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Identification of MyD88 as a novel target of miR-155, involved in negative regulation of *Helicobacter pylori*-induced inflammation

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ABSTRACT

MicroRNA-155 (miR-155) has been implicated as a central regulator of the immune system. We have previously reported that miR-155 negatively regulates *Helicobacter pylori* (*H. pylori*)-induced inflammation, but the molecular mechanism of miR-155 regulating the inflammation is not fully clear. Here, we identified myeloid differentiation protein 88 (MyD88) as a target gene of miR-155, and found that miR-155 decreased MyD88 expression at the protein but not the mRNA message level, suggesting that the miR-155-mediated inhibition is a post-transcriptional event. Furthermore, the overexpression of miR-155 led to significantly reduced IL-8 production induced by *H. pylori* infection. Thus, we have demonstrated that miR-155 can negatively regulate inflammation by targeting a key adaptor molecule MyD88 in inflammatory pathways.

1. Introduction

Helicobacter pylori is a gram-negative bacterium that plays an etiologic role in the development of gastritis, peptic ulceration, and gastric adenocarcinoma [1]. About 50% of the world's population is infected with *H. pylori*. In acute or chronic *H. pylori* infection, inflammation is thought to be a major determinant of both peptic ulceration and gastric malignancy [2], but the regulatory mechanisms of *H. pylori*-induced inflammation are still not well understood.

Recently, microRNAs (miRNAs) are a class of evolutionarily conserved, single-stranded non-coding RNA molecules of 19–24 nucleotides that control gene expression at a post-transcriptional level [3]. miRNAs are central regulators of various physiologic processes and their disruption is associated with human diseases [4,5]. Accumulating evidence has demonstrated that miRNAs play a key regulatory role in the innate immune response to pathogens and stimulus [6–9]. In our previous study, we first reported the increased expression of miR-155 in human gastric epithelial cells infected with *H. pylori*, and overexpression of miR-155 negatively regulated the release of proinflammatory cytokines [10]. To further investigate the molecule mechanism of the regulatory role of miR-155, we predicted the potential target genes of miR-155 using different prediction algorithms. Interestingly, a series of transcription factors and important signal molecules such as MyD88, basic-leucine zipper transcription factor1 (BACH1), IkB kinase ε (IKKε), fas-associated death domain protein (FADD), TAK1-binding protein 2 (TAB2) were found to be targeted by miR-155.

Among the potential targets of miR-155, one interesting hit is MyD88, an important adapter protein involved in most Toll-like receptors (TLRs) and interleukin-1 receptor (IL-1R) pathways [11]. Furthermore, *H. pylori* can induce proinflammatory cytokines including IL-12, IL-10, IL-6 and IL-1β via MyD88 in a TLR-dependent manner [12,13]. Hence, in this study we explored the possibility that MyD88 was down-regulated by miR-155, and searched for additional mechanisms through which miR-155 suppressed *H. pylori*-induced inflammation.

2. Materials and methods

2.1. Cell lines and H. pylori strains

Human gastric cancer cell line AGS and human embryonic kidney HEK-293 cells were routinely cultured in RPMI 1640

