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CLINICAL MICROBIOLOGY - RESEARCH PAPER

Helicobacter pylori infection induces abnormal expression of pro-angiogenic gene *ANGPT2* and miR-203a in AGS gastric cell line

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Abstract

Helicobacter pylori colonizes the stomach and induces an inflammatory response that can develop into gastric pathologies including cancer. The infection can alter the gastric vasculature by the deregulation of angiogenic factors and microRNAs. In this study, we investigate the expression level of pro-angiogenic genes (*ANGPT2*, *ANGPT1*, receptor *TEK*), and microRNAs (miR-135a, miR-200a, miR-203a) predicted to regulate those genes, using *H. pylori* co-cultures with gastric cancer cell lines. In vitro infections of different gastric cancer cell lines with *H. pylori* strains were performed, and the expression of *ANGPT1*, *ANGPT2*, and *TEK* genes, and miR-135a, miR-200a, and miR-203a, was quantified after 24 h of infection (h.p.i.). We performed a time course experiment of *H. pylori* 26695 infections in AGS cells at 6 different time points (3, 6, 12, 28, 24, and 36 h.p.i.). The angiogenic response induced by supernatants of non-infected and infected cells at 24 h.p.i. was evaluated in vivo, using the chicken chorioallantoic membrane (CAM) assay. In response to infection, ANGPT2 mRNA was upregulated at 24 h.p.i, and miR-203a was downregulated in AGS cells co-cultured with different *H. pylori* strains. The time course of *H. pylori* 26695 infection in AGS cells showed a gradual decrease of miR-203a expression concomitant with an increase of *ANGPT2* mRNA and protein expression. Expression of *ANGPT1* and *TEK* mRNA or protein could not be detected in any of the infected or non-infected cells. CAM assays showed that the supernatants of AGS-infected cells with 26695 strain induced a significantly higher angiogenic and inflammatory response. Our results suggest that *H. pylori* could contribute to the process of carcinogenesis by downregulating miR-203a, which further promotes angiogenesis in gastric mucosa by increasing *ANGPT2* expression. Further investigation is needed to elucidate the underlying molecular mechanisms.

Keywords *Helicobacter pylori* · *ANGPT2* · miR-203a · Angiogenesis · *ANGPT1*

Introduction

Helicobacter pylori is a Gram-negative microaerophilic bacterium that colonizes the human stomach. The infection causes inflammation, whose characteristics depend on the type of strain and on the host response against the infection

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[1]. Infection by *H. pylori* is initially presented as chronic gastritis in the antrum, which in some patients can proceed to chronic atrophic gastritis and, in some cases, result in gastric cancer (GC). The progression towards GC involves multiple alterations, including cellular proliferation and differentiation processes, degradation of the extracellular matrix, and angiogenesis [1–3].

Inflammation caused by *H. pylori* infection is initially characterized by the recruitment of neutrophils. This process is facilitated by an increase in the permeability of the vascular endothelium, allowing extravasation of inflammatory cells, proteins, and fluids. *H. pylori* is also a potent activator of nuclear factor-kB (NF-kB) in gastric epithelial cells [4]. Activation of NF-kB by *H. pylori* infection induces a variety of cytokines, angiogenic factors, and matrix metalloproteinases (MMPs) [5].

A major component of gastrointestinal inflammation is the disturbance of the vascular structure and function, as supported by endoscopic and histologic findings related to *H. pylori* infection, including erythema, edema, and vasodilation, as well as inflammatory cell infiltration [6]. Several studies have indicated that *H. pylori* infection can alter the structure and function of the gastric vasculature, and induce angiogenic growth factors, such as vascular endothelial growth factor A (VEGFA) and MMP-9 [7–9]. Neovascularization of the gastric mucosa was shown to be significantly higher in the antrum of *H. pylori*-positive than in *H. pylori*-negative cases in dyspeptic patients, and significantly upregulated expression of inflammatory and pro-angiogenic genes in gastric epithelial cells has been reported in different studies [10–12]. These data imply that *H. pylori* is capable of inducing angiogenesis in its host, which may play a critical role in the development and progression of cancer. However, the process by which the bacterium induces neovascularization in precancerous lesions and early GC development is not fully understood.

Angiogenesis is already present in early GC, but as the cancer progresses toward more advanced stages, angiogenesis becomes more pronounced [13]. Neovascularization not only provides nutrients and oxygen to the tumor cells, and carries away metabolic waste, but it also stimulates tumor growth through autocrine or paracrine modes of action [14, 15]. Endothelial cells differentiate and proliferate under the action of selected growth factors and cytokines, particularly VEGF and angiopoietins (ANGPT, ANG), which are also produced by cancer cells and macrophages (6). ANGPT-1 and ANGPT-2 share the Tie2 (*TEK* gene) receptor in endothelial cells, with opposite effects. ANGPT1 induces maturation and quiescence of vessels, whereas ANGPT2 destabilizes blood vessels for the sprouting of new capillaries [16].

