Genome-wide mRNA–miRNA profiling uncovers a role of the microRNA miR-29b-1-5p/PHLPP1 signalling pathway in Helicobacter pylori-driven matrix metalloproteinase production in gastric epithelial cells

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Abstract
Aberrant expression of microRNAs (miRNAs) is associated with tumour progression, extracellular matrix remodelling, and cell proliferation. miRNAs modulate host gene expression during infection by pathogens such as Helicobacter pylori, which is associated with varying degrees of gastric pathology. In order to gain insight into the regulation of gene expression by miRNAs during H. pylori infection of gastric epithelial cells and its likely downstream consequences, we analysed the transcriptomes and miRnomes of AGS cells infected with H. pylori. In silico analysis of miRNA–mRNA interactions suggested that miR-29b-1-5p was a likely regulator of pathways associated with gastric epithelial cell pathology. We validated PH domain leucine rich phosphatase 1 (PHLPP1), a negative regulator of the Akt signalling pathway, as a target of miR-29b-1-5p. In an in vivo mouse model, we observed that infection with H. pylori was associated with upregulation of miR-29b-1-5p and downregulation of PHLPP1. Transfection with either a mimic or an inhibitor of miR-29b-1-5p confirmed that downregulation of PHLPP1 upregulates Akt-dependent NF-κB signalling leading to activation of matrix metalloproteinases 2 and 9, players in the degradation of extracellular matrix during H. pylori infection. The secreted antigen HP0175 was associated with upregulation of miR-29b-1-5p, regulation of metalloproteinase activity, and migration of AGS cells. Our study suggests that targeting the miR-29b-1-5p/PHLPP1 signalling axis could be a potential host-directed approach for regulating the outcome of H. pylori infection.

KEYWORDS
helicobacter, infection, transcriptomics

1 INTRODUCTION

Helicobacter pylori is a microaerophilic spiral-shaped bacterial pathogen that colonises the gastric mucosa of more than half of the world's human population (Parkin, 2006). The spectrum of disease caused by H. pylori ranges from gastric or duodenal ulcers to mucosa-associated lymphoid tissue lymphoma and gastric carcinoma (Blaser et al., 1995; De Falcao et al., 2015; Malferttheiner, Chan, & McColl, 2009). The best studied virulence factors of H. pylori such as cytotoxin-associated gene A (CagA) are associated with the Type IV secretion systems. Mucosa-
associated lymphoid tissue lymphoma of the stomach is associated with \textit{H. pylori} strains expressing CagA (Eck et al., 1997). Although CagA has been extensively studied, other \textit{H. pylori} factors also influence cell signalling. These include vacuolating cytotoxin A, adhesin blood group antigen binding adhesin (BabA), urease, serine protease high temperature requirement protein (HtRa) (Backert, Neddermann, Maubach, & Naumann, 2016), \textit{H. pylori} neutrophil-activating peptide (de Bernard & D’Elios, 2010), and HP0175 (Kundu, 2013), the focus of our laboratory. Each of these factors modulates the course of infection by influencing signalling in the gastric epithelium and in immune cells. It is therefore necessary to acquire comprehensive knowledge about the pathways and processes regulated by the virulence factors of \textit{H. pylori}.

MicroRNAs (miRNAs) are small, noncoding RNAs of approximately 22 nucleotides (Bartel, 2009). They are usually transcribed as pri-miRNAs by RNA polymerase II, cleaved to precursor (pre-miRNAs) by the RNase III enzyme Drosha, exported to the cytoplasm, and further processed to mature miRNAs. The seed sequence of a miRNA shows complementarity to regions present in the 3’ untranslated regions (3’UTRs) of target mRNAs (Friedman, Farh, Burge, & Bartel, 2009). Binding of a miRNA to its target usually blocks translation and leads to mRNA degradation (Guo, Ingolia, Weissman, & Bartel, 2010). miRNAs regulate diverse biological processes including cellular differentiation, adhesion, angiogenesis, invasion, migration, and metastasis and therefore play crucial roles in diseases such as cancer (Tili, Croce, & Michaille, 2009). miRNAs can act either as oncogenes or tumour suppressors (Li et al., 2012; Xia et al., 2012; X. Zhao et al., 2013) and have been reported to regulate tumour growth, invasion, and metastasis in gastric cancer (M. Zhu et al., 2014; Zuo et al., 2015).

The human and mouse miR-29 family consists of miR-29a, miR-29b, and miR-29c. The precursor of the miR-29a/29b-1 cluster is located on chromosome 7 (7q32), whereas the precursor of the miR-29b-2/c cluster is located on chromosome 1 (1q32). The role of the miR-29 family in cancer has been reviewed by Z. Wang, Zhang, Li, Yu, and Ren (2013). miR-29a-3p regulates cell proliferation and migration (Z. Zhao et al., 2015). Mir-29a inhibits cell proliferation through downregulation of p42/3 in gastric cancer (Cui et al., 2011). A recent report has shown that repression of miR-29c expression is associated with gastric cancer (Matsu et al., 2013). The 3’ end of the miR-29b-1 precursor is clipped to generate miR-29b-1-3p with a seed sequence conserved with those of miR-29b-2-3p, miR-29a-3p, and miR-29c-3p. The 5’ end of miR-29b-1 is clipped to generate miR-29b-1-5p. However, in this case, the seed sequence is not highly conserved.

