U.PORTO
INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

The role of the ETS-1 transcription factor on matrix metalloproteinase (MMP)-10 expression in the context of *Helicobacter pylori* infection

Ana Filipa Araújo Freire

Dissertação de Mestrado em Oncologia

Porto, 2015

Ana Filipa Araújo Freire

The role of the ETS-1 transcription factor on matrix metalloproteinase (MMP)-10 in the context of *Helicobacter pylori* infection

Dissertação de Candidatura ao grau de Mestre em Oncologia submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

Orientador – Céu Figueiredo

Categoria – Professora Auxiliar

Afiliação – Faculdade de Medicina da Universidade do Porto

Co-orientador – Rui M. Ferreira

Categoria – Post Doc

Afiliação – Instituto de Patologia e Imunologia Molecular da Universidade do Porto

Este trabalho foi financeiramente suportado pela fundação para a Ciência e a Tecnologia (FCT) no âmbito do projeto com a referência, PTDC/BIA-MIC/116890/2010.

FCT
Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR

Este trabalho foi co-financiado pelo Fundo Social Europeu:

POPH
PROGRAMA OPERACIONAL **POTENCIAL HUMANO**

QREN
QUADRO DE REFERÊNCIA ESTRATÉGICO NACIONAL
PORTUGAL 2007.2013

UNIÃO EUROPEIA
Fundo Social Europeu

"And now here is my secret, a very simple secret:
It is only with the heart that one can see rightly;
what is essential is invisible to the eye."
Antoine de Saint-Exupéry, The Little Prince

AGRADECIMENTOS

Há um enorme número de motivos pelos quais todo este meu percurso, que culminou com esta tese de Mestrado, foi possível. Dedicação, persistência, alegrias, bons momentos, e até mesmo um bocadinho de stress e desespero quando as experiências não funcionavam..mas sem dúvida que nada disto teria sido possível e alcançável sem o enorme apoio e dedicação das pessoas que me rodeiam e me acompanharam neste caminho. É a todas essas pessoas que quero agradecer!

Antes de mais quero agradecer à minha orientadora Céu Figueiredo por me ter dado a oportunidade de fazer este trabalho no seu grupo. Obrigada por todo o apoio e dedicação, principalmente nos momentos mais difíceis. E acima de tudo, obrigada por me ter dado a oportunidade de fazer uma coisa que tanto gosto.

Ao meu Co-orientador Rui Ferreira, obrigada por me teres ensinado tanto sobre esta área e por teres estado sempre disponível para me ajudar em tudo o que precisei. Em parte, todo este trabalho só foi possível por todo o teu empenho e dedicação.

Como não poderia deixar de ser a todas as pessoas do grupo da *Helicobacter* (mesmo as que já não estão presentes!) Obrigada a todos! Obrigada ao Miguel que tanto me aturou e ajudou (principalmente para repicar bactérias). À Marina que muito me fez crescer e ser melhor. À Inês por me ter acolhido e ensinado muitas das técnicas que usei neste trabalho. À Raquel que infelizmente já não está no nosso grupo mas a quem devo muito – sem a tua ajuda com o último construct nunca teria conseguido fazer os ensaios de luciferase. À Joana por toda a ajuda e boa disposição. Ao Deiber por me ter aturado nestes últimos tempos e me ter feito começar a gostar de proteínas. Aos feupinhos Sílvia, Nuno e Joana por tornarem a sala das bactérias um local mais animado (mesmo sem rádio!). Por fim, ao Augusto e à Wendy, obrigada por tudo o que fizeram por mim.

Queria também agradecer a todas as pessoas do Cancer Genetics pelo bom ambiente e boa disposição. É sempre bom trabalhar num sítio onde somos tão bem acolhidos.

Um agradecimento especial à Ana Margarida e à Janine obrigado por estarem sempre presentes e pelas energias positivas que tanto me puxaram para cima quando mais precisei.

Um agradecimento não menos especial à Vânia a quem devo tanto e que nunca me deixou ir abaixo ou desistir. Obrigada pelos nossos almoços (ainda estás em dívida não te esqueças!) e por nunca teres duvidado de que eu era capaz.
À Ana Dias que de vez em quando até consegue arranjar um tempinho para mim (estou a brincar!) Obrigada por todo o apoio! À Patricia por toda a ajuda e principalmente por me teres ouvido quando mais precisei. À Lu que tanto me faz rir, obrigada por estares sempre disponível. Ao Hugo Pinheiro por estar sempre disponível para ajudar.
À Maria Lázaro, nem tenho palavras para agradecer tudo o que fizeste e tens feito por mim, és simplesmente incrível! Obrigada!

Queria também agradecer a todos os meus amigos com os quais eu fui crescendo e que, mais ou menos presentes, estavam sempre disponíveis e prontos para me fazerem rir. Um obrigado muito muito grande à Clara Pais que já me atura praticamente desde que nasci (que exagero!). Nem sei o que seria de mim sem ti. Devo-te mesmo muito do que sou. Espero um dia poder fazer por ti tudo o que tens feito por mim. À Cristina e ao Zé claro (senão ainda me matam =p) Obrigada pelos bons momentos, a sério só tu Cristina para me fazeres rir (tipo na aula de história da música lembras-te?). Aos novos amigos por acréscimo, Nando, Tiago e Isabel, Ticha e Pedro, etc., obrigada pelas jantaradas. À Mi, que mesmo só tendo conhecido há pouco tempo me trata como familia. Obrigada por tudo que tens feito por mim.

Aos meus amiguinhos do Cambridge que tanta força me deram. Obrigada Tomás, Cátia e Ana pelo apoio incondicional e pelos pequenos kinders, and of course to Catherine for all your kindness.

À minha familia pelo apoio incondicinal. Um obrigado muito especial aos meus pais que tanto passaram comigo mas que sempre acreditaram em mim. À minha avó Ninhas e ao avô Manuel que mesmo já não estando presentes me fizeram a pessoa que sou hoje.

And last but not least, ao meu mais que tudo, ao meu Icas. A quem eu devo muito…nem sei como te poderei agradecer por tudo o que tens feito por mim. Por todas as palavras certas nos maus momentos, por toda a paciência, por tanto me

fazeres sorrir e sentir que sou capaz de fazer tudo e enfrentar todos os obstáculos, por me deixares simplesmente ser eu mesma e ainda adorares isso em mim. Obrigada por fazeres parte da minha vida.

SUMMARY

Helicobacter pylori is a gram-negative bacterium that colonises the human gastric epithelium, and is the major etiological factor of gastric carcinoma. Several signalling pathways involved in carcinogenesis are triggered by *H. pylori* infection, leading to up-regulation of several matrix metalloproteinases (MMPs).

MMPs are zinc-dependent enzymes that regulate physiological as well as disease processes including inflammation and cancer.

Several MMPs are up-regulated in the context of *H. pylori* infection. One of the less explored MMPs, MMP-10, was found to be up-regulated by *H. pylori* in a whole genome cDNA microarray performed by our Group. In addition, MMP-10 was found to be overexpressed on epithelial derived cancers.

Studies have shown that the expression of MMPs may be regulated by transcription factors of the ETS family, namely ETS-1. Interestingly, data from our Group has shown that ETS-1 was also up-regulated by *H. pylori*.

Adding to that, data from the literature together with data from our Group has shown that receptor tyrosine kynases such as EGFR may be involved in modulating both ETS-1 and MMP-10 expression.

Since the mechanisms underlying MMP-10 activation in the context of *H. pylori* infection are still poorly understood, and several ETS transcription factors are known to be involved on the modulation of MMPs, the aim of this thesis is to evaluate the role played by ETS-1 transcription factor on the modulation of *H. pylori*-induced MMP-10 expression in gastric epithelial cells.

To accomplish that aim, this study started with the validation of previous results obtained by our Group by quantitative real-time PCR and western blot. The involvement of the ETS-1 on the modulation of MMP-10 expression by *H. pylori* was evaluated through siRNA gene silencing experiments and luciferase gene reporter assays, with three MMP-10 promoter deletion constructs.

The role of EGFR on the modulation of ETS-1 and MMP-10 expression was evaluated by the abrogation of EGFR with a specific pharmacological inhibitor.

Results showed that *H. pylori* infection increased mRNA and protein expression of both MMP-10 and ETS-1.

Results also showed that ETS-1 was involved in *H. pylori*-mediated MMP-10 expression and protein secretion.