MicroRNAs (miRNAs) are small, non-coding RNAs of 19–25 nucleotides in length that negatively regulate gene expression interacting with the 3′ untranslated region (3′ UTR) of target mRNAs to induce mRNA degradation and translational repression [17]. Consequently, a downregulation of the miRNA produces an upregulation in its target gene; therefore, the upregulation of the miRNA implies the downregulation of the target gene. MicroRNAs modulate multiple cellular activities, including cellular differentiation, cell-cycle control, angiogenesis, and migration. Also, the investigation of tumor-specific miRNA expression profiles has shown their deregulation in diverse cancers [18]. miRNAs have been identified to act as tumor suppressors or as oncogenes based on their modulating effect on the expression of their target genes [19]. miR-203a, miR-200a, and miR-135 are predicted to regulate *ANGPT2*. miR-203a has been reported as tumor suppressor [20–22]; miR-200a and miR-135 have been reported to have tumor suppressor or oncogene function depending on the context [23–25]. So far, there are no reports about the regulation of the *ANGPT2* gene by microRNAs in the context of *H. pylori* infection. In this study, we investigate the expression level of the pro-angiogenic genes *ANGPT1*, *ANGPT2*, and their receptor *TEK*, and microRNAs miR-203a, miR-200a, and miR-135, using GC-cell lines co-cultured with *H. pylori*.

Materials and methods

Cell culture

The gastric cancer cell lines AGS (ATCC, CRL-1739), MKN28 (JCRB0253), and MKN45 (RCB1001RIKEN, BRC Cell Engineering Division, Japan) were maintained in RPMI 1640 or Dulbecco's modified Eagle medium/F12-Glutamax growth media, supplied with 10% fetal bovine serum, 50 IU/mL penicillin, and 50 ug/mL streptomycin (All Gibco). Cells were kept at 37 °C in a humidified incubator with 5% CO_2. The experiments were performed with 400, 000 AGS, 600, 000 MKN28, and 200, 000 MKN45 cells, seeded in 6-well plates and cultured for 48 h before infection, until reaching 90% confluence.

H. pylori culture and *in vitro* infection model

The following *H. pylori* strains were used: 7.13 (kindly provided by Christine Varon, INSERM, University of Bordeaux, France), 26695 (ATCC 700392), and 60190 (ATCC 49503). Insertion mutants with inactivation of cagA (84183ΔcagA) or cagE (84183ΔcagE) were used together with the wild-type strain 84183 (ATCC 53726). These were a kind gift from Professor John Atherton (University of Nottinham, UK) and were constructed as described previously [26]. Strains were inoculated on Columbia agar plates containing

5% sheep blood and incubated at 37 °C for 2–5 days under microaerophilic conditions in a humidified CO2 incubator.

For co-culture experiments, bacteria were added to the gastric cells at a multiplicity of infection (MOI) of 100 bacteria per gastric cell. Cells were infected for 24 h. Additionally, AGS cells were infected for 3, 6, 12, 18, 24, and 36 h with *H. pylori* strain 26695. Prior to infections, cells were washed twice in PBS, and incubated for at least 3 h in antibiotics-free medium with no serum, and co-cultured until RNA and protein were extracted after the induction. As negative controls, monolayers without *H. pylori* were processed in the same way.

Studied genes and microRNAs

The results of a whole-transcriptomic microarray previously performed at IPATIMUP (deposited at Gene Expression Omnibus under accession number GSE70394) [27] to characterize changes in the gene expression of the human gastric carcinoma cell line AGS in response to *H. pylori* infection showed increased expression of *ANGPT2* gene. We chose to study miR-135a, miR-200a, and miR-203a as they showed to be downregulated in human gastric mucosa infected by *H. pylori* in previous reports [28], and are predicted to regulate the *ANGPT2* gene by algorithms as TargetScan [29] and miRbase [30]. We also chose to study ANGPT1 and Tie2 (*TEK*), as they are also related with ANGPT2 pathway.

Quantitative PCR for miR-135a, miR-200a, miR-203a, *ANGPT1*, *ANGPT2* and *TEK*

Total RNA was isolated from cells using miRvana kit (Ambion) or Trizol (Invitrogen) in accordance with the manufacturer's instructions. RNA from each sample was transcribed into cDNA using random primer or specific miRNA primers. TaqMan assays (Thermo Scientific) were used to quantify the expression of miRNAs and the other genes (Table 1). A total of 500 ng of cDNA was used for qRT-PCR amplification using the StepOne Plus thermal cycler (Applied Biosystems, Foster City, CA, USA) under the following conditions: 95 °C for 10 s, 95 °C for 5 s, and 60 °C for 30 s, run for 40 cycles. Levels of cDNA for the microRNAs were normalized by *RNU48* small RNA; for other genes, *GAPDH* was used as endogenous control. Ct values were obtained from independent triplicate co-cultures with three technical replicas of each sample. Fold-difference for infected vs. non-infected cells was estimated by the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Cells were lysed using lysis buffer solution (PBS 1×, 1% Triton X-100, 1% NP-40, Roche Complete™ Mini EDTA-free protease inhibitor cocktail and phosphatase inhibitor cocktail) (Merck, Darmstadt, Germany). Following centrifugation at 14, 000 *g* for 30 min, the proteins in the cell lysates and supernatants were quantified using the protein assay dye reagent concentrate (BioRad), and 30 µg of protein was separated by 7.5% SDS-PAGE, prior to being transferred onto a nitrocellulose membrane (BioRad). Following blocking with 5% skim milk in PBS buffer in 0.05% Tween 20 for 1 h at room temperature, the membranes were then separately incubated overnight at 4 ˚C with the following monoclonal antibodies: Rabbit ANGPT1 (1:500; Abcam ab102015) and Rabbit ANGPT2 (1:500, Abcam ab8452) and Rabbit GAPDH (1:1000, Abcam, ab9485). The secondary antibodies (dilution 1:10 000, horseradish peroxidase-conjugated anti-rabbit, Sigma-Aldrich A9169) were applied at room temperature for 1 h. Labeled bands were detected by enhanced chemiluminescence (Clarity ECL, BioRad, USA) and analyzed with the Chemidoc Imaging System (Bio-Rad). Protein levels were quantified using the Image Lab software, version 4.1 (Bio-Rad, USA).