From genome-wide transcriptomic profiling, we show in this report that miR-29b-1-5p is one of the highly upregulated miRNAs during the early hours of infection of gastric epithelial cells with \textit{H. pylori}. miR-29b-1-5p expression is dependent on the secreted antigen HP0175. The PH domain leucine rich repeat protein phosphatases (PHLPPs) 1 and 2 are Ser/Thr phosphatases which acts as negative regulators of the PI3K/Akt pathway (Gao, Furnari, & Newton, 2005). PHLPP1 plays an important role in cell survival, migration, cell death, and metastasis. Low expression of PHLPP1 has been associated with gastric cancer (Y. Wang, Shu, et al., 2013). In silico analysis of miRNA–mRNA interactions suggested that PHLPP1 is a putative target of miR-29b-1-5p. We have validated that PHLPP1 is a bona fide target of miR-29b-1-5p. Its expression is inversely correlated with the expression of miR-29b-1-5p. By virtue of targeting PHLPP1, miR-29b-1-5p activates the Akt2 kinase which is associated with increased NF-κB activation leading to enhanced production of matrix metalloproteases (MMPs) 2 and 9. As a consequence, miR-29b-1-5p regulates cell migration in response to challenge of gastric epithelial cells with \textit{H. pylori}. Our data further suggest a role of the secretory antigen HP0175 in the regulation of \textit{H. pylori}-induced MMP production and cell migration in a miR-29b-1-5p/PHLPP1 dependent manner.

2 | RESULTS

2.1 | Integrated analysis of differently regulated miRNAs and their putative targets

In order to gain insight into whether microRNAs are differentially regulated during the early hours of infection of gastric epithelial cells with \textit{H. pylori} and to obtain insight into the likely role of miRNAs in infection, we analysed the transcriptomes and miRNomes of AGS cells infected with \textit{H. pylori} at a multiplicity of infection (MOI) of 100 for 4 hr. The transcriptomic data sets are available in the NCBI Geo Database under accession number GSE108307. For the purpose of the present study, we focused on the differentially regulated miRNAs (Table S1) or mRNAs (Table S2) with a fold change ≥1.5 in infected (WT-100) cells compared with uninfected (UT) cells. miRNA–mRNA target pairs were predicted from the list of differentially expressed, inversely correlated miRNAs and mRNAs using miRDB 5.0 (http://www.mirdb.org/download.html). The target miRNAs were further analysed to identify their association with functional pathways probably linked to \textit{H. pylori} infection using the Ingenuity Pathway Analysis tool (Ingenuity systems, http://www.ingenuity.com). During early infection, functional processes associated with cell death and survival, cell viability, cell cycle progression, cellular growth, and proliferation were enriched among the miRNA target genes. All these are of likely relevance to pathological responses linked to infection of gastric epithelial cells by \textit{H. pylori}. (Table S3). We next generated a miRNA–mRNA interaction network with the miRNA–mRNA pairs identified in infected AGS cells (Figure 1), focusing on those miRNAs which are upregulated during infection. A total of 10 miRNAs, namely, miR-29b-1-5p, miR-671-5p, miR-3646, miR-31-3p, miR-3147, miR-3189-3p, miR-4257, miR4313, miR-629-3p, and miR-765 were identified from this network.

In this paper, we have focused on miR-29b-1-5p considering that it (a) was highly upregulated during the early hours of infection and (b) was associated with a large number of pathways of relevance to gastrointestinal diseases (Table S3). In silico analysis of miRNA–mRNA interactions showed that PHLPP1 is a putative target of miR-29b-1-5p (Figure 1), with expression levels inversely correlated with the expression of miR-29b-1-5p. PHLPP1 dephosphorylates Akt2, a member of the Akt family of serine threonine kinases (Grzechnik & Newton, 2016). PHLPP1 reportedly promotes pancreatic cell death (Nitsche et al., 2012) and is associated with poor prognosis of gastric cancer (Hou et al., 2015). In view of the above, we focused on the miR-
29b-1-5p/PHLPP1 miRNA/target pair for detailed investigation into their role in *H. pylori*-mediated signalling in gastric epithelial cells.

2.2 | hsa-miR-29b-1-5p is up regulated in *H. pylori*-infected AGS cells in an HP0175 dependent manner

We validated the upregulation of miR-29b-1-5p as an early event of *H. pylori* infection of AGS cells, by quantitative reverse transcription polymerase chain reaction (qRT-PCR). At an MOI of 100, miR-29b-1-5p was upregulated 4 hr post-infection (Figure 2a). We tested whether miR-29b-1-5p is differentially regulated during infection in vivo. Mice were infected intragastrically with the mouse-adapted strain of *H. pylori*, SS1. miR-29b-1-5p was upregulated in the pyloric part of the stomachs of infected mice, compared with the uninfected mice (Figure 2b). Considering that secretory antigens play important roles in regulating pathogen-induced host cell signalling, we tested the possible role of the secreted protein HP0175, in the regulation of gastric epithelial miRNA and mRNA expression during *H. pylori* infection. Using a knockout strain of *hp0175* (Basak et al., 2005), we observed that *H. pylori*-induced upregulation of miR-29b-1-5p was clearly compromised in the absence of *hp0175* (Figure 2a). We further tested the role of the virulence factor CagA during infection of AGS cells, by testing the expression of miR-29b-1-5p in a cagA-negative strain. miR-29b-1-5p upregulation in AGS cells occurred even in the absence of cagA (Figure S1). These results suggested that miR-29b-1-5p upregulation occurs in an HP0175-dependent, CagA-independent manner.

2.3 | PHLPP1 is a target of miR-29b-1-5p

In order to test whether PHLPP1 is a bona fide target of miR-29b-1-5p, the 3’UTR of PHLPP1 was predicted to contain one binding site complementary to the seed sequence of miR-29b-1-5p (Figure 2c) and was cloned downstream of a constitutive firefly luciferase open reading frame in pMIR Report. This construct was transfected in HEK 293 cells either in the absence or in the presence of miR-29b-1-5p mimic or control mimic, and luciferase activity was measured. Luciferase activity was downregulated in the presence of the 29b-1-5p mimic (left panel, Figure 2d). The specificity of the regulation of PHLPP1 by miR-29b-1-5p was confirmed by our observation that deletion of the miR-29b-1-5p binding site in the PHLPP1 3′UTR abrogated the ability of the miR 29b-1-5p mimic to repress luciferase activity (right panel, Figure 2d). These results strengthened the view that PHLPP1 is a bona fide target of miR-29b-1-5p.
2.4 | PHLPP1 is regulated in a miR-29b-1-5p-dependent manner during H. pylori infection

We next tested the regulation of PHLPP1 by analysing its levels after infection of AGS cells with H. pylori. PHLPP1 levels increased above basal levels shortly after infection with H. pylori but fell off within 4 hr of infection (Figures 2f and S2a). Further, we observed that PHLPP1 expression was lower in the stomachs of infected mice compared with uninfected ones (Figures 2g and S2b). The effect of miR-29b-1-5p on PHLPP1 expression was evaluated by transfecting cells with either a miR-29b-1-5p inhibitor or mimic prior to infection. PHLPP1 increased over time in cells transfected with a miR-29b-1-5p inhibitor compared with a control inhibitor (Figures 2h and S2c).

