemsa, and number of colonies with > 50 cells was counted.

**Statistical analysis**

Quantitative data are expressed as mean± SEM. Statistical analysis involved SPSS 13.0 (SPSS Inc., Chicago, IL) with two-tailed Student's *t* test or one-way ANOVA for analysis of more than 2 subgroups. Statistical significance was set at *P*<0.05.

**Results**

**Expression of FoxM1 is upregulated in human gastritis and primary GC**

We investigated the expression of FoxM1 in gastric specimens at different disease stages, including 20 normal, 12 superficial gastritis (SG), 12 atrophic gastritis associated with intestinal metaplasia (AG/IM) and 20 primary GC tissues (Supplementary Table 1). The mRNA expression of FoxM1 gradually increased from gastritis to cancer as compared with non-cancerous gastric tissues (*P*<0.01, Fig. 1A), so upregulation of FoxM1 was an early event in human gastric carcinoma. This overexpression was confirmed by IHC assay, which showed FoxM1 expressed in SG tissue (21.7% positive cells), AG/IM tissue (36.4% positive cells) and GC tissue (89.2% positive cells) as compared with non-cancerous gastric tissues (6.7% positive cells) (*P*<0.01, Fig. 1B, C). FoxM1 expression was not associated with patient age or sex (Supplementary Table 1).

*H. pylori* and its key virulence factor CagA promote FoxM1 expression in gastric
epithelial cell lines.

Next, we determined whether infection with \textit{H. pylori} (standard strain 26695) could affect FoxM1 expression in 2 gastric epithelial cell lines: poorly differentiated AGS and well-differentiated BGC-823 cells. Both the mRNA and protein levels of FoxM1 were significantly upregulated with \textit{H. pylori} infection time and dose dependently (Fig. 2A-D). Furthermore, to evaluate whether overexpression of FoxM1 by \textit{H. pylori} infection was strain specific, AGS and BGC-823 cell lines were infected with \textit{H. pylori} strains 26695, 11637 and SS1 (1:100) for 12 h. FoxM1 expression was substantially elevated after infection by all of the 3 strains as compared with the control (Fig. 2E, G). To determine which virulence factor was responsible for the increased FoxM1 expression, we treated cells with CagA (CagA full-length plasmid), VacA (centrifugation of \textit{H. pylori} liquid medium) and lipopolysaccharide (LPS; hot inactivation of \textit{H. pylori}) [28] and examined FoxM1 expression. In AGS cells, CagA promoted the mRNA and protein levels of FoxM1, with almost no impact with other treatments (Fig. 2F, H). These results were confirmed in cells with CagA overexpression (Fig. 2I, J). Thus, upregulation of FoxM1 may have been induced by \textit{H. pylori} infection through its component CagA.

\textit{H. pylori} and CagA were involved in cell proliferation by suppressing \textit{P27 Kip1} expression mediated by FoxM1

We previously reported that \textit{P27 Kip1} expression was suppressed by FoxM1 in GC cell lines[18]. Hence, we examined whether \textit{H. pylori} infection could inhibit \textit{P27 Kip1} expression by upregulating FoxM1. Both the mRNA and protein levels of \textit{P27 Kip1}
were significantly inhibited in AGS and BGC-823 cell lines after *H. pylori* infection and CagA transfection, separately (Fig. 3A, B and Fig. 4A, B). Furthermore, both *H. pylori* and CagA treatment reduced the luciferase activity of P27^Kip1^ promoter and expression of P27^Kip1^, which could be partially reversed by knockdown of FoxM1 with effective siRNA (Fig. 3 C, D, E and Fig. 4C, D), which suggests that inhibition of P27^Kip1^ induced by *H. pylori* or CagA was partially mediated by FoxM1 through p27^Kip1^ promoter activity. Finally, we examined the function of *H. pylori* infection and CagA transfection on cell proliferation by colony-formation assay in AGS cells. *H. pylori* promoted colony formation compared with control, and this promotion was reversed by silencing of FoxM1 (*P* < 0.05, Fig. 3F, G and Fig. 4E, F).