Inhibition of EGFR signalling pathway resulted in a significant decrease in ETS-1 mRNA and protein expression, as well as a decrease in MMP-10 mRNA and protein secretion.

Taken together, these results suggest that the up-regulation of MMP-10 expression by *H. pylori* is mediated by the ETS-1 transcription factor, in an EGFR dependent manner.

RESUMO

A bactéria gram-negativa *H. pylori* coloniza o epitélio gástrico, sendo um dos fatores etiológicos mais bem estabelecidos do carcinoma gástrico. A infeção por esta bactéria é responsável pela ativação de várias vias de sinalização envolvidas no processo de carcinogénese. Uma vez ativadas, estas vias promovem a indução da expressão de várias metaloproteinases (MMPs) da matriz extracelular.

As MMPs são enzimas dependentes de zinco que regulam vários processos fisiológicos bem como processos associados a doença, nomeadamente inflamação e cancro.

Várias MMPs encontram-se sobre-expressas após infeção por *H. pylori*. Um array de expressão realizado pelo nosso Grupo, mostrou que a MMP-10, uma das menos estudadas MMPs, se encontrava sobre-expressa após infeção por *H. pylori*. Adicionalmente, outros estudos reportaram a sobre-expressão da MMP-10 em modelos de cancro com origem epitelial.

Vários estudos mostraram que a expressão das MMPs pode ser regulada por fatores de transcrição da família ETS, nomeadamente o ETS-1. Curiosamente, dados do nosso Grupo demonstraram que o ETS-1 também se encontra sobre-expresso na presença de *H. pylori.*

Paralelamente, dados na literatura e do nosso Grupo revelaram que recetores de tirosina cinase, tais como o EGFR, podem estar envolvidos na modulação da expressão do ETS-1 bem como da MMP-10.

Uma vez que os mecanismos que levam à promoção da ativação da MMP-10 no contexto de infeção por *H. pylori* ainda não se encontram totalmente descritos, o objetivo desta tese é avaliar o papel do fator de transcrição ETS-1 na modulação da expressão da MMP-10 induzida por *H. pylori* em células epiteliais gástricas.

Este estudo teve início na validação de resultados previamente obtidos pelo nosso Grupo, por PCR quantitativo e western blot. O estudo do envolvimento do ETS-1 na modulação da expressão da MMP-10, induzida por *H. pylori*, recorreu a experiências de silenciamento do ETS-1 através de um siRNA dirigido a este gene, e a ensaios de luciferase, nos quais se utilizaram três construtos compostos por diferentes regiões truncadas do promotor da MMP-10.

O papel da ativação do EGFR na modulação da expressão do ETS-1 e da MMP-10 foi estudado tendo-se recorrido à inibição do EGFR por um inibidor farmacológico.

Os resultados mostraram que a infeção por *H. pylori* induziu um aumento na expressão de mRNA e proteína, tanto para o ETS-1 como para a MMP-10.

Os resultados demonstraram também que o ETS-1 está envolvido na modulação da expressão e secreção proteica da MMP-10 mediada por *H. pylori*.

A inibição da via de sinalização ativada pelo EGFR resultou na diminuição significativa da expressão do ETS-1, tanto em termos de mRNA como de proteína, bem como na diminuição da expressão dos níveis de mRNA e secreção proteica de MMP-10.

Tendo em conta os resultados obtidos, podemos concluir que a modulação da expressão da MMP-10 induzida após infeção por *H. pylori*, é mediada pelo ETS-1 através da ativação da via de sinalização do EGFR.

TABLE OF CONTENTS

ABBREVIATIONS

AP-1	activator protein 1
ATCC	american type culture collection
BabA	blood-group antigen-binding protein A
Bp	base pair(s)
BSA	bovine serum albumin
BSP	bone sialoprotein
cagA	cytotoxin-associated gene A
cag PAI	cag pathogenicity island
CDKs	cyclin-dependent kinases
cDNA	complementary DNA
ChIP	chromatin Immunoprecipitation assay
COX-2	cyclooxygenase-2
Crk	chicken tumour virus number 10 regulator of kinase
Csk	c-Src tyrosine kinase
CZEMSA	capillary zone EMSA
c-Abl	Abelson tyrosine kinase
c-Fos	FBJ murine osteosarcoma viral oncogene homolog protein
c-Jun	Jun proto-oncogene protein
c-Kit	mast/stem cell growth factor receptor
c-Met	MET proto-oncogene receptor tyrosine kinase
c-Src	tyrosine-protein kinase CSK
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EBS	ETS binding site
ECM	extracellular matrix components
EGF	epidermal growth factor
EGFR	EGF receptor
EHF	ETS homologous factor
ELF1	E74-like factor 1
ELK1	ETS like gene 1
EMSA	electrophoretic mobility shift assay
ERF	ETS2 repressor factor
ERG	ETS-related gene
ERK	extracellular signal regulated kinase
ESE	epithelium-specific ETS-like transcription factor
ETS	E26 transformation-specific
ETV	ETS variant 3
E-cadherin	epithelial cadherin
FAM	fluorescein amidite
FBS	fetal bovine serum
FLI-1	Friend leukemia integration 1 transcription factor

Flt-1	fms-like tyrosine kinase-1
GABP	GA-binding protein
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
Grb	growth factor receptor-bound protein
GTP	guanosine-5'-triphosphate
HER2	human epidermal growth factor receptor 2
HGF	hepatocyte growth factor
HRP	horseradish peroxidase
HUVECs	human umbilical vein endothelial cells
H. pylori	Helicobacter pylori
IARC	International Agency for Research on Cancer
iEGFR	EGFR inhibitors
IFN	type I interferon
IgG	immunoglobulin G
IL	interleukin
JNK	c-Jun N-terminal kinase
jun-B	transcription factor jun-B
kDa	kiloDalton
KDR	kinase insert domain containing receptor
LUC	luciferase reporter gene
MALT	mucosal-associated lymphoid tissue
MAPK	mitogen-activated protein kinases
MEM	minimal essential medium
MET	hepatocyte growth factor receptor
MGB	minor groove binder
MMP	metalloproteinase
MOI	multiplicity of infection
mRNA	messenger RNA
M-CSF	macrophage colony-stimulating factor
NEB	New England Biolabs®Inc.
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NOD1	nucleotide-binding oligomerization domain protein 1
NS	non statistically significant differences
OD	optical density
OipA	outer inflammatory protein A
PAI	pathogenicity island
PAR1b	polarity-regulating kinase partitioning-defective 1b
PBS	phosphate buffered saline
PBS-T	PBS-Tween
PCR	polymerase chain reaction
PEA	polyoma virus enhancer-A binding protein
PI3K	phosphatidylinositol 3-kinase
PlCγ	phospholipase C γ

PNT	Pointed domain
qRT-PCR	quantitative real time PCR
RARE	retinoic acid response element
Ras GAP1	Ras GTPase-activating protein 1
Rho	Ras homolog
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RTK	receptor tyrosine kinases
RUNX2	runt-related transcription factor 2
SabA	sialyl-Lewis X binding protein A
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	standard error
SEM	SE of the mean
SHP	Src homology-2 domain-containing phosphatase
siRNA	small interference RNA
SPDEF	SAM pointed domain-containing Ets transcription factor
SPI1	transcription factor PU.1
Sp-1	specificity protein 1
STAT	signal transducer and activator of transcription
T4SS	type IV secretion system
TCF	ternary complex factor
Tcf/Lef	T-cell factor-lymphoid enhancer factor
TEL	ETS-variant gene 6
Tie2	tyrosine kinase with immunoglobulin-like and EGF-like domains2
TIMP	tissue inhibitor of metalloproteinases
Tm	melting temperature
TNF-α	tumor necrosis factor α
TSA	tryptic soy agar
TSS	transcriptional start site
uPA	urokinase-type plasminogen activator
uPAR	uPA receptor
vacA	vacuolating cytotoxin gene A
VEGF	vascular endothelial growth factor

LIST OF TABLES

LIST OF FIGURES

to ETS-1 or a non-targeting siRNA. **(A, C)** ETS-1 and **(B, D)** MMP-10 relative mRNA levels were analysed by qRT-PCR and normalized to GAPDH expression. **(A, C)** ETS-1 protein present on cell lysates was evaluated by western blot and GAPDH was used as an internal control. **(B, D)** Conditioned medium was collected and concentrated and MMP-10 secretion was assessed by western blot. The coomassie brilliant blue was used to control protein loading. The results are expressed relatively to cells treated with a non-targeting siRNA, with mean ± SE; **(A-B)** n = 3 or **(C-D)** n =2 independent experiments. * $p < 0.05$, relative to non-silencing cells infected with *H. pylori*; NS non statistically significant differences relative to non-silencing cells infected with *H. pylori*. 38