On the other hand, PHLPP1 levels were significantly lower at all time points in cells transfected with a miR-29b-1-5p mimic prior to infection, compared with cells transfected with a control mimic (Figures 2i and S2d). These results confirmed the role of miR-29b-1-5p in regulating PHLPP1 levels during H. pylori infection.

2.5 | miR-29b-1-5p and PHLPP1 regulate Akt activation in H. pylori-infected AGS cells

Akt is a serine/threonine kinase that plays a major prosurvival role in cancers. Akt2, the target of PHLPP1, has been specifically associated with human cancer (Cheng et al., 1996; Cheng, Lindsley, Cheng, Yang, & Nicosia, 2005). Akt is principally activated through the receptor-mediated phosphatidylinositol 3-kinase-dependent signalling pathway. Termination of Akt signalling depends on the phosphatases,
phosphatase and tensin homologue (PTEN), and PHLPP. PTEN is a phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase. PTEN negatively regulates intracellular levels of phosphatidylinositol-3,4,5-trisphosphate in cells and functions as a negative regulator of Akt pathways and as a tumour suppressor (Maehama & Dixon, 1998). PHLPP family members PHLPP1 and PHLPP2 directly dephosphorylate Akt at serine 473, inactivating the kinase. PHLPP1 selectively dephosphorylates Akt2. Unlike in the case of PHLPP1, transfection of AGS cells with a miR-29b-1-5p mimic did not repress PTEN levels in H. pylori-treated cells (Figure S2e). We therefore focused on the role of the miR-29b-1-5p/PHLPP1 axis on Akt activation. H. pylori infection resulted in increased Akt phosphorylation in AGS cells transfected with a control inhibitor, whereas transfection of a miR-29b-1-5p inhibitor prior to infection attenuated H. pylori-induced phosphorylation of Akt on Ser 473 (Figures 3a and S3a). Overexpression of PHLPP1 was associated with repression of H. pylori-dependent phosphorylation of Akt on Ser 473 (Figures 3b and S3b), whereas overexpression of a catalytically inactive mutant of PHLPP1 (PHLPP1 (ΔC)) led to augmented Akt Ser 473 phosphorylation following challenge of AGS cells with H. pylori (Figures 3c and S3c). In harmony with this, knock down of PHLPP1 (Figures 3d and S3d) enhanced H. pylori-induced Akt Ser 473 phosphorylation (Figures 3e and S3e). Taken together, these results supported the view that the miR-29b-1-5p/PHLPP1 signalling axis plays a role in regulating the activation of Akt in H. pylori-infected gastric epithelial cells.

2.6 | miR-29b-1-5p enhances NF-κB signalling in H. pylori- challenged gastric epithelial cells

The transcriptional regulator NF-κB plays important roles in carcinogenesis, infection, immunity, and inflammatory responses. It also regulates cellular growth and apoptosis (Grivennikov, Greten, & Karin, 2010). Akt phosphorylates the upstream kinase of the NF-κB pathway, IkB kinase (IKK), to augment the transactivation potential and phosphorylation of the NF-κB subunit, p65 (RelA). It promotes metastasis and angiogenesis in an NF-κB-dependent manner. We tested whether the miR-29b-1-5p/PHLPP1 signalling axis regulates NF-κB activation, by virtue of regulating Akt activation. Under basal conditions, the NF-κB p50-p65 dimer is sequestered in the cytoplasm by the binding of IkB-α. IKK-dependent phosphorylation of IkB-α leads to its ubiquitination and degradation, facilitating cytoplasmic to nuclear translocation of p65. We observed that H. pylori-induced phosphorylation of IkB-α on Ser32 was attenuated in cells treated with a 29b-1-5p inhibitor compared with cells treated with a control inhibitor (Figures 4a and S4a), suggesting that miR-29b-1-5p positively regulates H. pylori-mediated activation of NF-κB in AGS cells. To further confirm the role of miR-29b-1-5p in NF-κB activation, we tested the translocation of the p65 subunit of NF-κB from the cytosol to the nucleus following H. pylori infection of AGS cells which had been transfected with control inhibitor or miR-29b-1-5p inhibitor. Western blotting showed that H. pylori increased nuclear translocation of p65 (compared with uninfected cells) when transfected with control inhibitor prior to infection, as evidenced by Western blotting (Figure 4b). On the other hand, transfection of miR-29b-1-5p inhibitor attenuated H. pylori-induced translocation of p65 to the nucleus, compared with cells transfected with control inhibitor (Figures 4b and S4b). Similar observations were made by immunofluorescence microscopy (Figures 4c and S4c). Nuclear translocation of p65 was also inhibited in H. pylori-treated cells overexpressing PHLPP1 compared with cells transfected with the empty vector (Figure 4d). Overexpression of PHLPP1 (ΔC; which lacks the catalytic activity of PHLPP1) could not