### FoxM1 is a direct target of hsa-miR-370

We used computer-aided algorithmic programs ([TargetScanHuman](http://www.targetscan.org), PicTar [http://pictar.mdc-berlin.de] and miRBase [http://www.mirbase.org]) to predict FoxM1 as a putative target of hsa-miR-370 (Fig. 5A). The mRNA level of miR-370 was gradually decreased from SG, AG/IM to GC samples, which indicates the importance of miR-370 during GC progression (*P* < 0.01, Fig. 5B). The effect of miR-370 on FoxM1 expression was evaluated with effective overexpressing and interfering plasmids of miR-370 (Fig. 5C). Both AGS and BGC cell lines transfected with pSilencer-miR-370 expression plasmid showed reduced mRNA and protein levels of FoxM1 (Fig. 5D, E). Meanwhile, FoxM1 was upregulated after plasmid knockdown of miR-370 (Fig. 5D and E). Next, we validated FoxM1 as a target of miR-370 by dual luciferase report assay. AGS cells were
co-tranfected with pSilencer-miR-370 and FoxM1 wild-type or mutant type 3’UTR plasmids. Co-transfection of hsa-miR-370 and wild-type 3’UTR plasmid reduced the luciferase activity by approximately 80% relative to the control ($P < 0.01$), whereas mutant 3’UTR co-transfection almost restored the luciferase activity ($P < 0.01$) (Fig. 5F). Thus, miR-370 directly targeted the binding site located at FoxM1 3’UTR and FoxM1 may be a direct target of miR-370.

**miR-370–FoxM1 pathway mediates *H. pylori*-induced cell proliferation**

In AGS and BGC-823 cells, the expression of hsa-miR-370 was inhibited by *H. pylori* infection and transfection of CagA full-length plasmid, respectively, which suggests that suppression of hsa-miR-370 by *H. pylori* may play an important role in gastric carcinogenesis (Fig. 6A, B). Next, we examined whether the miR-370–FoxM1 pathway was involved in *H. pylori*-induced GC cell proliferation. miR-370 could inhibit FoxM1 expression and suppress *H. pylori* and CagA-induced upregulation of FoxM1 in AGS cells (Fig. 6C, D), which implies the existence of a miR-370–FoxM1 pathway in *H. pylori*-induced cell proliferation.

Furthermore, the results were confirmed by colony-formation assay, which showed that miR-370 significantly inhibited *H. pylori* and CagA-induced cell proliferation ($P < 0.01$, Fig. 6E, F).

**FoxM1 is involved in *H. pylori*-induced gastritis in vivo**

The validation of our mouse model of gastritis induced by *H. pylori* infection was confirmed by hematoxylin and eosin staining (Fig. 7A). After administration of *H. pylori* for 8 months, 90% (18/20) of mice were validated as gastritis models in the *H.
*Helicobacter pylori* infection group as compared with 13% (2/15) in the control group (Supplementary Table 2). FoxM1 protein level was greater in the model than the control on IHC assay (Fig. 7A and B, *P* < 0.01). In addition, the mRNA level of mmu-miR-370 was reduced and that of FoxM1 increased in mucosal epithelial samples of *H. pylori*-induced gastritis (Fig. 7C and D, *P* < 0.01). The results in vivo revealed the involvement of FoxM1 and miR-370 in *H. pylori*-induced gastritis.

**Discussion**

In this study, we found overexpression of FoxM1 and reduced expression of hsa-miR-370 in human gastritis and GC. *H. pylori* and its key virulent factor CagA induced the expression of FoxM1 by inhibiting that of hsa-miR-370, which directly targeted FoxM1, resulting in cell proliferation for gastric carcinogenesis by reducing p27Kip1 promoter activity and its expression (Supplementary Fig.1).

Gastric carcinomas may occur on the background of chronic gastric inflammation caused by persistent infection of *H. pylori*[29]. Accumulating studies indicate that *H. pylori* may be involved in SG formation, which might later progress into AG/IM, or even GC. The chronic inflammatory status over decades increases the risk of GC with *H. pylori* considered a Group 1 human carcinogen [30]. Studies have focused on the mechanism by which *H. pylori* induces carcinogenesis, such as bacterium products that cause gastric mucosal damage[31], generation of oxidative stress[32], and favoring the formation of mutagenic substances[33]. Moreover, *H. pylori* may inhibit apoptosis and promote proliferation, invasion and angiogenesis of cancer cells via
CagA, the key virulence factor of *H. pylori*[34].