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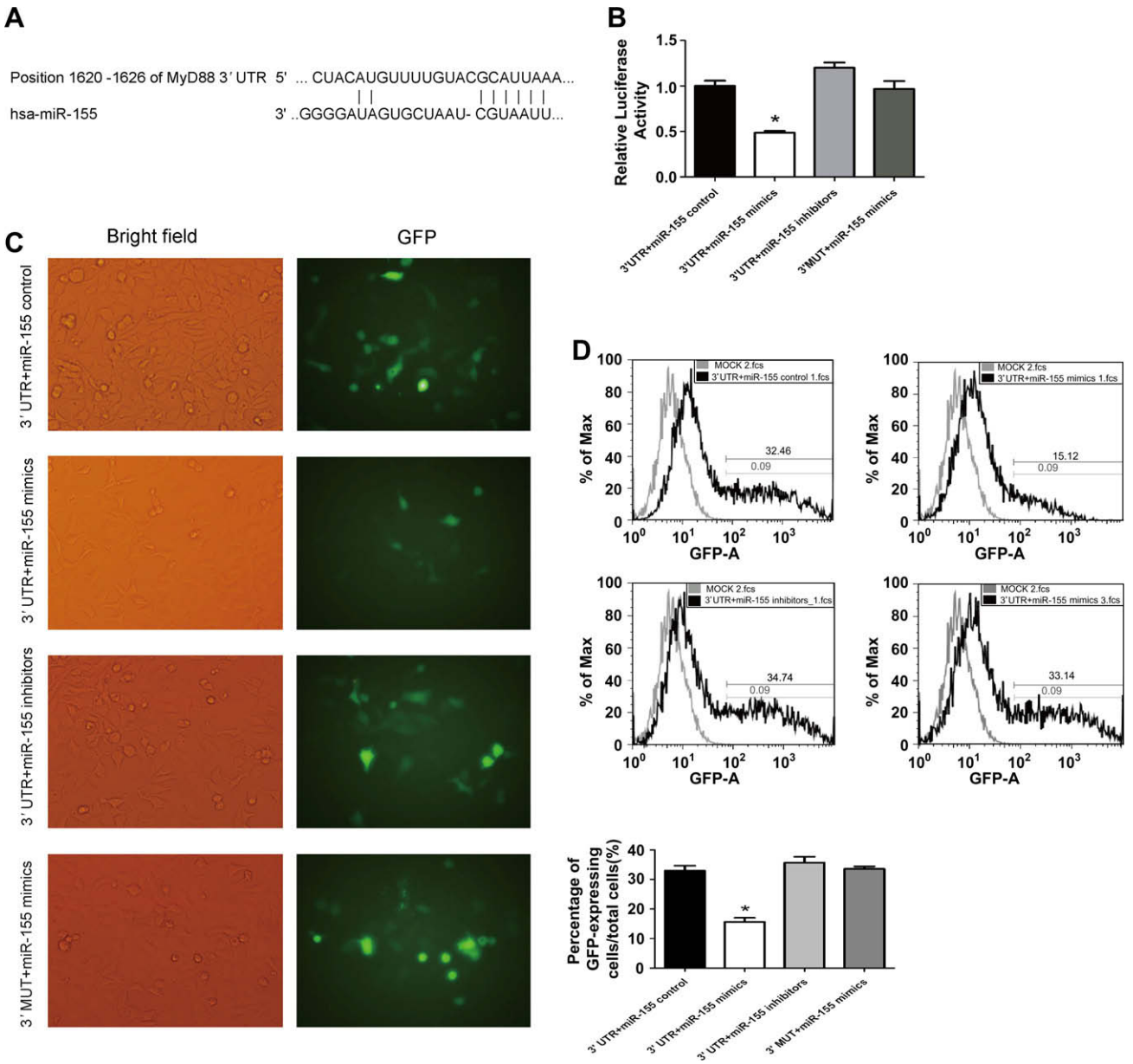

Fig. 1. Identification of MyD88 as a potential target of miR-155. (A) The region of the human MyD88 mRNA 3′UTR predicted to be targeted by miR-155 (TargetScan 4.2). (B) HEK-293 cells were transiently cotransfected with Luciferase report vectors, and either miR-155 mimics or miR-155 control. Luciferase activities were normalized to the activity of Renilla luciferase. (C and D) HEK-293 cells were cotransfected with the GFP report vectors, compared with the transfection of mimics, inhibitors or control of miR-155. Data are representative of at least three independent experiments.

medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin in a humidified incubator containing 5% CO_2 at 37 °C. The wild-type *H. pylori* strain 26695 was obtained from ATCC and grown as previously described [14].