![FIGURE 3](image-url)  miR-29b-1-5p and PH domain leucine rich phosphatase 1 (PHLPP1) regulate Akt activation. (a) AGS cells were transfected with control inhibitor or miR-29b-1-5p inhibitor and then infected with Helicobacter pylori at a multiplicity of infection of 100 for different periods of time. Phosphorylation of Akt was monitored by immunoblotting with pAkt antibody. (b, c) AGS cells were transfected with either empty vector (b, c) or plasmid overexpressing PHLPP1 (b) or mutant PHLPP1 (ΔC; c) followed by infection with H. pylori for different periods of time. Expression of phosphorylated Akt (pAkt) was monitored as described under panel a. (d, e) AGS cells were transfected with either control or PHLPP1 siRNA. Expression of PHLPP1 was monitored by immunoblotting with PHLPP1 antibody. For (e), infections were carried out with H. pylori for different periods of time and phosphorylation of Akt was monitored by immunoblotting with pAkt antibody. Equal protein loading was confirmed by reprobing of all blots with GAPDH antibody. Each blot is representative of three separate experiments. UI = uninfected; GAPDH = glyceraldehyde 3-phosphate dehydrogenase.
inhibit nuclear translocation of p65 in infected cells (Figure 4d). These findings confirmed that the regulation of the Akt signalling pathway by miR-29b-1-5p/PHLPP1 impacts the NF-κB pathway during infection of gastric epithelial cells by *H. pylori*.

2.7 | miR-29b-1-5p/PHLPP1 signalling regulates expression of MMP9 and MMP2

We next interrogated the role of miR-29b-1-5p/PHLPP1-dependent NF-κB activation on the regulation of molecules of likely relevance to pathogenesis. Metastasis and degradation of extracellular matrix is frequently associated with gastric cancer pathology. MMPs are the principal players in degradation of the extracellular matrix during *H. pylori* infection (Bergin et al., 2004). The transcription factors reported to regulate MMPs include E2F1, Sp1, and NF-κB (Li et al., 2014). We therefore tested whether miR-29b-1-5p/PHLPP1 signalling regulates MMP expression in response to challenge of AGS cells with *H. pylori*, focusing on MMP2 and MMP9, the two principal gelatinolytic MMPs of AGS cells. Gelatin zymography further showed that transfection of cells with a miR-29b-1-5p inhibitor prior to
infection attenuated MMP2 and MMP9 activity compared with cells transfected with control mimic (Figure 4e). In line with our hypothesis that miR29b-1-5p impacts Akt/NF-κB signalling by targeting PHLPP1, knock down of PHLPP1 increased MMP2 and MMP9 production in H. pylori-challenged cells (Figure 4f).

2.8 Akt2 contributes to MMP2 and MMP9 secretion and AGS cell migration in response to H. pylori

Activation of Akt plays a very crucial role in gastric cancer cell survival, proliferation, and metastasis. Considering that PHLPP1 specifically targets Akt2, we tested (a) whether PHLPP1 directly regulates Akt2 during H. pylori infection, (b) whether Akt2 regulates NF-κB activity, and (c) whether Akt2 specifically regulates the expression of MMPs. PHLPP1 knock down augmented Akt2 expression in H. pylori-treated AGS cells (Figures 5a and 5S), supporting the view that PHLPP1 regulates Akt2 in AGS cells during H. pylori infection. Knock down of Akt2 (Figures 5b and 5S) impaired nuclear translocation of p65 after infection with H. pylori (Figures 5c and 5S), suggesting that Akt2 regulates, at least in part, the activation of NF-κB in cells challenged with H. pylori. Western blotting showed that knock down of Akt2 repressed expression of MMP9 (Figures 5d and 5S). Gelatin zymography confirmed that Akt2 knock down attenuates gelatinolytic activity of

**FIGURE 5** Akt2 regulates activation of matrix metalloproteinases (MMPs) 2 and 9 and cell migration. AGS cells were transfected with (a) either control siRNA or PH domain leucine rich phosphatase 1 (PHLPP1) siRNA; or (b) control siRNA or Akt2 siRNA. Cells were lysed and immunoblotted with Akt2 antibody. (c, d, e) AGS cells were transfected with either control or Akt2 siRNA as described, followed by infection with Helicobacter pylori at a multiplicity of infection of 100. (c) Nuclear translocation of p65 was followed (after 3 hr of infection) by immunofluorescence microscopy as described under Figure 4c. (d) Expression of MMP9 in cell lysates was followed by immunoblotting with MMP9 antibody. (e) MMP activity was assayed by gelatin zymography as described under Figure 4f. (f–h) AGS cells were transfected with (f) either control inhibitor or miR-29b-1-5p inhibitor; or (g) control siRNA or PHLPP1 siRNA; or (h) control siRNA or Akt2 siRNA. Transfected cells were left either uninfected (−) or infected (+) with H. pylori at a multiplicity of infection of 100 and subjected to a wound healing assay to monitor the migration of cells. Results are representative of three independent experiments. UI = uninfected; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; DAPI = 4,6-diamidino-2-phenylindole
MMP9 and MMP2 in *H. pylori*-infected cells (Figure 5e). Taken together, these results established that the miR-29b-1-5p/PHLPP1/Akt signalling axis regulates MMP2 and MMP9 activation in *H. pylori*-challenged AGS cells.

### 2.9 miR29b-1-5p/PHLPP1/Akt2 regulates migration of AGS cells challenged with *H. pylori*

Matrix remodelling activity is directly linked to cell migration. Wound healing assays are routinely used to analyse cell migration in cancer cells. We tested the migration of AGS cells treated with *H. pylori* in the absence or presence of miR-29b-1-5p inhibitor. The migration ratio was significantly lower for cells which had been transfected with the miR-29b-1-5p inhibitor compared with cells which had been transfected with a control inhibitor (Figures 5f and S5e). Our observations suggested that miR29b-1-5p is an important regulator of MMPs and consequently cell migration during *H. pylori* infection. As expected, knock down of PHLPP1 augmented migration ratios in *H. pylori*-treated AGS cells (Figures 5g and S5f). Finally, wound healing assays showed that the migratory ability of AGS cells treated with *H. pylori* was diminished when Akt2 was knocked down (Figures 5h and S5g). In summary, miR-29b-1-5p/PHLPP1-regulated Akt2 plays an important role in regulating NF-kB activation, the activation of MMPs, and cell migration during *H. pylori* infection.