Despite the importance of *H. pylori*, a susceptible host is also required in gastric tumorigenesis. Some typical pathways have been verified in *H. pylori* infection-related GC and include NF-κB and Wnt/β-catenin signaling pathways[35]. However, the involvement of the transcription factor FoxM1, shown upregulated in GC samples in our previous studies[18], remained unknown. As a typical cell-cycle related transcription factor, one of the key roles of FoxM1 in carcinogenesis is to promote tumor proliferation[9]. In addition, upregulation of FoxM1 is a frequent event in different tumor types, and FoxM1 deficiency triggers reduced cellular proliferation or even growth arrest [10-14], which suggests that FoxM1 may play an important role in tumorigenesis and progression. Our results revealed that overexpression of FoxM1 occurred from gastritis to GC progression and *H. pylori* induced FoxM1 expression *in vivo* and *in vitro*, so FoxM1 might take part in the early stage of GC. Because of the genetic heterogeneity present within *H. pylori* genomes, infection outcomes vary for different *H. pylori* strains and their virulence factors, such as CagA, VacA and LPS.

In this study, we found that all 3 *H. pylori* strains, 26695, 11637 and SS1, increased the expression of FoxM1 in different gastric cells, further indicating the key role of FoxM1 in *H. pylori*-induced pathological process. However, only CagA was able to promote FoxM1 upregulation. Microdissection and microarray were used to determine the differentially expressed mRNA levels with progression of SG, AG/IM and GC; data analysis revealed the involvement of FoxM1 in this process (data not
shown). In short, we found that upregulation of FoxM1 existed in the early stage of GC associated with *H. pylori* infection, which suggests an important role of FoxM1 in this progress.

Cell-cycle check-point genes play a pivotal role in gastric carcinogenesis[36]. P27$^{kip1}$, the negative regulator of cell cycle, is commonly downregulated in GC. It has been considered a tumor suppressor and could serve as a candidate molecular marker for early gastric carcinoma[37]. In the diffuse GC subtype, P27$^{kip1}$-negative–C-MYC-positive was the most frequent combination and was associated with more pathogenic *H. pylori* strains[38]. Furthermore, transcription of P27$^{kip1}$ was verified to be inhibited by CagA via a PI3K/Akt1 pathway[39]. In agreement with these observations, we found inhibited P27$^{kip1}$ mRNA and protein levels with *H. pylori* CagA treatment by upregulating FoxM1 expression, which decreased P27$^{kip1}$ promoter activity in AGS and BGC-823 cells. However, P27$^{kip1}$ inhibition and colony number were only partially reversed after knockdown of FoxM1 by its specific siRNA, which suggests other molecules involved in this process. One study showed that *H. pylori* decreased P27$^{kip1}$ expression through delta opioid receptor-mediated inhibition of histone acetylation within the P27$^{kip1}$ promoter[40]. Other research confirmed the involvement of cytoplasmic mislocalization of P27$^{kip1}$ induced by *H. pylori* in GC[41]. These findings also link low gastric P27$^{kip1}$ expression with gastric carcinogenesis induced by *H. pylori* infection. Also, they could explain why P27$^{kip1}$ expression could not be completely reversed by FoxM1 silencing: *H. pylori*-induced FoxM1 may be a critical but not the only way for
downregulation of \( p27^{kip1} \) for cell proliferation. Two oncogenes, \( c\text{-}myc \) and \( hTERT \), can be induced by \( H.\ pylori \) and are the direct target genes of FoxM1[9, 42]. We also found substantial induction of the protein levels of \( c\text{-}myc \) and \( hTERT \) \textit{in vivo} and \textit{in vitro} after \( H.\ pylori \) infection (data not shown), which further indicates that FoxM1 plays a key role in gastric tumorigenesis induced by \( H.\ pylori \) infection. We failed to induce GC after 8 months’ \( H.\ pylori \) infection in animal models. However, we elucidate that \( H.\ pylori \) and its key virulent factor CagA take part in inflammation-related GC through up-regulation of FoxM1.

MiRNAs are important negative regulators of post-transcriptional gene expression and involved in several biological processes, including tumorigenesis, by regulating tumor suppressors or oncogenes. Numerous deregulated miRNAs are involved in gastric cancer and include miR-146a, miR-155, miR-21, miR-27a, miR-106-93-25, miR-221-222 clusters and the miR-200 family [23-25, 43-46]. A growing number of studies also suggest the involvement of miRNAs in various steps of gastric carcinogenesis: from gastritis to metastatic disease[47]. In this study, we found reduced hsa-miR-370 expression in different disease stages, and the expression could be downregulated by \( H.\ pylori \) infection and CagA treatment in gastric cell lines. Moreover, FoxM1 was validated as a direct target of miR-370. Overexpression of hsa-miR-370 inhibited the cell proliferation induced by \( H.\ pylori \) infection or CagA treatment by silencing FoxM1, which suggested a tumor-suppressive role of miR-370 in \( H.\ pylori\)-induced gastritis toward GC.