Table 1 List of TaqMan assays for analyzing gene and microRNA expression by RT-qPCR

Gene/microRNA	Taqman assay ID
ANGPT1	Hs00919202_m1
ANGPT2	Hs001048042_m1
GAPDH	Hs02786624_g1
TEK (Tie2)	Hs00945150_m1
miR-135a	002232
miR-200a	001011
miR-203a	000507
RNU48	001006

In vivo chicken embryo chorioallantoic membrane (CAM) assay

The CAM assay was used to evaluate in vivo, the angiogenic activity of *H. pylori*-treated cells in comparison with non-infected cells. Filtrated supernatants of three independent infections of AGS cells with 26695 strain were tested in two CAM independent experiments, as previously described [31]. Briefly, fertilized chicken (*Gallus gallus*) eggs obtained from commercial sources (Pintobar, Braga, Portugal) were incubated horizontally at 37.8 °C in a humidified atmosphere and referred to embryonic day (E). On E3, a square window was opened in the shell after removal of 2–2.5 ml of albumen to allow detachment of the developing CAM. The window was sealed with transparent adhesive tape, and the eggs returned to the incubator. The experimental setting was designed to decrease intra-animal variability; thus, both test conditions were applied into the same egg. Accordingly,

at E10 and under sterile conditions, two silicon rings were placed in distinct areas of the same CAM, and 10 µl of supernatants, derived from infected and non-infected cells, was applied in each of the inoculation sites, delimited by the rings. The eggs were re-sealed and returned to the incubator for an additional 3 days. At E13, after removing the rings, the CAM containing the inoculation area was excised from the embryos, photographed ex ovo under a stereoscope, at 20 × magnification (Olympus, SZX16 coupled with a DP71 camera). The number of new vessels (less than 20 µm diameter) growing radially towards the ring area was counted blindly to the tested condition. All experiments using chick embryos were carried out in accordance with the Directive 2010/63/EU of the European Parliament and of the Council (22 September 2010) on the protection of animals used for scientific purposes, as well as the National Regulations (Decreto-Lein.º113/2013). Accordingly, experiments with chick embryos do not require approval from any licensing committee.

Statistical analysis

The Mann–Whitney test was used to compare between 2 groups (non-infected vs. infected) and the Kruskal–Wallis test was used to compare between groups. For analysis of the CAM assay experiments, the paired Student *t* test was used. Data is presented as mean ± standard deviation (SD). The analysis was performed using the GraphPad Prism 9 software (San Diego, CA, USA).

Results

H. pylori infection enhances expression levels of *ANGPT2* mRNA in AGS, MKN28, and MKN45 cells

A previous whole-transcriptomic microarray to characterize changes in the gene expression of AGS cells in response to *H. pylori* an upregulation of ANGPT2 upon infection [27]. Based on those results, here, we chose to study the *ANGPT1/2* and Tie2 pathway in gastric cell lines in the context of *H. pylori* infection.

First, the effect of *H. pylori* infection on *ANGPT2* expression in vitro after 24 h of co-culture was addressed in GC cells. In AGS and MKN28 cells, *ANGPT2* expression was upregulated in response to infection with distinct *H. pylori* strains, namely, 26695, 60190, 84183, and 7.13 strains ($p < 0.001$, Fig. 1a; $p < 0.005$, Fig. 1b). We additionally tested the effect of *H. pylori* 26695 and 7.13 strains on MKN45 cells, and it was found that *ANGPT2* mRNA was also upregulated in this cell line 24 h.p.i ($p < 0.001$; Fig. 1c).

ANGPT2 induction is CagA-independent

To assess if the induction of *ANGPT2* depends on the bacterial virulence factor CagA, we performed infections with mutants deficient in *cagA* (not expressing CagA) or in *cagE* (not expressing a functional type IV secretion system – T4SS). There were no differences in the induction of *ANGPT2* expression between the wild-type and both 84183 mutants (Fig. 1a, $p = 0.933$; 1b, $p = 0.800$).