### 2.10 Role of HP0175 in miR-29b-1-5p/PHLPP1-dependent MMP induction and cell migration

We tested whether HP0175 directly regulates the induction of miR-29b-1-5p, because miR-29b-1-5p levels were lower in the hp0175 knockout strain compared with the wild type. HP0175 induced miR-29b-1-5p expression (Figure 6a). We observed higher expression of PHLPP1 in AGS cells treated with the hp0175 knockout strain compared with wild type *H. pylori* (Figures 6b and S6a). At the same time, treatment of cells with recombinant HP0175 repressed PHLPP1 levels in AGS cells compared with the untreated cells (Figures 6c and S6b). A cagA-negative strain elicited a time-dependent decrease in PHLPP1 (Figure S6c,d) similar to that observed in the case of the wild type, in harmony with its ability to upregulate miR-29b-1-5p. These results suggested that PHLPP1 downregulation occurs in an HP0175-dependent, CagA-independent manner. Gelatin zymography showed that HP0175 induces MMP2 and MMP9 activity in AGS cells (Figure 6d); whereas MMP9 and MMP2 activities were downregulated in AGS cells treated with the hp0175 knockout strain compared with the wild type *H. pylori* (Figure 6e). Finally,
wound healing assays also supported a role of HP0175 in cell migration (Figure 6f).

3 | DISCUSSION

*H. pylori* is associated with a wide spectrum of diseases which include gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma. The outcome of *H. pylori*-associated disease is likely regulated by the balance of mechanisms controlling apoptosis versus cellular survival, cytoskeletal changes associated with extracellular matrix remodelling, and the activity of extracellular matrix degrading proteinases (MMPs). Several studies document the potential role of miRNAs in *H. pylori*-associated gastric cancer (Belair, Darfeuille, & Staedel, 2009). For example, Z. Zhang et al. (2008) have reported that miR-21 expression promotes cell proliferation and migration in the gastric epithelium. Matsushima et al. (2011) have reported that 31 miRNAs are differentially expressed between *H. pylori*-infected and uninfected gastric mucosa. *H. pylori* downregulates miR-449, an miRNA that targets Met, a proto-oncogene that encodes the hepatocyte growth factor receptor, and is associated with invasion and metastasis (Bou Kheir et al., 2011; Lizé, Klímke, & Dobbelstein, 2011). Numerous miRNAs overexpressed in gastric cancer function as oncomiRs by targeting members of the Bcl-2 protein family, miR-25, miR-93, and miR-106b inhibit apoptosis by preventing expression of the proapoptotic protein, Bim (Kan et al., 2009). Noto and Peek (2011) have summarised the differential regulation of a set of miRNAs both following *H. pylori* infection and in gastric cancer. These miRNAs modulate cell cycle progression, apoptosis, and the host inflammatory response. Rossi et al. (2016) have identified a set of miRNAs (miR-103a-3p, miR-181c-5p, miR-370-3p, miR-375, and miR-223-3p) that are downregulated in *H. pylori*-infected gastric mucosa compared with normal mucosa.

Despite what is already documented in literature, we still lack comprehensive knowledge of the temporal expression of miRNAs during *H. pylori* infection and their effects on regulatory networks of consequence to *H. pylori*-linked gastric pathology. In spite of the questions that remain while attempting to correlate results obtained from model systems in vitro and in vivo, in vitro models continue to be used in order to obtain mechanistic insight into the likely role of miRNAs during infection. Here, we have used the gastric epithelial cell line AGS to specifically evaluate the early changes in miRNA profiles during *H. pylori* infection and to mechanistically understand how these changes could influence the outcome of infection. Among the differentially regulated miRNAs, several have previously been reported to play important roles in various cancers such as breast cancer, hepatocellular carcinoma, colorectal cancer, and bladder cancer or have been suggested to serve as biomarkers for cancer. For example, miR-765 regulates human hepatocellular carcinoma proliferation by downregulating inositol polyphosphate-4-phosphatase, Type II (INPP4B) expression (Xie et al., 2016); miR-31-3p expression is linked to progression-free survival in patients with KRAS positive metastatic colorectal cancer. (Manceau et al., 2014); miR-671-5p targets FOXM1 (Forkhead box M1) and inhibits epithelial to mesenchymal transition in breast cancer (Tan et al., 2015), and miR-3646 contributes to breast cancer cell proliferation, migration, and invasion by modulating G2/M transition. (Tao et al., 2016).

Bioinformatic analysis of the differentially regulated miRNAs and miRNAs in AGS cells 4 hr after infection with *H. pylori* enabled us to narrow down on the miR-29 family member miR-29b-1-5p, an miRNA not linked to gastric pathology till date. A preliminary report has shown that knockdown of miR-29b-1-5p suppresses growth in the bladder urothelial cell line, T24 (Xu et al., 2013). Based on the functional pathways linked to miR29b-1-5p, it appeared to be of likely relevance to infection. The results of the present study bring to light a probable role of miR-29b-1-5p in regulating the outcome of *H. pylori* infection and link the secretory antigen HP0175 to *H. pylori*-induced miR-29b-1-5p upregulation. We have validated that PHLPP1 is a bona fide target of miR29b-1-5p. To the best of our knowledge, the present study is the first to report that PHLPP1 is a direct target of miR29b-1-5p. We have shown that transfection of a miR29b-1-5p inhibitor or overexpression of PHLPP1 attenuated activation of Akt, linking the miR29b-1-5p/PHLPP1 axis to the regulation of Akt activity. This axis assumes significance in the light of a recent report linking the downregulation of Akt2 (the isoform targeted by PHLPP1) to negative regulation of cell growth, apoptosis, migration, and invasion of *H. pylori*-positive gastric cancer tissues (F. Wang et al., 2017). In the downstream of Akt activation, we have linked the miR29b-1-5p/PHLPP1 axis to the activation of NF-κB (Figure 6g).