Figure 8. *H. pylori*-induced ETS-1 modulates MMP10 expression via EGFR pathways. AGS cells were treated with 5µM of the pharmacological inhibitor AG1478, or DMSO, for 1 hour and then infected with *H. pylori* strain 26695, at a multiplicity of infection of 100, for a 4 hours period. **(A)** ETS-1 and **(B)** MMP-10 relative mRNA levels were analysed by qRT-PCR and normalized to GAPDH expression. **(A)** Cells lysates were analyased by western blot using p-EFGR, EGFR and ETS-1 antibodies. p-EGFR was used as control of EGFR inhibition and GAPDH as a loading control. **(B)** Conditioned medium was collected and concentrated and MMP-10 secretion was assessed by western blot. The coomassie brilliant blue was used to control protein loading. The results are expressed relatively to non-infected cells treated with DMSO, with mean ± SE; n = 3 independent experiments.* $p < 0.05$, relative to non-infected cells treated with DMSO. 40

Figure 9. ETS-1 is required for MMP-10 promoter activation. MMP-10 promoter constructs (Full-length MMP-10 Promoter Construct, Construct 1, Construct 2 and Construct 3) were transiently transfected into AGS cells. Transiently transfected cells were **(A)** infected for 4 hours with *H. pylori* strain 26695 or **(B)** treated with EGF (50 ng/ml) for 4 hours, and a reporter gene assay was performed. The no treatment condition represents the basal MMP-10 promoter activity. The scheme on the left shows the ETS-1 transcription factor binding sites present on each MMP-10 promoter construct. For each construct, the individual effect of *H. pylori* and EGF on each MMP-10 promoter constructs was assessed by comparison with the respective no treatment conditions. Additionally, each of the constructs was compared with the Full-length MMP-10 Promoter Construct of their respective condition. The results are expressed as mean ± SE; n = 3 independent experiments. For each condition, statistically significant differences were obtained comparing each construct with the respective empty vector. Apart from Full-length MMP-10 Promoter Construct on the EGF condition, no statistically significant differences were obtained when comparing each construct with the respective no treatment condition. § $p < 0.05$ relative to the no treatment condition; * $p < 0.05$ relative to the *H. pylori* or EGF Full-length MMP10 Promoter Construct, respectively; # $p < 0.05$ relative to the no treatment Full-length MMP10 Promoter Construct. ETS-1 BS (yellow boxes) represents the ETS-1 transcription factor binding sites on

Introduction

1. *Helicobacter pylori* infection

Helicobacter pylori (*H. pylori*) are a spiral-shaped gram-negative bacteria that selectively colonises the human's gastric epithelium. These microaerophilic bacteria were first isolated in 1983 by Marshall and Warren in the luminal surface of the gastric epithelium of patients with chronic gastritis (1).

Present in nearly half the world's population, *H. pylori* is thought to have co-evolved with humans for thousands of years, with genetic studies pointing out that humans have been colonised by these bacteria for at least 58,000 years (2, 3). The infection occurs in childhood via several routes, such as fecal-oral, gastro-oral and oral-oral routes, and persists throughout life, causing disease mainly in adults if not treated (2, 4, 5). *H. pylori* strains are extremely diverse among different individuals but one single individual might be colonised by different *H. pylori* strains. Moreover, within a single host, variants from those strains can also be observed (6-8). The bacteria successful persistence in this niche is now attributed to strategies viewed as essential, such as DNA and protein repair pathways, combined with other factors, namely bacteria genetic diversity and attenuation of chemical radical production by the host cells (5). Within the gastric mucosa, *H. pylori* can be found within the mucus layer or attached to the epithelial cell surface (9-11). It has also been observed that *H. pylori* can be present in intercellular spaces located below the tight junctions (12). Although the majority of infected individuals remain asymptomatic throughout their lives, all develop chronic gastric inflammation (13), and, in some cases, the infection might lead to more severe diseases (14). The colonisation and persistence of *H. pylori* in the gastric mucosa causes acute and latter chronic inflammation whose severity and pathological consequences are determined by a combination of bacterial virulence, host genetic and environmental factors (15, 16).

1.1. *H. pylori* virulence factors

In order to overcome the acidic conditions of the gastric lumen and to successfully colonise the gastric mucosa, *H. pylori* produces urease that hydrolyses urea into ammonia and carbon dioxide, raising the gastric pH. Besides this, it has also been observed that ammonia is capable of causing different cell alterations and might also help in the recruitment of neutrophils and monocytes in the mucosa and in the production of proinflamatory cytokines (17, 18).

Other virulence factors are the adhesins BabA and SabA, and outer membrane inflammatory protein (OipA), involved in the adherence of *H. pylori* to the gastric mucosa and in the induction of host inflammatory response (Figure 1) (19).
Another important factor for successful colonisation of *H. pylori* is the presence of polar flagella, which confers motility capacity to penetrate through the mucus layer. Added to this, by hydrolising urea, *H. pylori* is able to reduce the viscoelasticity of the mucus layer, which facilitates its motility (20).
Apart from those described above, the pathogenicity island (*cag* PAI), the cytotoxin associated protein (CagA) and the vaculating cytotoxin (VacA) are three of the most well studied *H. pylori* virulence factors.
VacA is a multimeric high-molecular weight secreted toxin that is associated with increased peptic ulcer and gastric cancer risk (21). This protein was initially identified on the basis of its ability to induce vacuolation in culture epithelial cells (22) and is encoded by a chromosomal gene, present in all *H. pylori* strains, named *vacA*. Aside from that, this pore-forming cytotoxin is capable of inducing multiple cellular activities such as the induction of membrane channel formation, cytochrome c release from mitochondria, binding to cell-membrane receptors and activating a proinflammatory response, leakaging of ions and small molecules by disruption of intercellular junctions, inhibition of T-cell activation and proliferation (19, 23-27).
Despite being present in all strains, there is a marked genetic diversity within the *vacA* gene. This diversity is attributed to variations in *vacA* gene structures within the signal (s) region, the middle (m) region and the intermediate (i) region, which is located between the s and m regions, and each region is stratified into two allelic types (21, 28). The combination *vacA* s1/i1/m1 allele is strongly related to peptic ulcer disease and gastric carcinoma (15, 28, 29).
The *cag* PAI is a 40-Kb DNA region that comprises between 27 and 30 genes, some of them being involved in the synthesis of functional components of a type IV secretion system (T4SS). This *H. pylori* virulence determinant is present in approximately 60-70% of the *H. pylori* strains in Western countries and in aproximately 100% of the East-Asian *H. pylori* strains (30, 31). *H. pylori* strains that harbour this *cag* island increase the risk of severe gastritis, atrophic gastritis and gastric cancer compared with strains that lack the *cag* PAI (31-33). The T4SS system is a needle-like structure, induced by host cell contact, that allows the delivery of effector molecules into the host cells (34). So far, only two bacterial products have been reported to be delivered through the T4SS system: the CagA

and peptidoglycan components (35). The later are recognised by the intracytoplasmic pathogen-recognition NOD1 which leads to the activation of the NF-kB pathway, production of IL-8 and type I interferon (IFN), activation of Pi3K and cell migration (36-38) .

Upon delivery into the gastric epithelial cells, CagA localises close to the plasma membrane and undergoes tyrosine phosphorylation by c-Src and c-Abl kinases at EPIYA (Glu-Pro-Ile-Tyr-Ala) motifs (39-43). Thyrosine-phosphorylated CagA can interact with several host molecules such as tyrosine phosphatase SHP-2, Csk, Grb2, Crk, PI3K, Shp-1, Ras-GAP1 and Grb7, which lead to the induction of actin-cytoskeleton rearrangements ("hummingbird phenotype"), cell proliferation and motility (41-46). Non-phosphorylated CagA can also exert effects in the host cells by interaction with c-Met, E-cadherin, PlCγ, Grb2 and PAR1b, leading to the disruption of cell-cell junctions, loss of cell polarity, and pro-inflammatory and mitogenic responses(47-50) .