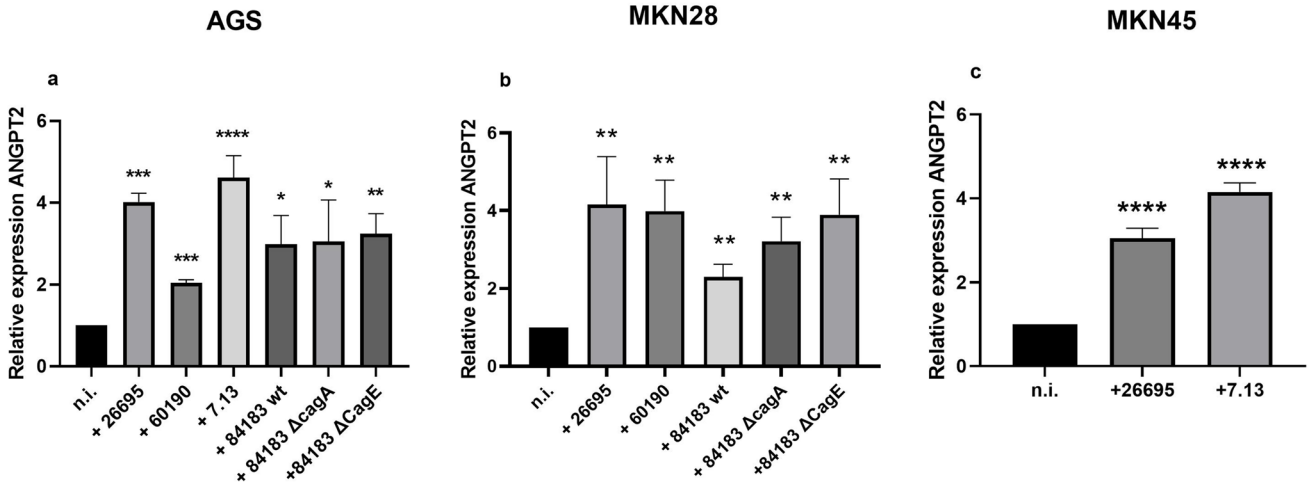

Fig. 1 *ANGPT2* mRNA expression increased in *H. pylori*-treated AGS (**a**), MKN28 (**b**), and MKN45 (**c**) gastric cell lines in a CagA-independent pathway. AGS, MKN28, and MKN45 gastric cells were incubated with *H. pylori* at a MOI of 100 for 24 h, with *H. pylori* 26695, 60190, 7.13, 84183wt, 84183ΔcagA, and 84183ΔcagE strains. Cells were analyzed to measure the relative expression levels of *ANGPT2* mRNA compared with *GAPDH* mRNA, in infected cells compared with non-infected AGS cells.****$p < 0.0001$; ***$p < 0.001$; **$p < 0.005$; *$p < 0.05$, compared to non-infected cells

ANGPT1 and TEK expression was not detected in gastric cell lines

The expression of *ANGPT1* and *TEK* in AGS, MKN28, and MKN45 cells was also assessed. In AGS and MKN28 cells, *ANGPT1* and *TEK* expression was detected at extremely low levels, and there was no change upon *H. pylori* infection. In MKN45 cells, the expression of *ANGPT1* and *TEK* was not detected at all in infected or non-infected cells, 24 h.p.i (data not shown). The protein ANGPT1 was not detected by Western blot analysis in cell lysates or in supernatants of AGS cells, on infected nor non-infected cells, at any time of infection with *H. pylori* 26695 (data not shown). These results suggest that AGS, MKN28, and MKN45 cells do not express the *ANGPT1* and *TEK* genes.

H. pylori infection alters expression levels of microRNAs in AGS and MKN28 cells

In concordance with the biological function of microRNAs, if a microRNA is the regulator of a gene, its downregulation implies the upregulation of its target gene, or *vice versa* [18]. According to this, as we found that *ANGPT2* is upregulated in response to *H. pylori* infection, miR-135a, miR-200a, and miR-203a must be expected to be downregulated. In MKN28 and AGS cells, miR-135a and miR-200a were upregulated in response to infection by *H. pylori* (Fig. 2a and b) (data not shown for miR-135a). Also, in MKN28 cells, *H. pylori* infection upregulated miR-203a (Fig. 2c). On the other hand, *H. pylori* infection downregulated miR-203a and upregulated *ANGPT2* expression in AGS cells (Fig. 2d). Consequently, it was decided to continue studying miR-203a and *ANGPT2* in AGS cells infected with 26695 strains.

Kinetics of expression of *ANGPT2* and *miR-203a* in *H. pylori* infected cells

In order to analyze the dynamics of the expression levels of *ANGPT2* and miR-203a, we performed a time course experiment of AGS infected with *H. pylori*. The increase in *ANGPT2* mRNA expression started at 18 h.p.i., and stabilized at 24 h, when the expression of *ANGPT2* reached a fold difference of almost 5 × relative to non-infected cells ($p < 0.0001$) (Fig. 3a and d). On the contrary, the expression of miR-203a decreased gradually, becoming evident at 12-h post-infection (h.p.i.) (Fig. 3c and d). To assess whether *H.*