Metastasis and degradation of the extracellular matrix are associated with pathology in the gastric epithelium. MMPs 2 and 9 are linked to the remodelling of the gastric epithelium (H. Zhang et al., 2006). Considering that miR29b-1-5p facilitated the activation of NF-κB in *H. pylori*-infected cells, we tested its role in activation of MMPs 2 and 9. Downregulation of MMP2 and MMP9 activity during *H. pylori* infection by expression of a miR29b-1-5p inhibitor confirmed our contention that miR29b-1-5p regulates remodelling of the gastric epithelium by augmenting MMP2 and MMP9 activity. Wound healing assays further showed that migration of AGS cells is regulated by miR29b-1-5p and PHLPP1. The *H. pylori* virulence factor CagA has been reported to modulate the expression of several miRNAs (Y. Zhu et al., 2012). Here, we show that miR-29b-1-5p upregulation is CagA-independent. HP0175 is a 29 kDa secreted protein and is among one of five antigens of *H. pylori* preferentially recognised by antibodies of patients with gastroduodenal ulcer rather than dyspepsia patients (Atanassov et al., 2002; McAteer, Fry, & Berg, 1998). It is associated with a higher grade of gastric inflammation (Oghai et al., 2016). It is a peptidyl-prolyl cis trans isomerase which induces apoptosis in gastric epithelial cells (Basak et al., 2005; Halder et al., 2015) through toll-like receptor 4-dependent signalling (Basak et al., 2005). Our earlier studies have shown that depending on the time and/or the dose of infection, HP0175 modulates host cell signalling to trigger either autophagy (Halder et al., 2015) or apoptosis (Basak et al., 2005) in gastric epithelial cells. Here, we show that early during infection, HP0175 is associated with augmented expression of miR29b-1-5p. Other independent studies have shown miR-29b-1-5p expression in human triple negative breast cancer cells (Ferrante et al., 2017). miR-29b-1-5p expression is repressed in an hp0175 knockout strain compared with the wild type, and recombinant HP0175 induces miR-29b-1-5p expression. Concomitantly, HP0175 represses PHLPP1.
expression. Using the hp0175 knockout strain, we provide evidence that HP0175 is involved in the activation of MMPs 2 and 9 and migration of AGS cells. A recent report has shown that peptidyl prolyl isomerase is one of the genes specifically associated with H. pylori-linked gastric cancer (Gong et al., 2011). The present study adds to the growing body of evidence linking HP0175 to epithelial cell damage and suggests that it could be a potential therapeutic target. These studies also suggest that targeting miR-29b-1-5p could offer therapeutic benefits in gastric cancers.

4 | EXPERIMENTAL PROCEDURES

4.1 | Reagents

Antibody against PHLPP1 was purchased from Abcam (ab 71972), p-Akt (Ser 473; #4060), Akt2 (#3063), p-lkBα (Ser 32; #9241), p65 (#8242), and horseradish peroxidase-tagged secondary antibodies were obtained from Cell Signalling Technology. TATA binding protein and glyceraldehyde 3-phosphate dehydrogenase antibodies were from Santa Cruz Biotechnology. Alexa 488-conjugated anti-rabbit secondary antibody was from Invitrogen (Molecular Probes). Gelatin for zymography was purchased from Amresco. hsa-miR29b-1-5p mimic, control mimic, hsa-miR29b-1-5p inhibitor, and control inhibitor were purchased from Ambion. Transfection reagent Lipofectamine 2000 was from Invitrogen. H. pylori selective supplement, Dent, was from Oxoid. Isovitalex, Brain Heart Infusion Agar, Brain Heart Infusion Broth, and Blood Agar were obtained from Difco, BBL. PHLPP1 overexpression plasmid (DU15247) was obtained from the MRC PPU Reagents and Services (University of Dundee, Scotland) PHLPP1 (ΔC; # 22931) plasmid was purchased from Addgene. c-Myc-Ago2 construct was a gift from Jidong Liu, Memorial Sloan-Kettering Cancer Center, NY. PHLPP1-ΔC contains a deletion of the PDZ-binding motif (amino acids 1023–1025; Gao et al., 2005).

4.2 | Cell culture

The human gastric epithelial cell line AGS (ATCC CRL-1739) a human gastric adenocarcinoma cell line, was obtained from the National Centre of Cell Science (Pune, India) and maintained in Ham’s F-12 (Invitrogen) medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO2.

4.3 | Bacterial growth and infection

H. pylori 26695 or its isogenic hp0175 mutant (H. pylori KO; Halder et al., 2015), mouse-adapted SS1 strain, and CagA negative strain of H. pylori (clinically isolated from endoscopic sample of a ulcer patient in Kolkata; Kundu et al., 2006) were grown in Brain Heart Infusion Agar containing 10% heat-inactivated fetal calf serum, Isovitalex, and Dent under microaerophilic conditions at 37 °C. Kanamycin (20 μg/ml) was added for the growth of hp0175 mutant. AGS cells were incubated at the indicated MOIs for different periods of time, washed, and used for further experiments.

4.4 | H. pylori infection in mice

C57/BL6 mice used for this study were housed under clean conditions with free access to water and food. All experiments were designed strictly following the guidelines of the Animal Ethics Committee of the Bose Institute, and all animal experiments were approved by the Institutional Animal Ethics Committees (Bose Institute, Kolkata; IAEC/B/84/2017). Animals of both control and experimental groups were kept separately and were fasted overnight before each inoculation and sacrifice.

For H. pylori infection, mouse-adapted H. pylori strain SS1 was grown for 36 hr and harvested in PBS. Mice were inoculated by orogastric gavage with about 108 colony-forming unit/mouse/inoculation twice in a 3-day period. Groups of mice either inoculated with SS1 strain or only PBS were kept separately with free access to food and water. One week after final inoculation (10 days post primary inoculation), mice from each group were fasted overnight and then sacrificed. The pyloric part of both control and inoculated mice stomach was collected for further studies.

4.5 | Expression and purification of HP0175

N-terminal His tagged HP0175 was purified by Ni2+ NTA (Qiagen) affinity chromatography as described earlier (Basak et al., 2005). For all experiments, HP0175 was used at a concentration of 1 μg/ml.