Recent reports have shown that non-phosphorylated CagA can counter-regulate the effects of the VacA toxin on the host cells (51, 52).

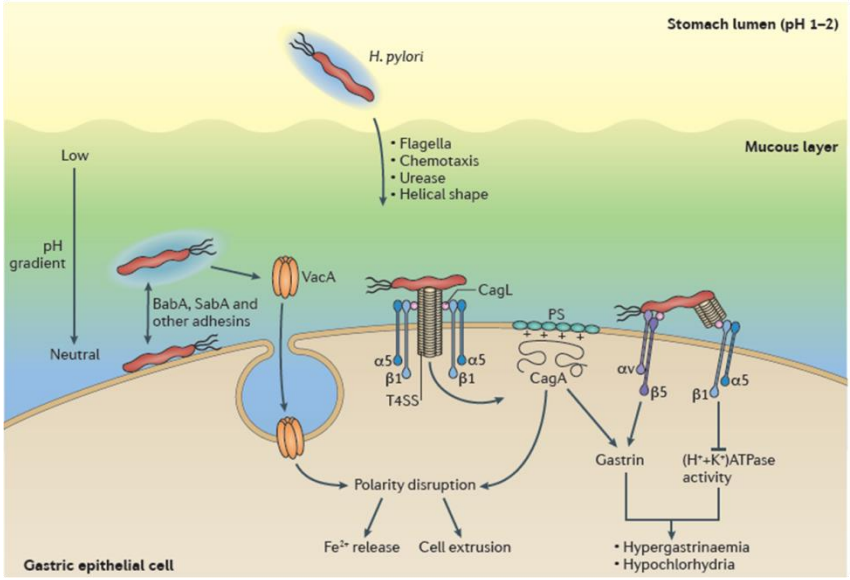

Figure 1. Overview of *H. pylori* virulence factors-induced host cell alterations. Adapted with permission from Macmillan Publishers Ltd: *Nature Reviews in Microbiology* (5), © *2013.*

1.2. Clinical outcomes of *H. pylori* infection

The clinical outcome of *H. pylori* infection is widely variable. All infected individuals develop an inflammatory response to the bacteria that results in the establishment of chronic gastritis, which remains asymptomatic in the majority of infected individuals (53, 54). The persistent infection can lead to an increased risk of developing more severe diseases such as peptic ulcer disease, gastric mucosal-associated lymphoid tissue (MALT) lymphoma, or gastric cancer (55).

1.2.1. *H. pylori* and gastric cancer

Gastric cancer is considered the fifth most common cancer worldwide, with almost one million new cases diagnosed each year (56). Despite being a multifactorial disease, the infection with *H. pylori* is considered the strongest risk factor for this malignancy. As a result, the International Agency for Research on Cancer (IARC) has classified this bacteria as a type I carcinogen (57).

H. pylori has been associated with two distinct histological variants of gastric cancer, each having distinct pathological and epidemiological features: intestinal-type and diffuse-type gastric cancer (58). The latter, consists of individually infiltrating neoplastic cells that do not form glandular structures (16), whereas the intestinal-type follows through well-defined histological steps that starts with chronic gastric inflammation induced by *H. pylori*, which can lead to atrophic gastritis, intestinal metaplasia, dysplasia, and finally gastric cancer (16, 58).

Throughout the years, studies on animal models and in humans have been conducted to provide evidence of the role played by *H. pylori* infection in the promotion of gastric carcinoma (59-61). To reinforce this correlation, randomised prospective studies have shown that eradication of *H. pylori* significantly reduced the presence of premalignant lesions in the gastric mucosa, demonstrating its involvement in the early stages of gastric carcinogenesis (61, 62).

Although present in the stomach of, at least, half the world's population, only a very small group of carriers might develop gastric carcinoma. The genetic diversity among *H. pylori* strains and the bacterial virulence factors play a vital role in determining the infection outcome, combined with host responses enhanced by dietary and environmental risk factors. Host genetic polymorphisms that enhance the expression of IL-1β or TNF-α have been found to confer an increased risk of developing atrophy, hypochlorhydria and gastric cancer (63-65). Regarding

environmental factors, tobacco (66), high dietary salt consumption (67), and consumption of processed meat (68) are associated with an increased risk for development of gastric cancer. *H. pylori* strains that contain the *vacA* alleles s1/i1/m1 are associated with an increased risk for gastric epithelial injury and gastric cancer (15, 21, 23, 29). Similarly, patients infected with *cag* PAI positive strains have higher risk for developing gastric cancer compared to those patientes infected with strains that lack the *cag* island (55).

Several studies had tried to clarify the association between *H. pylori* infection and the increased risk of developing gastric carcinoma. However, the molecular mechanisms through which gastric carcinoma arises are still not clearly understood. It has been observed that *H. pylori*, when in close contact with gastric epithelial cells, is able to disturb cell molecular pathways and, due to inflammation, inducing mutations in the host genome that might not be repaired and, therefore enhance the risk for disease progression (64, 65, 69-71).

2. Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a large family of zinc-dependent enzymes that share a common structure, most of them containing four distinct functional domains: signal peptide, propeptide, catalytic domain and hemopexin-like domain. The catalytic domain, as well as the propeptide are highly homologous between all MMPs, being the first one responsible for the proteolytic activity (72). Apart from being involved in the degradation of extracellular matrix (ECM) components, these enzymes are also involved in other functions, such as regulation of apoptosis, regulation of cell growth, alteration of cell motility, among others (73). MMPs are divided into four subfamilies: collagenases, stomelysins, gelatinases, and membrane-type MMPs, depending on the substrate specificity and their structure (74).

In general, the majority of MMPs are secreted in an inactive form (zymogen) and are later activated in the extracellular space. MMPs contain a highly conserved cysteine residue in the propeptide that has the ability to interact with the Zn^{2+} ion present in the catalytic domain. This covalent interaction between the Zn^{2+} ion and the cysteine residue retains these enzymes in the latent, catalytically inactive state. Whenever this bond is disrupted, physically or chemically, the pro-enzyme becomes activated, in a process named “cysteine switch” (74).

The activity of MMPs is tightly regulated at the transcription level, compartmentalization, pro-enzyme activation or inactivation and by their natural inhibitors TIMPs (Tissue Inhibitor of Metalloproteinases) (75).

2.1. MMPs in cancer

MMPs have been implicated in cancer for more than 40 years. Although originally associated with tumour invasion and metastasis due to the role played in ECM degradation (76), it is now clear that MMPs are also involved in several steps of cancer development (77, 78). MMPs' function is far more complex than initially thought. They are able to mediate a wide range of biological effects on their surrounding tissue, which include activation of growth factors, suppression of tumour cell apoptosis, destruction of chemokine gradients developed by the host immune response, or release of angiogenic factors (77, 79).
MMPs were found to be upregulated in almost every type of human cancer but, unlike classic oncogenes, this upregulation is not due to gene amplification or activating mutations but probably due to transcriptional changes induced by growth factors, hormones, cytokines, and cell-cell matrix interactions (77, 80).

2.1.1. MMPs, *H. pylori* infection and gastric cancer

It is now well established that *H. pylori* infection predisposes to gastric cancer. However, the responses of epithelial cells to *H. pylori* infection that might lead to the subsequent progression to cancer are still largely unknown.
Different MMPs have been reported to be overexpressed in gastric cancer (81) and in the early stages of gastric carcinogenesis (82). Added to that, it has been shown that *H. pylori* up-regulates the expression and activity of several MMPs, both in the gastric mucosa and in gastric epithelial cell lines (83, 84), leading to gastric damage (85). Among those, MMP-1, -2, -7 and -9 are the most well-studied.
Concerning MMP-1, it has been reported that *H. pylori* stimulates MMP-1 expression *in vitro*, as well as, in human gastric biopsies (85, 86). Up-regulation of MMP-1 in gastric epithelial cells was found to be dependent on *H. pylori* strains that harbour CagA and the outer inflammatory protein A (OipA) (85-87), and involves activation of JNK and ERK1/2 signalling pathways(85, 86), under the regulation of PKC (88). Moreover, induction of MMP-1-mediated *H. pylori* has been

strongly associated with stimulation of gastric epithelial migration and invasion (85, 88).