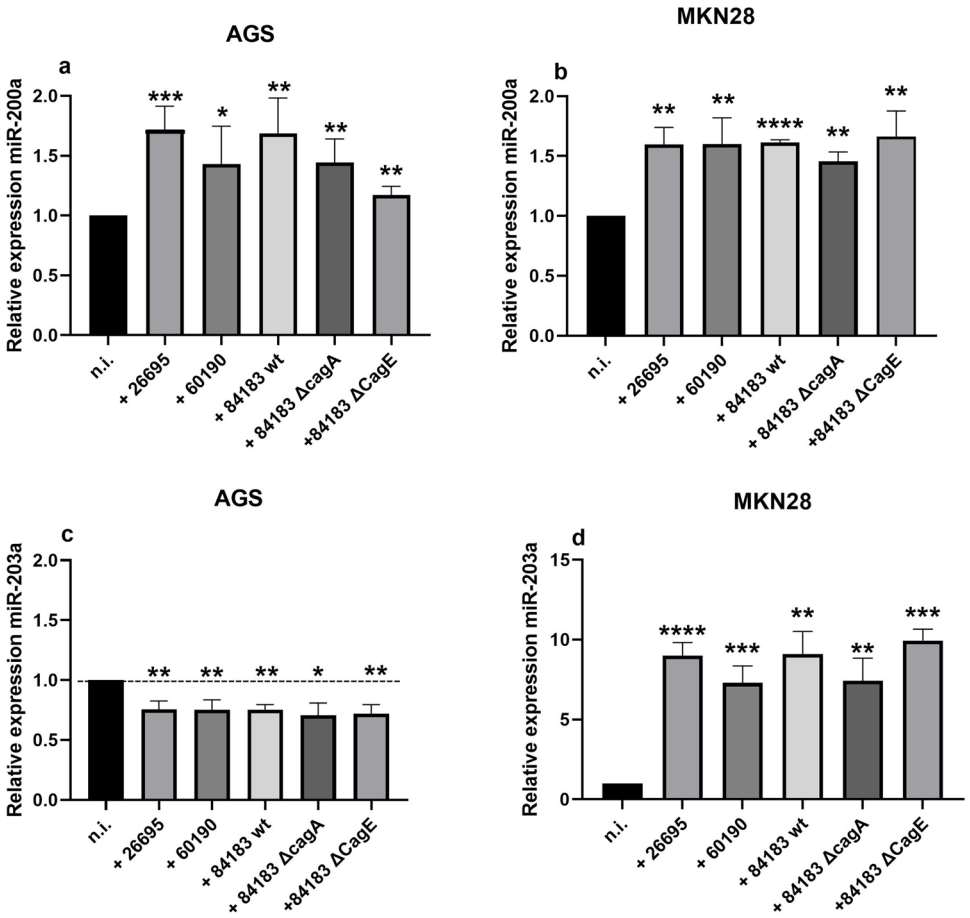

Fig. 2 *H. pylori* alters the expression of microRNAs *in vitro*. **a** and **b** miR-200a expression is increased in *H. pylori*-treated MKN28 and AGS cell lines. **c** and **d** miR-203a is upregulated in MKN28 and downregulated in AGS cell lines. AGS and MKN28 gastric cells were incubated with *H. pylori* at a MOI of 100 for 24 h, with 26695, 60190, 84183wt, 84183ΔcagA, and 84183ΔcagE strains. Cells were analyzed to measure the relative expression levels of microRNAs compared with RNU48 RNA, in infected cells compared with non-infected cells. ****$p < 0.0001$; ***$p < 0.001$; **$p < 0.005$; *$p < 0.05$, compared to non-infected cells