2.2. Plasmid construct

The construction of Luciferase report vectors and GFP reporter vectors for miR-155 target, MyD88, was performed according to the instructions (details shown in Supplementary data).

2.3. Luciferase assay and GFP repression experiments

HEK-293 cells were transfected with Luciferase reporter vector or GFP reporter vector using Lipofectamine 2000 (Invitrogen Life Technologies) (details shown in Supplementary data).

2.4. siRNA assay

siRNAs transfections were performed with Lipofectamine 2000 reagent (Invitrogen). AGS cells were transfected with 100 nM siRNA for 24 h, followed by *H. pylori* infection (details shown in Supplementary data).

2.5. Quantitative RT-PCR

Quantitative RT-PCR analyses for the mRNA and miR-155 were performed by using PrimeScript RT-PCR kits (Takara) and TaqMan miRNA assays (Ambion) as described [10] (details shown in Supplementary data).

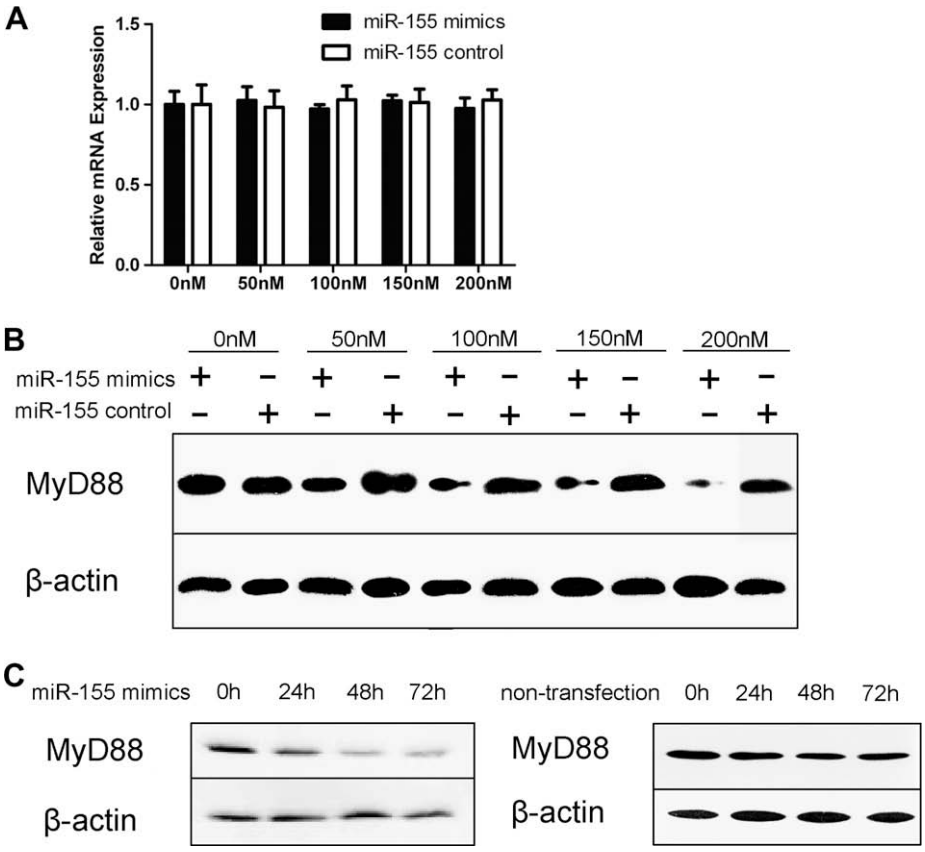

Fig. 2. MyD88 expression is repressed by miR-155 in a dose- and time-dependent manner. (A and B) AGS cells were transfected with miR-155 mimics and control in a different concentration for 24 h followed by qRT-PCR and Western blot analysis for MyD88. (C) AGS cells were transfected with miR-155 mimics at 100 nM with the extension of time followed by Western blot analysis for MyD88 (left), and non-treated cells at the indicated time were control group (right). Data are representative of at least three independent experiments.