H. pylori-mediated MMP-7 expression has been reported in gastric epithelial cells, both *in vivo* and *in vitro*, and is dependent on the presence of an intact *cag* PAI (83, 89, 90). The signalling pathways underlying the *H. pylori*-mediated MMP-7 expression are still controversial. Several signalling pathways including the β-catenin signalling and activation of the PEA3 member of the ETS transcription factor (89), aberrant activation of p120-catenin (91), activation of ERK 1/2 by specific components within the *cag* pathogenicity island (83) or even small GTPases of the Rho family through activation of NF-κB and AP-1 transcription factors (92), have been shown to contribute to the induction of *H. pylori*-mediated MMP-7 expression. This enhanced MMP-7 expression mediated by *H. pylori* infection has been strongly correlated *in vivo* with development of epithelial damage, mainly gastritis, and *in vitro* with cell migration and invasion (83, 89, 92).

H. pylori-mediated MMP-2 and MMP-9 overexpression has also been reported (87, 93-96). However, the mechanisms underlying their regulation are slightly different (96). There is no consensus regarding the involvement of *cag* PAI in the regulation of these two MMPs. Some authors argue that this regulation depends on the presence of *cag* PAI, (97, 98), whereas others claim that this regulation is *cag* PAI-independent (95). Nevertheless, concerning MMP-9, it is claimed that the CagA phosphorylation is important for *H. pylori*-mediated MMP-9 expression (98). The pathways through which *H. pylori*-mediates MMP-2 and MMP-9 overexpression are also controversial. Regarding *H. pylori*-mediated MMP-9 expression an intracellular signalling pathway that involves IκB kinase and nuclear factor κB-inducing kinase (87, 94, 96, 98), *via* COX-2 (87), or CagA phosphorylation and thus activation of SHP-2 (98) have been pointed out as important for the induction of MMP-9 expression. Added to that, the infection-associated cytokines TNF-α, IL-1β, IL-6 and IL-21 are reported to induce MMP-9 secretion (94, 95).

Regarding the mechanisms underlying the regulation of MMP-2 and MMP-9 in *H. pylori* infection, their increase activity has been strongly associated with inflammation and cell invasion (87, 94, 97).

2.2. Transcriptional regulation of MMP genes

In normal conditions, the expression of most MMPs in tissues is low, only being induced when remodelling of ECM is required. It is now well established that MMP gene expression is primarily regulated at the transcriptional level, through different transcriptional control mechanisms that integrate signals from multiple pathways, and further modulated by epigenetic modifications, namely methylation and acetylation, which lead to chromatin remodelling (72, 74, 99). The promoter regions of MMPs' genes harbour several conserved *cis*-acting elements which allow the regulation of gene expression by a diverse set of *trans*-activators (72, 99). These functional *cis*-acting elements in MMP promoters include the activator protein (AP)-1, the polyoma virus enhancer-A binding protein-3 (PEA3), STAT, NF-κB, SMAD, Tcf/Lef-1, RUNX2, Sp-1and RARE (74, 99, 100). Based on the composition of *cis*-response elements, the MMP promoters can be divided into three groups (Figure 2) (99). The first group (MMP-1, -3, -7, -9, -10, -12, -13, -19 and -26) includes the majority of MMP promoters and comprise a TATA box at -30 bp from the transcriptional start site (TSS) and an AP-1 binding site at -70 bp. These promoters can also contain an upstream PEA3-binding site that co-operates with an AP-1-binding site to induce MMP transcription (Figure 2). The MMP promoters in the second group (MMP-8, -11 and -21) contain a TATA box but lack a proximal AP-1-binding site (Figure 2).

Finally, the promoters in the third group (MMP-2, -14 and -28) lack both a TATA box and an AP-1-binding site and comprise multiple transcription sites. The expression of MMPs in this group is mostly regulated by the SP-1 family of transcription factors, which bind to a proximal GC box (Figure 2) (72, 99, 100).

It is important to reiterate that functional-related MMPs differ greatly in the composition of transcription factor binding sites (as described above). In contrast, MMPs that are commonly co-regulated upon certain stimulation share common sites and a high similarity in their promoter binding sites (99, 100).

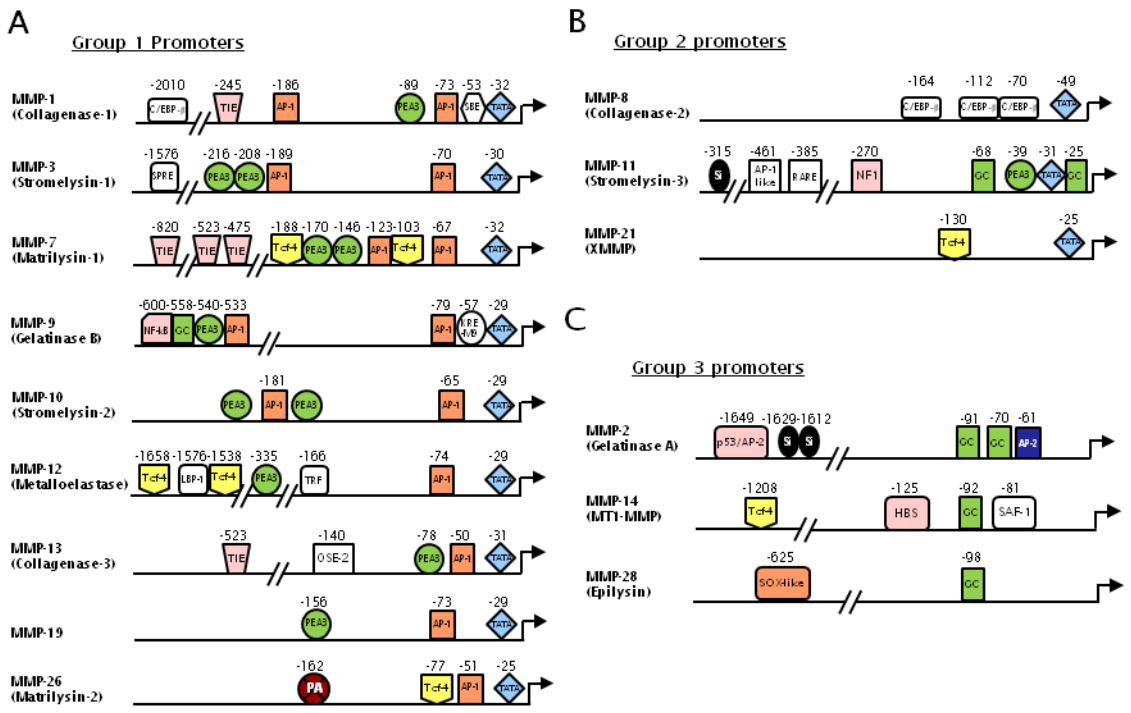

Figure 2. Functional regulatory *cis*-elements in human MMP promoters. Adapted with permission from John Wiley and Sons: *Journal of Cellular Physiology* (99) *© 2007.*

In this work, we will discuss in more detail two of the main transcriptional binding sites involved in the induction of MMP expression, AP-1 and PEA2 sites, in the section below.

2.2.1. AP-1 and PEA3-binding sites

AP-1 is the critical transcription factor binding site that regulates the activity of many MMP promoters, located near the TATA box. Variations in the composition of the AP-1 proteins (c-fos, c-jun, jun-B) along with the juxtaposition of other binding sites, namely PEA3, are responsible for different MMP gene specificity (72). Taking as an example the regulation model for MMP-1 promoter, the stimulation by inflammatory cytokines or growth factor, like EGF, leads to MAPK signalling activation which increases the levels of c-Fos, c-Jun, JunB and/or PEA3, and enhanced MMP1 *trans*-activation. It has been suggested that c-Jun has the capability to induce MMP-1 expression independently, while jun-B needs other AP-1 proteins, such a as c-Fos, for the induction of this MMP (99, 101).

The PEA3 binding site, is present in many MMP promoters located close to the AP-1 binding site with which it frequently co-operates in the promotion of MMP activity (80, 99). Moreover, it has been reported that multiple PEA3 binding sites are able to bind members of the ETS family of transcription factors through a highly conserved ETS domain that recognises a purine rich PEA3 element (102, 103), and thus induce MMP expression. These effects however, require the presence of proximal AP-1 site.