In summary, our study demonstrates that the miR-370–FoxM1 pathway is involved in
the progress of gastritis towards GC induced by *H. pylori* infection by affecting the expression of P27^Kip1^, which suggests potential application in early intervention and treatment of gastric cancer.

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Figure legends:

Fig. 1 FoxM1 was overexpressed in human specimens of gastritis and gastric cancer. (A) Quantitative RT-PCR (qRT-PCR) analysis of relative mRNA level of FoxM1 in gastric biopsy specimens. *P<0.01. Each point represents one sample. (B)
Quantitative immunohistochemistry results of percentage of FoxM1-positive cells in gastric tissues. *P<0.01. (C) Immunohistochemistry staining of FoxM1 expression in normal (upper left panel), superficial gastritis (SG; upper right panel), atrophic gastritis (AG; lower left panel) and cancerous (lower right panel) gastric tissues.

**Fig. 2** The mRNA and protein levels of FoxM1 were upregulated by *Helicobacter pylori* and its key virulent factor CagA in gastric epithelial cell lines. (A, C) AGS and BGC-823 cells were infected with *H. pylori* [multiplicity of infection (MOI) 1:100 (cell:*H. pylori*)] for 6 h and 12 h. qRT-PCR and western blot analysis of mRNA and protein levels of FoxM1 over time, *P<0.01* vs con. (B, D) Cells were infected by *H. pylori* with MOI at 1:25, 1:50 and 1:100 for 12 h. qRT-PCR and western blot analysis of mRNA and protein levels of FoxM1 expression, *P<0.01* vs con. (E, G) qRT-PCR and western blot analysis of mRNA and protein levels of FoxM1 by infection with all 3 *H. pylori* stains 26695, 11637 and SS1 at MOI 1:100 for 12 h, *P<0.01* vs con. (F, H) qRT-PCR and western blot analysis of mRNA and protein levels of FoxM1 with CagA, lipopolysaccharide (LPS) and VacA treatment in AGS cells, *P<0.01* vs con. (I, J) CagA full-length plasmid was transfected into AGS and BGC-823 cells for 48 h. qRT-PCR and western blot analysis of mRNA and protein levels of FoxM1 with CagA transfection, *P<0.01* vs pcDNA3.1 Con. Data are mean ± SEM of 3 independent experiments.

**Fig. 3** *H. pylori* was involved in the proliferation of gastric epithelial cells by inhibiting p27<sup>Kip1</sup> promoter activity through FoxM1. (A, B) mRNA and protein levels of p27<sup>Kip1</sup> in AGS and BGC-823 cells infected with *H. pylori* 26695 (MOI 1:100) for 12 h, *P<0.01* vs con. (C, D, E) Luciferase activity of P27<sup>Kip1</sup> promoter and P27<sup>Kip1</sup> expression in AGS cells with *H. pylori* (1:100) infection (*Hp*) for 12 h, control siRNA
transfection for 48 h (Cs) and FoxM1 silencing with specific siRNA for 48 h (Fs). Luciferase activity was normalized to that of Renilla of the TK plasmid. *$P<0.01$ vs con and $^\#P<0.05$ vs *H. pylori* infection. (F, G) The foci number of AGS cells after *H. pylori* (MOI 1:100) infection for 12 h and FoxM1 silencing, *$P<0.01$ vs con and $^\#P<0.01$ vs *H. pylori* infection. Data are mean ± SEM of 3 independent experiments.

**Fig. 4** CagA induced the proliferation of gastric epithelial cells by inhibiting P27$^{kip1}$ promoter activity through FoxM1. (A, B) qRT-PCR and western blot analysis of mRNA and protein levels of the expression of P27$^{kip1}$ in AGS and BGC-823 cells with CagA full-length plasmid transfection for 48 h, *$P<0.01$ vs pcDNA3.1 Con. (C, D) Luciferase activity of P27$^{kip1}$ promoter and P27$^{kip1}$ mRNA expression with CagA transfection for 48 h (CagA), control siRNA transfection for 48 h (Cs) and FoxM1 silencing with specific siRNA for 48 h (Fs). Luciferase activity was normalized to that of Renilla of the TK plasmid. *$P<0.01$ vs pcDNA3.1 Con and $^\#P<0.05$ vs CagA plasmid treatment. (E, F) The foci number of AGS cells with *H. pylori* (1:100) infection (Hp), CagA transfection (Cs) and FoxM1 silencing with specific siRNA (Fs), *$P<0.01$ vs pcDNA3.1 and $^\#P<0.05$ vs CagA plasmid treatment. Data are mean ± SEM of 3 independent experiments.