2.6. ELISA

The production of IL-8 in the culture supernatants was measured by ELISA as specified by the manufacturer (R&D systems) (details shown in Supplementary data).

2.7. Western blot

MyD88 protein was analyzed by Western blot using MyD88 monoclonal antibody (Santa Cruz) (details shown in Supplementary data).

2.8. Flow cytometry

Cells were subjected to flow cytometric analysis on a FACSCalibur (BD Biosciences), and data were analyzed with CellQuest software (BD Biosciences) (details shown in Supplementary data).

2.9. Statistical analysis

The results are expressed as mean ± S.D. from at least three separate experiments performed in triplicate. The differences between groups were determined using two-tailed Student's t-test using SPSS 11.5 software. Statistical differences were declared significant at $P < 0.05$ level. Statistically significant data are indicated by asterisks ($P < 0.05$ (*), $P < 0.01$ (**)).

3. Results

3.1. MyD88 is a target of miR-155

When screening the targets of miR-155, MyD88 was identified as a putative miR-155 target gene by using TargetScan (version 4.2, www.targetscan.org) (Fig. 1A). Subsequently, we generated two Luciferase report vectors that contain the putative miR-155 binding sites within 3′UTR and mutant 3′UTR (3′MUT), as shown in Fig. 1B, the relative luciferase activity was reduced by 50% following cotransfection with miR-155 mimics compared with transfection with miR-155 control. In contrast, no change of luciferase was observed in cells transfected with 3′MUT constructs or miR-155 inhibitors. This result was subsequently confirmed by GFP repression experiments. As shown in Fig. 1C and D, GFP fluorescence was significantly reduced in cells cotransfected with GFP report vectors containing binding sites and miR-155 mimics, while no obvious reduction of GFP was observed in cells transfected with miR-155 inhibitors or 3′MUT constructs. Taken together, above results suggest that miR-155 targets the predicted site in the MyD88 gene.

3.2. miR-155 mediates MyD88 expression via translational suppression

Because miRNAs may down-regulate the target genes through mRNA degradation or inhibition of translation, we tried to determine which mechanism results in the suppression of MyD88 by miR-155 through measuring the mRNA and protein levels of