3. The ETS family of transcription factors

The discovery of this large family of conserved genes began with the identification of the v-ets oncogene of the avian leukemia virus, E26, in 1983 (104). Since then, almost 30 members of the family have been indentified in mammals which are shown to encode nuclear transcription factors for regulation of gene expression (105). All ETS family members have a conserved sequence that encodes the winged helix-turn-helix DNA-binding domain (ETS domain), that is able to recognise a unique DNA sequence containing GGAA/T (ETS Binding Site, EBS or PEA3 site) in the promoter region of target genes (105, 106). Based on their ETS domain sequence homology, the ETS factors can be classified into 12 subgroups: ETS, ERG, PEA3, ETV, TCF, GABP, ELF1, SPI1, TEL, ERF, SPDEF, and ESE (107). Some ETS proteins also have a second domain, the Pointed (PNT) domain, important for protein-protein interactions and oligomerisation (106).

3.1. ETS factors regulation and target genes

The ETS functional activity can be modulated at different levels. The transcriptional regulation depends on combinatorial interaction between multiple proteins, namely other transcription factors. Numerous transcription factors have their DNA binding site closer to EBS (108, 109). The binding of an ETS protein, depending on the precise sequence context, near other transcription factors can lead to the activation and/or repression of specific target genes (108). For example, the interaction between ETS factors and the AP-1 transcriptional complex lead to an increase transcriptional activity of promoters containing AP1—EBS binding sites, such as MMP-1, which activate cellular responses (106). Regarding post-transcriptional modification, ETS factors can be controlled by phosphorylation, acetylation, sumoylation, ubiquitinylation, and glycosylation (107). However,

phosphorylation seems to be the best-studied. The mitogen-activated protein kinases (MAPK) ERK, JNK and p38 are the best characterised ETS modulators. ERKs are activated in response to mitogenic signals, whereas JNKs and p38 respond to stress signals (107, 110). Specific ETS factors, namely ETS-1, ETS-2, ELK1, among others, can be phosphorylated by MAPKs, leading to an increased transcriptional activation (110). It was found that the phosporylation of a MAP kinase site near the PNT domain leads to a positive transcriptional regulation of ETS-1 and ETS-2 activities (106). Added to that, phosphorylation can also affect the sub-cellular localisation of ETS proteins (111).

Concerning the target genes of ETS factors, over 700 genes have been reported, most of them essencial in several biological processes, namely cellular proliferation, control response to various signalling cascades, differentiation, development, transformation, apoptosis, adhesion, tissue remodelling, and ECM composition (107, 109).

3.2. ETS factors and Cancer

Many tumours cause a deregulation of signal transduction pathways, which results in constitutive and often ligand independent activation (106, 109). ETS factors, being end effectors of these pathways, have their function significantly altered, which in turn affects the expression of key regulators of proliferation, differentiation, and metastasis, such as RTKs (receptor tyrosine kinases; e.g., HER2), the M-CSF receptor, MET, c-kit, and the VEGF receptor (109, 112). Furthermore, ETS factors are known to act as positive or negative regulators of genes that control critical tumour progression processes, namely cellular hematopoiesis, apoptosis, adhesion, migration, invasion, tissue remodelling, ECM composition, angiogenesis and other signalling cascades (109). An important example of upregulated ETS target genes are the ECM-degrading proteins, like MMP-1 and MMP-9 (107). Figure 3 gives an overview of ETS factors activity in cancer. It has also been shown that ETS factors can directly regulate the expression of cytokines and chemokines, affecting both tumour stroma and tumour cells via HGF/c-Met (111). Added to that, there has been growing evidence suggesting a critical role in the regulation of stromal activation by ETS family members, through their capability to regulate ECM-degrading enzymes, such as MMPs and collagenases, crucial for the establishment of a reactive stroma, promoting the metastatic process (113).

It is claimed that the oncogenic and tumour suppressing activities described above are, on the one hand, induced by point mutations (e.g., ETS-1 and SPI-1), increased (e.g., ETS-1, ETS-2, ERG) or decreased (e.g., SPDEF, EHF) expression, genomic amplification (e.g., ETS-1, ETS-2, ERG), or rearrangements (e.g., ETV-6, FLI-1, ERG) (106). On the other hand, it has been suggested that those activities are coordinated by the target genes of ETS factors (106, 108, 109). Among these are the genes responsible for cell proliferation (like cyclins and cdks), cell motility (HGF), invasion (MMPs, PAI, uPA and uPAR (urokinase receptor); TIMPs), extravasation (MMPs, Integrins), micro-metastasis (e.g., Osteopontin, Osteonectin and BSP), as well as establishment and maintenance of distant site metastasis and angiogenesis (neovascularization and neoangiogenesis (integrin β3, VEGF, Flt-1/KDR, Tie2)) (109).

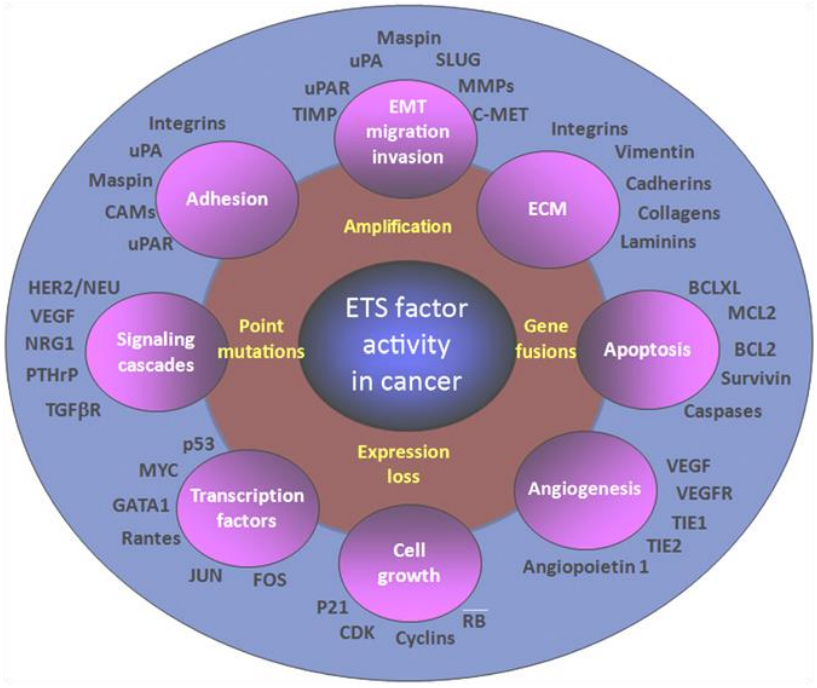

Figure 3. ETS factors activity in cancer. Adapted with permission from Elsevier: *Advances in Cancer Research* (107) © *2013*.

3.3. ETS-1 transcription factor

ETS-1 transcription factor, considered the founding member of the ETS family, is usually localised in the nucleus, and found to be expressed in a large variety of different cell types and tissues, playing roles in both physiological and pathological processes, such as embryogenesis, wound healing, and tumour progression/invasion (114, 115).

The role of ETS-1 in tumour invasion relates to the transcriptional activation of enzymes involved in ECM degradation, namely serine proteases, MMPs, TIMPs, and other matrix proteins (116).

Expression of ETS-1 is not ubiquitous in embryonic and adult tissues (117, 118). Its expression is restricted to specific invasive processes which include the formation of new blood vessels during normal and pathological development. In embryos, expression of ETS-1 is limited to endothelial cells and blood islands of the yolk sac, whereas, in adults, it is expressed in endothelial cells during tumour angiogenesis, wound healing and reendothelialization following the denuding injury (115).

3.3.1. ETS-1 and the regulation of the extracellular matrix

Several studies have demonstrated the co-expression of ETS-1 and matrix degrading proteases, particularly in invasive tumours, in various human tissues (119). Examples include co-expression of ETS-1, MMP-1, MMP-3, and uPA in lung carcinomas and angiosarcoma of the skin (116); production of MMP-1, MMP-9 and MMP-3 along with ETS-1 in ovarian carcinoma cells and stromal fibroblasts (120, 121); and the correlation between ETS-1 expression and uPA in lung and brain tumours (122, 123). Also, in breast cancer endothelial or hepatoma cells, when overexpressed, ETS-1 induces the production of MMP-1, MMP-3, MMP-9, and upA (115, 117, 124). In meningiomas, ETS-1 was shown to be up-regulated along with MMP-2 and MMP-9 (125)

The role of ETS-1 in regulating MMPs in tumours is further evidenced by the presence of a single nucleotide polymorphism in the MMP-1 promoter region which creates an ETS binding site (126).