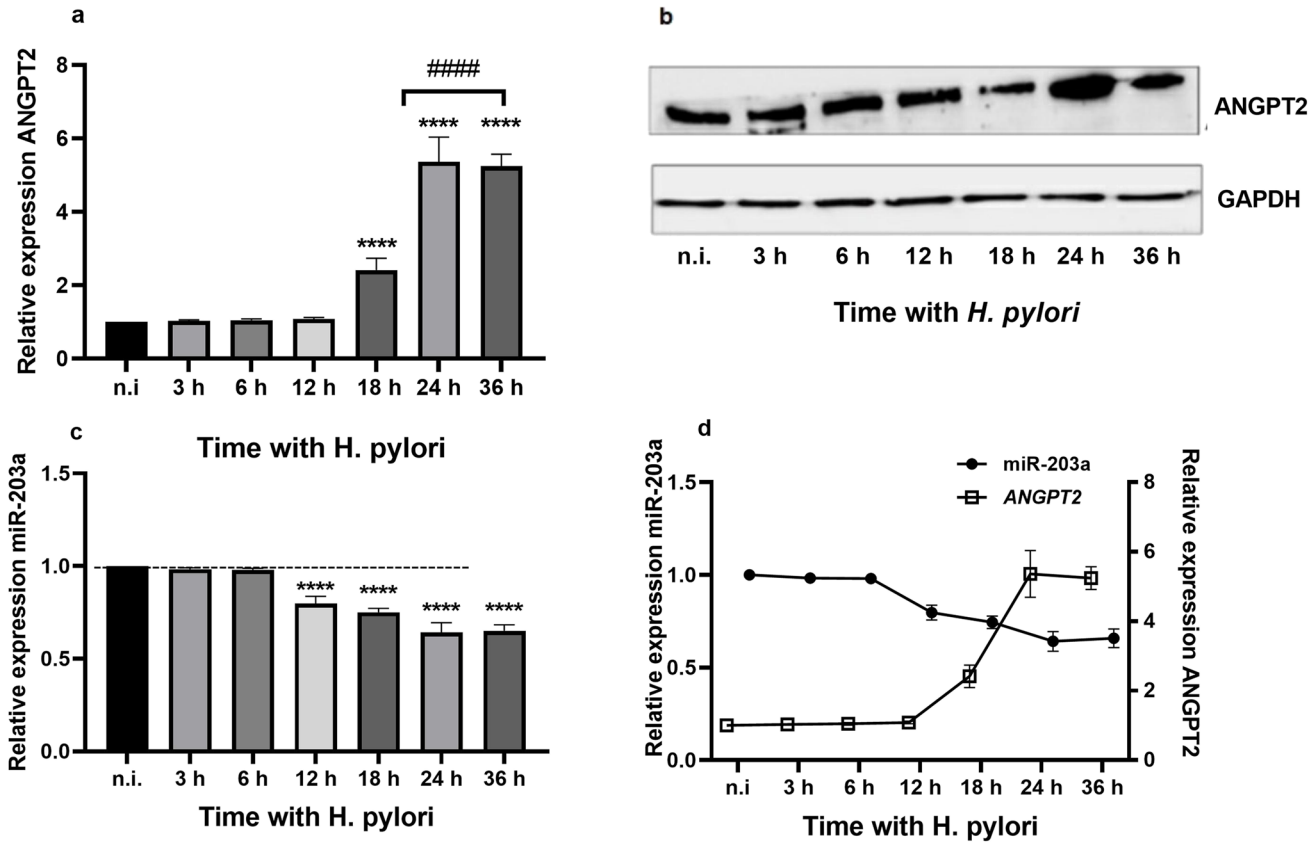

Fig. 3 Time-dependent induction of *ANGPT2* mRNA and protein levels, and miR-203a, by *H. pylori* 26695 in AGS cells. **a** *ANGPT2* mRNA expression increased overtime in *H. pylori*-treated cells, reaching significance after 18 h in comparison to the initial time point (0 h). **b** Western blot analysis of *H. pylori* induced ANGPT2 protein expression in AGS cells over time. **c** miR-203a expression was downregulated over time in *H. pylori*-treated cells, reaching significance after 12 h.p.i. until 36 h.p.i. **d** Concurrently with the downregulation of miR-203a by *H. pylori*, there was an increase in the expression of ANGPT2. AGS gastric cells at 90% confluence were incubated with *H. pylori* at a MOI of 100 for 3, 6, 12, 18, 24, and 36 h. Cells were analyzed to measure the relative expression levels of *ANGPT2* mRNA compared with *GAPDH* mRNA, in infected cells compared with non-infected AGS cells at the same time points. #### or ****$p < 0.0001$. Protein levels were determined in total cellular protein extracts by Western blotting

pylori upregulation of *ANGPT2* also occurred at the protein level, its expression was studied by Western blot, where a significant increase of the ANGPT2 protein was detected at 24 h.p.i (Fig. 3b).

H. pylori infection increases angiogenesis *in vivo*

To evaluate the effect of *H. pylori* infection in the potential angiogenic response of AGS cells, the in vivo chick embryo chorioallantoic membrane (CAM) model was used. The angiogenic response of supernatants from AGS cells infected for 24 h with *H. pylori* 26695, versus uninfected cells, was quantified by counting the number of neovessels recruited to the inoculation site. Conditioned media derived from *H. pylori*-infected AGS cells elicited a significantly higher angiogenic response than supernatants of non-infected cells ($p = 0.004$; Fig. 4a). Although not defined as a primary readout of the CAM assay, it was also possible to evaluate the inflammatory/reaction area of the CAM inoculation sites. Using the Cell Sens Olympus software, the reaction areas were quantified, and CAM sites treated with supernatants derived from *H. pylori*-infected AGS cells showed a significantly higher response than uninfected cells ($p < 0.0001$; Fig. 4b).

Discussion

The gastric carcinogenic process is characterized by numerous genetic and epigenetic changes, including oncogene activation, loss of tumor suppressor and DNA repair genes, and overexpression of angiogenic, inflammatory, and growth factors [1, 6, 32–34]. miRNAs may play a role in this process, by inhibiting the expression of target genes involved