4.6 | Transfections

Gastric epithelial cell line AGS was plated on 35-mm plates at a density of 3 × 105 cells 1 day before transfection. Cells were transfected with either control mimic or miR-29b-1-5p mimic (40 nM); control inhibitor or 29b-1-5p inhibitor (40 nM) or with different plasmids using Lipofectamine 2000, according to the manufacturer’s instructions. After 24 hr, cells were infected with H. pylori as indicated. Cotransfections of miR-29b-1-5p mimic and PHLPP1 3’UTR in HEK 293 cells were carried out similarly.

4.7 | Gene silencing

PHLPP1 or Akt2 siRNA was purchased from Eurogentech, and gene silencing was carried out using the Lonza nucleofection kit according to the manufacturer’s instructions. Briefly, 106 cells were electroporated using 50-nM siRNA. Cells were grown either for 48 or 72 hr prior to infection with H. pylori. Silencing was checked by western blotting of lysates.

4.8 | Extraction from tissues

Pyloric part of both inoculated and control mice stomach was collected in PBS containing protease inhibitor cocktail (Santacruz biotechnology) and stored at −20 °C. Prior to sample preparation, tissue sections were weighed, and approximately 20–25 mg of tissue was used for sample preparation. About 20 μl/mg radioimmunoprecipitation assay buffer (RIPA) lysis buffer along with 1 mM phenylmethylsulfonfyl fluoride (PMSF), 2 mM Na3VO4, and protease inhibitor cocktail were added to the tissues, and tissue extraction was done using homogenisation at 4 °C. After proper
homogenisation, tissue extracts were collected by high speed centrifugation at 13,000 r.p.m at 4 °C for 15 mins. Clear supernatant was collected in new tube, and 100-μg tissue extracts were used for western blotting with PHLPP1 antibody.

4.9 | Preparation of nuclear extracts

Nuclear extracts form cells were prepared as described earlier (B. C. Y. Wong et al., 2003). Confluent cells in 35-mm plates were treated with indicated effectors for various time periods, and approximately 2 x 10^5 cells were used for each time point. After treatment, cells were washed twice with ice-cold PBS and resuspended in 300 μl of buffer A (10 mM Hepes pH 7.9, 1.5 mM MgCl2, 10 mM Kcl, 0.5 mM dithiothreitol (DTT), 0.5 mM PMSF, and protease inhibitor cocktail; Santacruz Biotechnology). The cells were allowed to swell on ice for 20 mins, and then 12.5 μl of 10% NP-40 was added to lyse the cells gently. After 2 min, cells were centrifuged at 13,000 rpm for 10 min, and clear cytosplasmic fraction was collected. The nuclear pellet was resuspended in 60 μl of extraction buffer (20 mM Hepes, pH 7.9, 1.5 mM MgCl2, 400 mM NaCl, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT 0.5 mM PMSF, and protease inhibitor cocktail) and agitated for 45 min at 4 °C, and nuclear debris were spun down at 13,000 rpm for 15 min. Clear supernatants were collected as nuclear extract for further studies.

4.10 | Western blotting

After infections, cells were washed with ice-cold PBS and lysed at 4 °C in lysis buffer (50 mM Tris–HCl pH 7.8, 150 mM NaCl, 1% Nonidet P-40, 0.5% Na-deoxycholate, 1% SDS, 1 mM Na3VO4, and protease inhibitor cocktail [Roche Applied Science]) for 30 min at 4 °C. The lysates were separated by SDS-PAGE followed by electrophoretic transfer on polyvinylidene difluoride membranes. The blots were developed using the LumiGLO chemiluminescence detection system (Cell Signalling Technology).

4.11 | Cloning of the 3’UTR of PHLPP1 in pMiR-report and luciferase assays

The 3’UTR of PHLPP1 was amplified genomic DNA of human origin, using the sense and antisense primers 5’-ACACTAGTCATGAAGGC ATCACCGGAGGC-3’ (A), and 5’-TAGAAGCTTGGGTATGTAAAT GGCCTAATG-3’ (B).

The resulting PCR product was cloned between the Spel and HindIII sites of the vector pMiR-Report (Promega) harbouring the firefly luciferase gene. Site-directed mutagenesis of the seed region in the 3’UTR of PHLPP1 was performed by overlap extension PCR. The initial rounds of PCR were performed using primer A (forward) and the reverse primer: 5’-TGTGAGTAAACCAATGGTGCCCT GGGA-3’; and the forward primer 5’-TCCCGGCTCATTGGGT TTACTCCACA-3’ and primer B (reverse). The two products were taken in equimolar ratio for the final round of PCR using the primer pair A and B. This product was cloned in pMiR Report and sequenced. The 3’UTR construct was cotransfected with miR-29b-1-5p mimic (40 nM) or control mimic into HEK-293 cells along with a β-galactosidase expressing construct. Cells were incubated for 24 hr after transfection and lysed, and luciferase activity was measured using the Luciferase assay kit (Promega), according to the manufacturer’s protocol. Luciferase activity was normalised by measuring β- galactosidase activity using the β-galactosidase assay kit (Promega).

4.12 | Quantitative real time PCR for analysis of miRNA

Total RNA was extracted from 10^6 AGS cells using the mirVana miRNA isolation kit (Ambion) according to the manufacturer’s instructions. cDNA for miRNA was prepared using the Taqman cDNA synthesis kit using specific primers from Applied Biosystems. Quantitative Real Time PCR was carried out using Taqman Universal Mastermix and specific primers for hsa-miR-29b-1-5p (Assay ID 002165) and U6 snRNA (Assay ID 001973) from Life Technologies, on a 7500 Real Time PCR system (Applied Biosystems). Expression of U6 was used for normalisation, and relative expression was analysed using the comparative ΔΔCt method. The fold change was expressed as 2^{-ΔΔCt}.