Several studies have highlighted the importance of RTKs in the relationship between ETS-1 and MMPs. In breast cancer cells, it was observed that the human

epidermal growth factor receptor-1 (HER2) induces MMP-1 expression while cooperating with ETS-1 (127). Added to that, one or more ETS binding sites have been identified in the promoter regions of several RTKs, including the epidermal growth factor receptor (EGFR) and the hepatocyte growth factor receptor (c-MET), suggesting an involvement of ETS-1 in their transcriptional regulation (128).

Aims

MMPs are a group of zinc-dependent endopeptidases involved on the regulation of cell-matrix composition. By regulating the cell-matrix integrity and composition, these proteins are able to participate in several normal and pathological processess, namely in cancer progression (73, 74).

Invasion and metastasis are prominent features of malignant tumours, and the degradation of the ECM is one of the key steps involved in these processes (79, 80). *In vitro* and *in vivo* studies have shown that several MMPs were found to be up-regulated in a context of *H. pylori* infection, pointing to a role of this bacterium in inducing ECM degradation (83, 93, 95, 129-131). MMP-10, one of the lesser studied MMPs, is overexpressed in several human tumours of epithelial origin (132-135), and its expression has been linked to poor clinical prognosis (135, 136). Previous results from our Group have shown that MMP-10 is overexpressed in response to *H. pylori* infection. However, the mechanism underlying this up-regulation is not well established.

A whole genome cDNA microarray, also performed by our Group, showed that apart from MMP-10, the ETS transcription factor ETS-1, was found to be up-regulated upon *H. pylori* infection.

Due to the strong association between ETS family members and MMPs regulation, and since the relationship between ETS-1 transcription factor and MMP-10 modulation has never been studied, the general aim of this thesis was to evaluate the role played by ETS-1 on *H. pylori*-mediated up-regulation of MMP-10.

For that, the initial approach was to validate the results from the cDNA microarray by quantivative real time PCR and western blot, using two *H. pylori* strains. To better elucidate the expression pattern of ETS-1 transcription factor and MMP-10 throughtout time, a time course of infection analysis was performed. The involvement of ETS-1 on the regulation of MMP-10 expression, was addressed by performing experiments with siRNA gene silencing and luciferase gene reporter assays, using different MMP-10 promoter deletion constructs.

Finally, previous results have shown a correlation between EGF stimulation and activation of ECM proteins (137-139) by ETS family members (127, 140). In order to study the involvement of EGFR on the modulation of ETS-1 and MMP-10 expression in the context of *H. pylori* infection, a series of experiments with a chemical inhibitor for this tyrosine kinase receptor were performed.

Materials And Methods

1. Cell culture and bacteria growth

Human gastric carcinoma AGS cell line (ATCC CRL-1739) was maintained in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and 1% antibiotics (200 μg/ml streptomycin and 200 IU/ml penicillin; Gibco) at 37°C, under a 5% humidified atmosphere.
H. pylori strains 26695 (ATCC 700392) and 60190 (ATCC 49503) were maintained for 24 or 48 hours in tryptic soy agar (TSA) supplemented with 5% sheep blood (BD Bioscience) and incubated at 37°C under microaerobic conditions.

2. Co-culture of AGS cell line with *Helicobacter pylori*

48 hours prior the infection with *H. pylori*, AGS cells were grown in complete medium (6-well plates) in order to reach 80% confluency. 1 to 3 hours before the infection, the medium was replaced by serum- and antibiotic-free medium (RPMI 1640).
The *H. pylori* strains were cultured for 24 hours in TSA supplemented with 5% sheep blood, collected from the agar plate with an inoculation loop and resuspended in 1ml of RPMI 1640. The bacterial density was measured by absorbance at 600nm (OD =0.1 corresponds 2x106 bacteria/μL). All infections were performed at a multiplicity of infection (MOI) of 100. After the infection period, the conditioned media were collected and the cells were lysed either for protein or RNA extraction.

3. Small interference RNA (siRNA) transfection

AGS cells were grown for 24 hours in complete medium (6-well plates) in order to reach 50% confluency. Before the transfection, the medium was replaced for serum- and antibiotic-free medium. The transient transfection was performed using Lipofectamine 2000 (Invitrogen) with 50nM of siRNA RNA targeting ETS-1 (ON-TARGETplus SMARTpool - Human ETS-1 L-003887-00-0005; GE Healthcare Dharmacon) or with control (non-targeting) siRNA (ON-TARGETplus Non-targeting siRNA #1 D-001810-01-05; GE Healthcare Dharmacon). The siRNAs and the Lipofectamine were previously eluted in Opti-MEM® (Gibco).
At 16 hours of transfection, the transfection medium was replaced with RPMI 1640 supplemented with 10% FBS and antibiotics-free and, 30 hours later, the cells were

infected with *H. pylori* (24 hours or 4 hours). The efficiency of siRNA silencing was evaluated after each infection period by qRT-PCR and western blot.

4. Cell treatment with chemical inhibitors

100% confluent cells were incubated for 3 hours in serum- and antibiotic-free medium. Pharmacological inhibitor AG1478 (Cayman Chemicals), at a concentration of 5 μM, was then added to the medium. One hour later, the cells were infected with *H. pylori* for a 4 hour period. The controls were treated in a similar way but replacing the volumes of inhibitor and bacteria for an equivalent volume of dimethyl sulfoxide (DMSO) and serum- and antibiotic-free medium. When the incubation period ended, the conditioned media were collected and the cells were lysed both for protein and RNA extraction.

5. RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using TripleXtractor reagent (Grisp). cDNA was synthesized from 500 ng of RNA using Random Primers, NZY Ribonuclease Inhibitor (Nzytech) and NZY Reverse Transcriptase (Nzytech). Quantitative real-time PCR was performed using the TaqMan Mix (Applied Biossystems) and the following TaqMan probes (Applied Biosystems): ETS-1 (Assay Hs00428293_m1), MMP-10 (Assay Hs00233987_m1). The relative gene expression was normalized to mRNA of GAPDH (Human GAPDH Endogenous Control (FAM Dye/ MGB Probe, Non-Primer Limited) and calculated by the $2^{-\Delta\Delta Ct}$ method. The reactions were performed in a 7500 Real Time PCR System (Applied Biosystems), in triplicates, with 0.5 μL of cDNA template in a final volume 10 μL.

6. Protein extraction and western blot

After the infection period, the cells were washed with cold phosphate buffered saline (PBS), pH 7.4, and then lysed with cold Catenin lysis buffer (1% Triton X-100, 1% NP-40 in PBS, pH 7.4) supplemented with phosphatase inhibitor cocktail 2 (Sigma) and protease inhibitor cocktail (Roche). Cells with cold lysis buffer were incubated 15min on ice, scraped and centrifuged at 20818 g for 10min at 4°C. The soluble proteins were quantified using the Bio-Rad DC Protein assay (Bio-Rad) but only 25 μg of total protein extract were used for immunoblot analysis. Proteins

were separated by standard SDS-PAGE using the Precision Plus Protein Standard Dual Color protein marker (Bio Rad) to determine the molecular weights. Next, the proteins were transferred onto a nitrocellulose membrane (GE Helthcare) and stained with Ponceu S (Sigma), to monitor transfer efficancy. The membranes were blocked for 1 hour with 5 % non-fat milk or bovine serum albumin (BSA) in PBS + 1% Tween-20 (PBS-T), and blotted overnight with the primary antibody in 5% non-fat milk or 5% BSA in PBS-T. Before the incubation with the secondary antibody, unbound primary antibody was washed for four times with PBS-T. The membranes were incubated for 2 hours with the secondary antibody in PBS-T with 5% non-fat milk and then washed six times with PBS-T. The detection was performed using Luminata Forte Western HRP Substrate (Millipore). The loading control used was GAPDH (glyceraldehyde-3-phosphate dehydrogenase). The protein bands obtained were analysed and quantified using the Quantity One software (Bio-Rad).

7. Antibodies

The table bellow presents the relevant information about the antibodies used for western blot analysis.