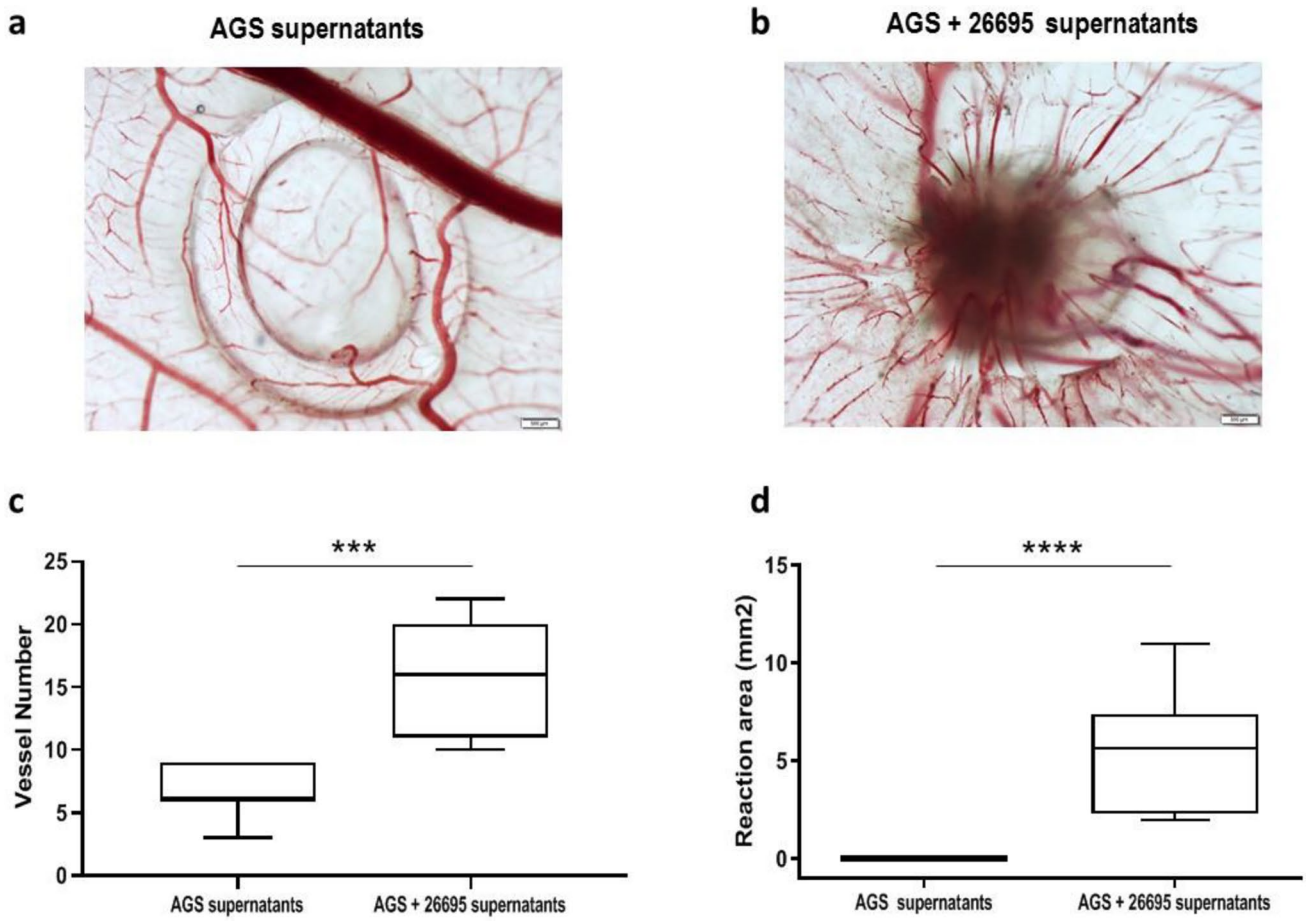

Fig. 4 Chicken embryo choriollantoic membrane (CAM) assay. **a**, **b** Stereomicroscope images of excised wounded CAMs representative of the two conditions tested pairwise, in the same egg, supernatants of *H. pylori* 26695-infected AGS cells and supernatants of uninfected AGS cells, 3 days after inoculation (scale bar = 100 µm); **c** quantification of newly formed blood vessels (< 20 µm in diameter) and **d** reaction areas at the inoculation sites. Supernatants of *H. pylori* 26695-infected AGS cells induce a significantly increased angiogenic (**c**) and inflammatory (**d**) responses in the CAM assay. Data regards 12 eggs (12 pairs of samples) from two independent experiments. ***$p < 0.001$, ****$p < 0.0001$ (pair *t* test)

in the signaling pathways. Previous studies using different in vitro models have indicated that the expression of some pro-angiogenic factors is altered by *H. pylori* infection, in gastric cancer cells and in endothelial cells [8, 10, 11, 31, 35]. Using an in vitro co-culture system, this study describes the changes in the expression of the pro-angiogenic gene *ANGPT2* and its predicted regulator miR-203a, in response to infection by *H. pylori*.

In the presence of VEGFA, ANGPT2 destabilizes established blood vessels through the interruption of Tie2 signaling, inducing removal of pericytes, and triggering the permeability of endothelial tubes [36, 37]. ANGPT2 is expressed in endothelial cells and macrophages, but also in cancer cells in vitro and in vivo. *ANGPT2* mRNA and protein have been reported as upregulated in several gastric cancer cell lines such as AZ521, SOH [38], NCI-87 [39], AGS, and SGC7901 [40]. Here, we consistently observed an upregulation of *ANGPT2* mRNA in AGS, MKN28, and MKN45 cells, upon infection with different *H. pylori* strains, showing that this effect is cell line and bacterial strain is independent. Furthermore, upregulation of *ANGPT2* by *H. pylori* is independent of the bacterial virulence factors CagA and T4SS. We additionally observed that *H. pylori* induces increasing *ANGPT2* mRNA and protein expression levels in time course experiments performed in AGS cells.

In agreement with our findings, Leite et al. [31] showed that MKN74 gastric cells infected with *H. pylori* 60190 triggered a strong angiogenic response, increasing the expression of numerous pro-angiogenic proteins, including ANGPT2, interleukin-8, VEGFA and VEGFC, platelet-derived growth factor, fibroblast growth factor-1, -2, and -4, and plasminogen activator urokinase (uPa), among

others. In contrast to our findings, Kim et al. [41] reported that *ANGPT2* mRNA was constitutively expressed in AGS cells but was not regulated by *H. pylori*, although data on *ANGPT2* expression upon infection was not shown. Such differences may be related to various aspects of the experimental setting, including differences in the *H. pylori* strain used.

The CagA oncoprotein, which is delivered into the host cell by the T4SS, is among the best-studied *H. pylori* virulence factors with impact on multiple host cellular responses. Our data suggest that *ANGPT2* upregulation by *H. pylori* is mediated by factors other than CagA or the T4SS. *H. pylori* has numerous other virulence factors that could be involved in the induction of ANGPT2, some of which are factors associated with gastric pathology, including the cytotoxin VacA, the adhesins BabA and SabA, and the outer membrane protein OipA, reviewed elsewhere [42]. Actually, Olivera-Severo et al. [35] showed an increase in ANGPT2 protein and in other pro-angiogenic factors upon exposure of AGS cells to purified *H. pylori* urease from strain 26695. They further demonstrated that *H. pylori* urease induced formation of tube-like structures by HUVECs and intense neovascularization in the in vivo CAM assay model. Similarly, when we evaluated the angiogenic potential and inflammatory response of conditioned medium of *H. pylori*-infected cells in the CAM assay, we verified significant increases in the number of newly formed vessels and in the inflammatory reaction area. This suggests that pro-angiogenic factors are secreted to the medium directly by *H. pylori* or indirectly by the host cells upon infection.