**Fig. 5** Hsa-miR-370 directly targeted to FoxM1 *in vitro*. (A) The sequence with hsa-miR-370 directly binding to the 3’-UTR of FoxM1 (from TargetscanHuman). (B) qRT-PCR analysis of the expression of hsa-miR-370 in gastric biopsy specimens, *$P<0.01$ vs normal tissues. Each point represents one sample. (C) In AGS and BGC-823 cells, hsa-miR-370 was overexpressed by pSilencer-miR-370 and inhibited
by pSuper-anti-miR-370, respectively, *$P<0.01$ and #$P<0.05$ vs con. (D, E) mRNA and protein levels of FoxM1 in AGS and BGC-823 cells with overexpression (370) and inhibition (inhibitor) of miR-370, respectively, *$P<0.01$ and #$P<0.05$ vs con. (F) Relative luciferase activity with reporter plasmids (pGL3-FoxM1-wild-3'UTR and pGL3-FoxM1-mutant-3'UTR) cotransfected with pSilencer-miR-370 into AGS cells. Luciferase activity was normalized to that of Renilla of the TK plasmid, *$P<0.01$ vs pSilencer+3'-UTR Con, #$P<0.01$ vs miR-370+3'-UTR. Data are mean±SEM of 3 independent experiments.

**Fig. 6** *H. pylori* and CagA inhibited hsa-miR-370 expression and thus induced FoxM1 expression for cell proliferation. (A) Hsa-miR-370 expression after *H. pylori* infection (MOI 1:100) for 12 h, *$P<0.01$ vs con. (B) Hsa-miR-370 expression with CagA transfection for 48 h, *$P<0.01$ vs pcDNA3.1 Con. (C, D) qRT-PCR and western blot analysis of mRNA and protein levels of FoxM1 with overexpression of hsa-miR-370 (370) with *H. pylori* infection and CagA transfection, *$P<0.01$ vs pSilencer (Psil), #$P<0.01$ vs con. (E, F) The foci number of AGS cells after *H. pylori* infection and CagA transfection, with pSilencer-miR-370 (Psil) and has-miR-370 (370) treatment, *$P<0.01$ vs pSilencer (Psil), #$P<0.01$ vs con. Data are mean ± SEM of 3 independent experiments.

**Fig. 7** FoxM1 was upregulated and mmu-miR-370 was downregulated in *H. pylori*-induced gastritis in mice. (A) Hematoxylin and eosin (HE) staining and immunohistochemistry (IHC) assay of FoxM1 in mucosal epithelial tissue with control and *H. pylori* infection. (B) Proportion of FoxM1-positive cells as determined
immunohistochemically. *P<0.01 vs con. qRT-PCR analysis of relative mRNA level of (C) FoxM1 and (D) Mmu-miR-370 expression in mucosal epithelial tissue cells, *P<0.01 vs con. Each point represents one sample. Data are mean ± SEM of 3 independent experiments.
Fig. 3

A

Relative expression of p27 mRNA

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<td>con</td>
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<tr>
<td>hp</td>
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B

Time

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P27

Actin

C

Relative luciferase activity

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D

Relative expression of p27 mRNA

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E

P27

Actin

F

Con

Hp

Hp+Cs

Hp+Fs

G

Fod number 300 cells

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Fig. 4

A

Relative expression of p27 mRNA

AGS

BGC-823

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B

P27

Actin

AGS

BGC-823

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C

Relative luciferase activity

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D

Relative expression of p27 mRNA

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E

F

Foci number per 300 cells

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Fig. 6

A

Relative expression of hsa-miR-370

AGS  

B

Relative expression of hsa-miR-370

AGS  

C

Relative expression of FoxM1 mRNA

Psil 370  

D

Table: FoxM1 and Actin

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E

Psilencer

miR-370

F

Foci number / 400 cells

Psil 370  

Con

Hp

CagA
Fig. 7

A

Con

Gastritis

HE

600X

IHC

600X

B

% of FoxM1 positive cells

Con

Gastritis

C

Relative expression of FoxM1

Con

Gastritis

D

Relative expression of rmn-mR-370

Con

Gastritis

*
# Molecular Cancer Research

## Fork head box M1 is overexpressed in Helicobacter pylori-induced gastric carcinogenesis and is negatively regulated by hsa-miR-370

Yimin Feng, Lixiang Wang, Jiping Zeng, et al.

*Mol Cancer Res* Published OnlineFirst April 10, 2013.

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