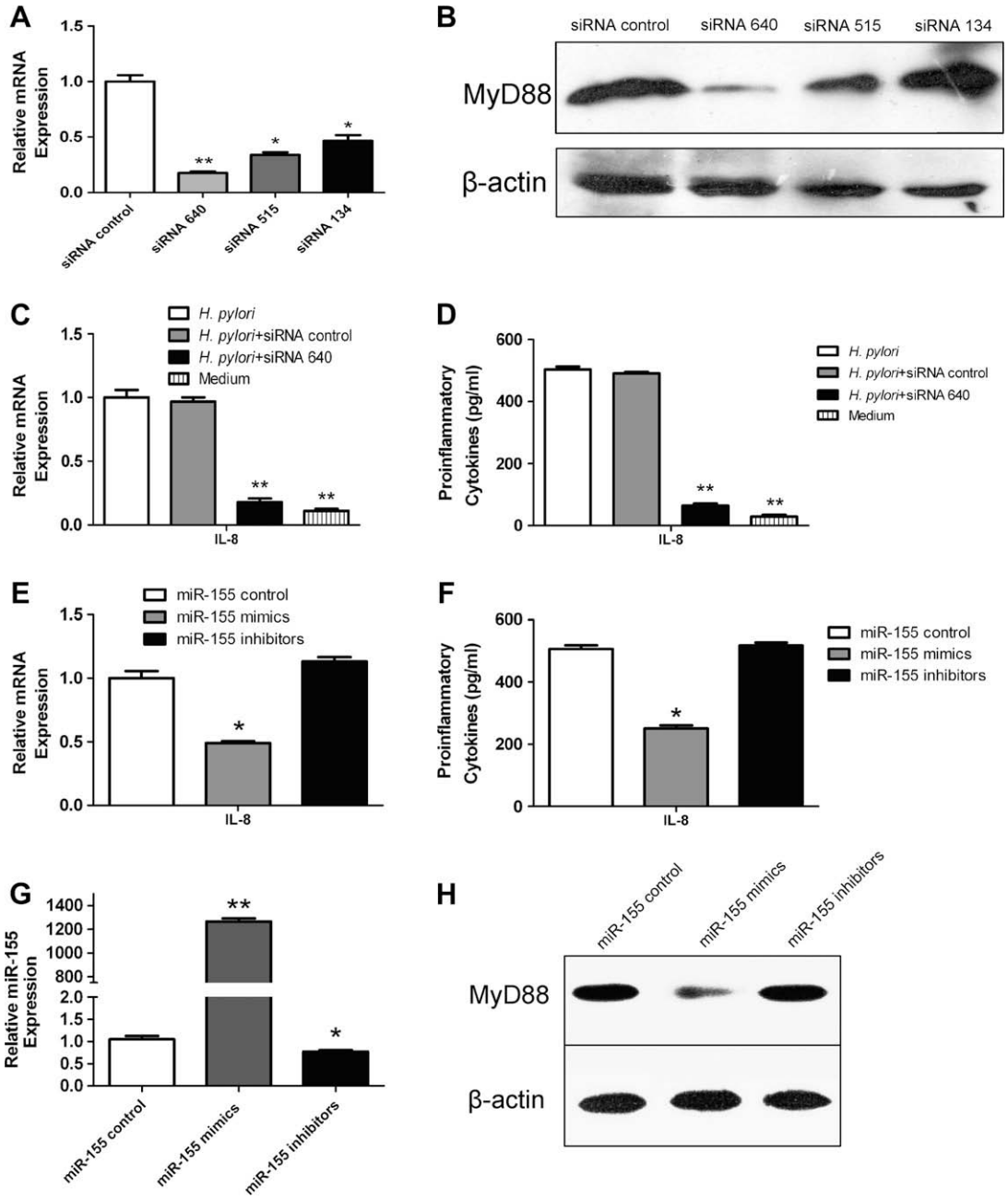

Fig. 3. Inhibition of MyD88 signaling by miR-155 or siRNA decreases the production of IL-8. (A and B) AGS cells were transfected with three siRNAs targeting MyD88 (100 nM) for 24 h. The mRNA and protein levels of MyD88 were determined. (C and D) AGS cells were transfected with siRNA-640 or a control oligonucleotide at 100 nM for 24 h followed by *H. pylori* infection or medium. The mRNA and protein levels of IL-8 were determined. (E and F) AGS cells were transfected with miR-155 mimics, inhibitors, or control at 100 nM for 24 h followed by *H. pylori* infection. The mRNA and protein levels of IL-8 were determined. The expression of miR-155 and MyD88 was analyzed, respectively, by qRT-PCR and Western blot analysis (G and H). Results are mean ± S.D. of triplicate samples and are a representative experiment of three separate experiments.

MyD88 in AGS cells transfected with miR-155 mimics or miR-155 control. As shown in Fig. 2A, miR-155 has no effect on the mRNA expression level of MyD88. In contrast, overexpression of miR-155 mimics decreased MyD88 protein content in a dose and time-dependent manner (Fig. 2B and C). Above data suggest that miR-155 might down-regulate the target protein MyD88 through inhibition of translation.