As we found an interesting upregulation of *ANGPT2* gene and protein in response to *H. pylori* infection, we hypothesized that in our model it could be possible that other angiogenesis-related factors could be altered by the infection. In contrast with the results of Wang et al., who reported the expression of both *ANGPT1* and *TEK* mRNA and protein in AGS, MKN45, and other gastric cells [40], we could not detect the expression of *ANGPT1* or *TEK* mRNAs in any of the studied gastric cell lines. ANGPT1 is an activator of tyrosine kinase receptor Tie2 responsible for a quiescent vascular phenotype and is known as an endothelial survival and vascular stabilization factor, necessary for the maturation of newly formed vessels [43]. It is produced by pericytes and immune cells [37, 44] and is also expressed in several cancer cell lines and tissues [45, 46]. As the ANGPT2 and ANGPT1 proteins antagonize for the same receptor, our observation that in the same gastric cell line there is an upregulation of ANGPT2, but no expression of ANGPT1, suggests that a gain in ANGPT2 activity over ANGPT1 might be an initiating factor for tumor angiogenesis. The tumor angiogenic environment in vivo is characterized by a deregulation of ANGPT1/ANGPT2 ratio, with a bias towards ANGPT2 [40]. Overproduction of ANGPT2 drives the destabilization of existent vasculature to create new vessels [36]. ANGPT1 is also overproduced but is insufficient for the adequate maturation of new abnormal vessels, which have few pericytes and become tortuous and leaky [36, 44, 47]. Elevated plasma levels of ANGPT2, but not ANGPT1, have been associated with metastasis and worse prognosis in cancer patients, as well as with resistance to anti-VEFG therapy [48]. This suggests that an early high production of ANGPT2 rather than ANGPT1 may favor tumoral fitness.

Previous reports, and our bioinformatics analyses, predicted miR-203a as a regulator of the *ANGPT2* gene. Our work is the first report for a significant downregulation of miR-203a in response to *H. pylori* infection in AGS cells, which coincided with the upregulation of *ANGPT2* gene, in a time-dependent manner. miR-203a is expressed specifically in epithelial cells and is considered as a tumor suppressor, as it has been shown to be downregulated in vitro and in vivo in several cancers, including colorectal, lung, and esophageal cancer [20, 21, 49]. Significant downregulation and hypermethylation of miR-203a gene promoter were found in patients with advanced GC, associated with poor patient survival [50]. miR-203a was also shown to be aberrantly downregulated in *H. pylori*-positive tissues and cancer-derived cell lines, including AGS and MKN45, also regulating the *CASK* oncogene [51]. Furthermore, the reduced expression of miR-203a was shown to increase angiogenesis in vitro and in vivo in human hepatocellular carcinoma, via the *HOXD3* gene, through the upregulation of the VEGFR signaling pathway [52]. These data, together with ours suggest that miR-203a, as other microRNAs, have numerous targets and participate in several carcinogenic pathways, and could therefore be a key master regulator of angiogenesis, invasion, and metastasis.

The results presented here suggest that *H. pylori* could contribute to the process of carcinogenesis by downregulating miR-203a, which further promotes angiogenesis in gastric mucosa by increasing *ANGPT2* expression, favoring the process of chronic inflammation that has important functional implications for the etiology of GC. The molecular mechanism by which miR-203a could regulate the development of *H. pylori*-associated GC, by *ANGPT2* deregulation, is not yet clear. It has been demonstrated that NF-κB can promote angiogenesis, invasion, and metastasis in several types of cancer [53]. It has been observed that miR-203a is downregulated in a NF-κB-dependent mechanism in Epstein Barr virus-related nasopharynx cancer [49]. Although in this study we did not examine the pathways or mechanisms involved, it can be hypothesized that *H. pylori*-dependent activation of NF-κB could deregulate miR-203a and upregulate *ANGPT2* (Fig. 5). Further investigation is needed to clarify this question.

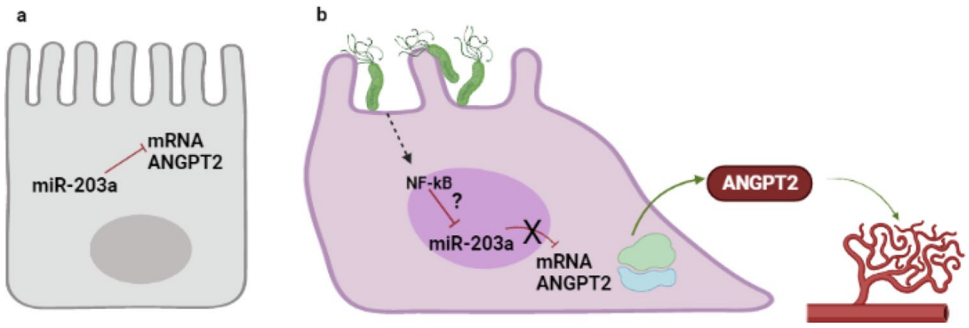

Fig. 5 Model for *H. pylori*-induced downregulation of miR-203a and upregulation of *ANGPT2* in AGS cells. **a** Uninfected AGS cells show a basal expression of ANGPT2 and miR-203a. **b** In an analogous way to the EBV infection model [49], *H. pylori* infection may activate the NF-κB pathway that could then downregulate miR-203a which would allow the translation of ANGPT2 mRNA, by its destabilizing effect in near vasculature. Created with BioRender.com

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Data Availability Datasets are available on request.

Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

Conflict of interest The authors declare no competing interests.

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