4.13 | RNA isolation and analysis of miRNA form mouse tissue

Pyloric part of both control and infected mice stomach was collected in RNA later solution (Ambion) and stored at −20 °C until further use. RNA was extracted from tissue sample using mirVana miRNA isolation kit (Ambion) according to manufacturer’s instruction. The qRT-PCR for miR-29b-1-5p quantification was carried out according to a two-step manufacturer’s protocol (Applied biosystems) using specific primers for murine miR-29b-1-5p (Assay ID 002497) and snoR-142 (Assay ID 001231) using 2 μg total RNA as template.

4.14 | Fluorescence microscopy

For immunostaining, AGS cells (1.5 x 10^5) were grown on cover slips. After infection with H. pylori, cells were prefixed with 500 μl of 10% neutral buffered formalin solution for 2 min. The culture medium was replaced by 500 μl of 10% formalin solution and incubated for 20 min at room temperature followed by permeabilisation with 0.05% TritonX-100 (v/v) in PBS for 15 min. Fixed cells were blocked in 2% BSA (w/v) in PBS for 30 min, incubated with primary antibody (p65; 1:500) at 4 °C, overnight, washed extensively with PBS, and finally stained with Alexa fluor 488-conjugated goat-anti rabbit antibody (1:750). After washing with PBS, the coverslips were mounted on glass slides using Prolong gold and slow fade gold antifade reagent (Molecular Probes, Invitrogen) and observed using a Zeiss AxioImager A1 fluorescence microscope.

4.15 | Gelatin zymography

AGS cells were plated in 35-mm plates at a seeding density of 3 x 10^5 cells per plate using HAM-F12 K nutrient medium and supplemented with 1% fetal bovine serum, 24 hr before treatments. Prior to treatments, medium was changed with serum and antibiotic-free Roswell Park Memorial Institute (RPMI) 1640 medium. Cells were infected with H. pylori or treated with HP0175 for the indicated periods of time, and the conditioned media were collected. Conditioned media
were loaded in appropriate volumes in 1% gelatin containing 8% SDS polyacrylamide gels and separated by electrophoresis.

After electrophoresis, gels were washed in renaturation buffer (2.5% Triton X-100 in 1X Tris buffered saline) at room temperature for 30 min and then incubated in developing buffer (50 mM Tris–HCl pH 7.8, 200 mM NaCl, 5 mM CaCl₂, 0.02% Brij 35) for 30 min at room temperature with gentle agitation and then at 37 °C overnight. Coomassie brilliant blue R-250 gel stain was used for gel staining. Gelatinolytic clear bands were observed against a blue background upon destaining.

4.16 | Immunoprecipitation of c-myc-Ago2

Association of PHLPP1 with miR29b-1-5p was tested by immunoprecipitation with c-myc-Ago2 as described by Fasanaro et al. (2009). Briefly, 3 × 10⁵ AGS cells were plated in 35-mm plate and transfected with either 29b-1-5p mimic or control mimic along with c-myc-Ago2 construct. After 24 hr, cells were lysed with lysis buffer (25 mM Tris–HCl, pH 7.4, 150 mM KCl, 5 mM EDTA, 0.5% NP-40, 5 mM DTT, 1 mM PMSF, Protease inhibitor cocktail, and 100 units/ml RNasin plus) for 30 min at 4 °C. Yeast tRNA (AM7119, Promega) was added to the lysates followed by incubation with protein A/G agarose or c-myc-agarose for 3 hr at 4°C. The agarose beads containing immune complexes were washed with IP wash buffer (300 mM NaCl, 50 mM Tris–HCl, pH 7.4, 5 mM MgCl₂, and 0.1% Triton X-100). Residual wash buffer was removed by washing with PBS. The beads were then resuspended in Qiazol (Invitrogen), and RNA was purified using the miRVana purification Kit (Ambion). Association of PHLPP1 mRNA was quantitated by qRT-PCR.

4.17 | Wound healing assay

Migration of AGS cells was analysed using a wound healing assay. Briefly, 10⁵ cells were plated in each chamber of a 4-well chamber slide prior to transfection with miRNA inhibitor or with siRNA. Cells were allowed to grow to 95% confluency. A wound was made using a 200 μl of pipette tip followed by washing with serum-free medium for removal of cell debris. Images were thereafter captured in a time-dependent manner under a phase contrast microscope, and the wound area was measured (t = 0). The cells were incubated either with media or in the presence of H. pylori (MOI 100) or purified HP0175 (1 μg/ml) at 37 °C, 5% CO₂, and allowed to migrate into the wound area for up to 6 hr. The wound area was then imaged. Migration ratio was determined using the following formula: [Wound distance at t = 0] – [(Wound distance at t = 6 h)] / [Wound distance at t = 0] (Lan, Tang, Jin, & Sun, 2016).

4.18 | Bioinformatic analysis

We have generated microarray data of AGS cells, either not infected or infected with H. pylori at an MOI of 100 for 4 hr at the Genomics Facility of Genotypic Technology, Bangalore. Two biological and two technical replicates were performed. Analysis of the microarray data was done using GeneSpring GX12.0. Raw signal intensities were normalised to the 75th percentile by percentile shift normalisation. Values flagged “Absent” were filtered out, and probe sets with “Present” or “Marginal” intensity values were retained. The data were then subjected to Student’s t test, with Benjamini–Hochberg false discovery rate multiple testing correction, with a significance level of p value ≤ .05 and a fold-change value ≥ 1.5 (compared with uninfected cells), to obtain the differentially expressed genes and miRNAs in H. pylori-infected AGS cells. miRNA–mRNA target pairs were predicted from the sets of differentially expressed inversely correlated miRNAs and miRNAs in H. pylori-infected AGS cells using the database miRDB 5.0 (N. Wong & Wang, 2015).

Functional enrichment analysis of the miRNA target genes was done using Ingenuity Pathway Analysis (https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis/). The miRNA–mRNA interaction network was constructed using Cytoscape v2.8.2 (Smoot, Ono, Ruscheinski, Wang, & Ideker, 2011).

4.19 | Statistical analysis

Student’s t test was carried out for comparison between two groups, and p value less than .05 was considered as statistically significant.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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