3.3. Inhibition of MyD88 by miR-155 reduces the H. pylori-induced proinflammatory cytokine IL-8

In our previous study, we have demonstrated that miR-155 negatively regulated the release of proinflammatory cytokines. Based on the previous reports [15,16], we chose IL-8 as the typical proinflammatory cytokine released by AGS cells in response to *H. pylori*. To further address the possibility that the inhibition of MyD88 by miR-155 is responsible for the reduction of *H. pylori*-induced IL-8, we assessed the impact of MyD88 silencing by RNA interference. As shown in Fig. 3A and B, the mRNA and protein levels of MyD88 were significantly decreased in AGS cells transfected with three siRNAs (640, 515 and 134), and silence effect of siRNA-640 was the most significant. Subsequently, MyD88 silencing resulted in the drastically decrease of IL-8 (Fig. 3C and D), similar to the effect observed in AGS cells with enforced expression of miR-155 mimics (Fig. 3E and F). Moreover, to obtain further experimental evidence for the possibility, we examined the expression of

miR-155 and MyD88 in AGS cells upon infection in the presence of miR-155 control, mimics or inhibitors. As shown in Fig. 3G and H, the inhibition of MyD88 was in agreement with overexpression of miR-155, whereas no significant change in the levels of MyD88 with miR-155 inhibitors was found. Overall, these results suggest that overexpression of miR-155 mimics can reduce *H. pylori*-induced IL-8 by targeting MyD88.

4. Discussion

Recently, miR-155 has been indicated to play a key role in the regulation of normal immunity or inflammation response [6,17–20]. Expression of miR-155 in monocytes or macrophages is strongly induced by a broad range of stimuli including bacterial lipopolysaccharide (LPS), IFN-β polyriboinosinic–polyribocytidylic acid (poly IC) or tumor necrosis factor-α (TNF-α) [6–8]. In addition, the up-regulated miR-155 expression has been found in synovial fibroblasts and tissue from rheumatoid arthritis patients [21]. However, the relationship between bacterial infection and miRNAs is just beginning to be explored. In our previous studies, we demonstrated the increased expression of miR-155 and miR-146a in human gastric epithelial cells infected with *H. pylori*, and the miRNAs play a potential role in a negative feedback loop to modulate the inflammation during *H. pylori* infection [10]. Therefore, the mechanism by which miR-155 modulate the immune response to *H. pylori* is needed to be further investigated.

Although a number of target genes of miR-155 are predicted in silico, not all predicted targets have been found to be responsive to the miRNAs upon experimental validation [22]. To date, about 26 genes have been experimentally validated in predicted targets of miR-155, including many transcriptional regulatory proteins, protein receptors, kinases, nuclear and DNA binding proteins [3]. For example, TAB2, an adaptor in the TLRs/IL-1 signaling cascade, was found to be a direct target of miR-155 [23]. JARID2/Jumonji, a cell cycle regulator and part of a histone methyltransferase complex, was also targeted by miR-155 [24]. In this report, we identified that MyD88 was a novel target of miR-155, and miR-155 might down-regulate the target protein MyD88 through inhibition of translation.

MyD88 is a key downstream adapter for most Toll-like receptors and interleukin-1 receptors [25]. Most TLRs, upon recognition of discrete pathogen-associated molecular patterns (PAMPs), activate MyD88 leading to the nuclear translocation of transcription factors, such as NF-κB and AP-1, and thus transcriptionally regulate host-cell responses to pathogens [26]. Thus far, many of the studies on innate immune responses to *H. pylori* in epithelial cells have focussed on TLRs [27,28]. Furthermore, MyD88 has been indentified as an important signal transducer in *H. pylori*-infected human epithelial cells [29]. In this report the results of RNA interference suggest that overexpression of miR-155 mimics was able to negatively regulate *H. pylori*-induced proinflammatory cytokine IL-8 via targeting of MyD88.

It is known that an individual miRNA has the ability to modulate multiple genes and many genes can be regulated by more than one miRNA, therefore the function of miR-155 during *H. pylori* infection may be more complex than we can imagine. The immune response of *H. pylori* infection is complex and fascinating. Our work on trying to understand the miRNA response of gastric epithelial cells to *H. pylori* may provide insight into the unique character of the response to infection by this organism. Furthermore, *H. pylori*-induced miRNAs could become a new therapeutic drug in *H. pylori*-related inflammation and malignant diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.02.063.

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