Table 1. Antibodies used for immunoblot analysis

	Target Molecules	Serum	Molecular Weight (kDa)	Dilutions	Supplier
Primary antibodies	Phospho-EGFR	rabbit	175	1:1000 BSA	Cell Signaling
	EGFR	rabbit	175	1:1000 Milk	Cell Signaling
	MMP-10	goat	54	1:1000 Milk	Cell Signaling
	ETS-1	rabbit	52	1:1000 BSA	Cell Signaling
	GAPDH	mouse	37	1:10000 Milk	Santa Cruz
Secondary antibodies	Goat IgG (HRP)	donkey	-	1:3000 Milk	Santa Cruz
	Mouse IgG (HRP)	sheep	-	1:20000 Milk	GE Healthcare
	Rabbit IgG (HRP)	donkey	-	1:5000 Milk	GE Healthcare

8. Preparation of conditioned media

The conditioned media collected from infected and non-infected cells were centrifuged at 6000 g for 3 minutes at 4°C and filtered through a 0.2 μm pore size filter (Advantec). For protein analysis with SDS-PAGE, the conditioned media were concentrated using Amicon ultra-0.5 centrifugal filter devices, with a 30 KDa cut-off (Millipore).

9. DNA isolation from AGS cells

Total DNA was extracted using the Genomic DNA Purification Kit (Fermentas), according to the manufacturer's instructions, and the concentration and purity were determined using a NanoDrop Spectrophotometer ND-100 and by gel electrophoresis.

10. Prediction of ETS-1 transcription factor binding sites in the MMP-10 promoter region

The MMP-10 human promoter sequence reported on this study was obtained using the Eukaryotic Promoter Database (http://epd.vital-it.ch/). For the purpose of this study, we only considered 948 bp, upstream the ATG, of the promoter sequence obtained.
The prediction of ETS-1 transcription factor binding sites on MMP-10 promoter was performed resorting to tree online databases: ALGGEN-PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3), JASPAR Core Database (http://jaspar.genereg.net/) and TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html). The obtained results from these tree databases were crossed in order to reach a stronger prediction of the ETS-1 binding sites. Only two ETS-1 binding sites predicted in at least two databases were considered for further studies.

11. MMP-10 promoter cloning and promoter deletion constructs

The MMP-10 full-length promoter construct as well as all deletion constructs for promoter characterization (Figure 4) were generated by PCR amplification using primers containing a specific restriction enzyme site (Table 3). PCR reactions were

performed using the AmpliTaq Gold polymerese (Applied Biosystems) with the following program: 95°C for 9 minutes, followed by 35 cycles of 95°C for 30 seconds, 58°C for 45 seconds, and 72°C for 45 seconds, with a final extension at 72°C for 10 minutes. The PCR products were purified with NZYGelpure kit (Nzytech) and the concentration and purity were determined by NanoDrop Spectrophotometer ND-100 and electrophoresis.

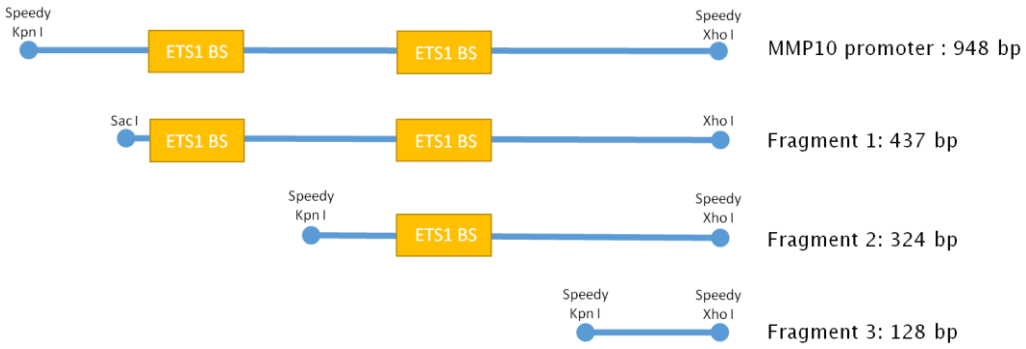

Figure 4. MMP-10 promoter deletion fragments scheme with their corresponding restriction enzymes. The blue line represents the MMP-10 human promoter (not scaled), whereas the yellow boxes represent the ETS-1 transcription factor binding sites within the MMP-10 promoter predicted by the cross of tree online databases (ALGGEN-PROMO, JASPAR and TFSEARCH).

Table 2. Restriction enzymes used in this study. (') stands for restriction enzyme recognition site.

Restriction enzyme	Sequence (5'->3')	Supplier
Speedy Kpn I	GGTAC'C C'CATGG	Nzytech
Speedy Xho I	C'TCGAG GAGCT'C	Nzytech
Sac I-HF	GAGCT'C C'TCGAG	New England Biolabs
Xho I	C'TCGAG GAGCT'C	New England Biolabs

Table 3. Oligonucleotides used in the study. The underlined sequences represent the restriction enzyme recognition sites.

Primers	Oligonucleotide sequence (5'-3')	Tm (°C)
Oligonucleotides used in the generation of promoter constructs		
MMP-10 promoter_Forward	CAGT**GGTACC**GTTATTGCAGACTTACTGTG	70.2
MMP-10 promoter_Reverse	AGCT**CTCGAG**TCTCACTGCCCTTACCTTC	69.2
F1_Forward	ATAG**GAGCTC**GACCACGACTATATTTGG	72.0
F1_Reverse	AGCT**CTCGAG**TCTCACTGCCCTTACCTTC	69.2
F2_Forward	CTAT**GGTACC**CAAAACATTTGGCAT	67.1
F2_Reverse	AGCT**CTCGAG**TCTCACTGCCCTTACCTTC	69.2
F3_Forward	CCAT**GGTACC**GATGTAGGCTGTATCACCA	72.5
F3_Reverse	AGCT**CTCGAG**TCTCACTGCCCTTACCTTC	69.2
Oligonucleotides used in DNA-sequencing		
pGL4.10_Forward	GAACATTTCTCTGGCCTAAC	57.8
pGL4.10_Reverse	GCCGAGGCCAGATCTTGA	66.7
pGEM_T7	TAATACGACTCACTATAGGG	50.9
pGEM_SP6	ATTTAGGTGACACTATAGAA	47.7

Two different approaches were used to clone the amplified fragments into the pGL4.10[luc2] firefly luciferase vector (Promega). The MMP-10 full-length promoter fragment and the Fragment 1 were double-digested with the restriction enzymes (Table 2): Speedy Kpn I and Speedy Xho I, and Sac I-HF and Xho I, respectively (Figure 4); for 1 hour at 37°C, and purified with NZYGelpure kit. The resulting fragments were cloned into a previous double-digested pGL4.10[luc2] vector, in 1:6 vector-insert proportion, using the Quick Ligation Kit (NEB), according to the manufacturer's instructions, and then transformed into DH5α *Echerichia coli* (*E. coli)* competent cells. The plasmid extraction and purification was performed using NZYMiniprep and NZTMaxiprep (Nzytech).

The MMP-10 Fragments 2 and 3, after amplification, were cloned into a pGEM-T Easy Vector Systems (Promega), according to the manufacturer's instructions. The resulting plasmids were then double-digested with the restriction enzymes Speedy Kpn I and Speedy Xho I (Table 2 and Figure 4), for 1 hour at 37°C, and purified with

NZYGelpure kit. The next steps, cloning into the pGL4.10[luc2] vector, transformation and plasmid extraction and purification, were performed as above described.

12. Transient transfection and luciferase gene reporter assays

AGS cells ($2.5x10^5$ cells/well) were grown, in 24-well plates, in complete medium for 24 hours. After the incubation period, the cells were co-transfected with the MMP-10 full-length promoter construct/the truncated MMP-10 promoter constructs (pGL4.10[luc2] vector containing F1/F2/F3 MMP-10 promoter fragments, as described previously) mixed with *Renilla* luciferase vector (pGL4.73[hRluc/SV40]; Promega) in a ratio 0.5µg expression vector: 0.1 µg *Renilla* vector, using Lipofectamin 2000 (1 µg of DNA : 1.5 µL of Lipofectamine 2000) in Opti-MEM. Cells were incubated with the transfection mixture for 6 hours followed by medium replacement (RPMI 1640 supplemented with 10% FBS, antibiotics free) and 16 hours incubation. After 16 hours, the medium was replace for RPMI 1640 serum- and antibiotics-free medium and, 3 hours after, the cells were infected with *H. pylori* or treated with EGF (50 ng/ml; Sigma) for a 4 hours period.
The Luciferase activity was estimated in total cell extracts using the Dual-Glo Luciferase Assay System (Promega), according to the manufacturer's instructions, with a 1450 MicroBeta TriLux Microplate Scintillation and Luminescence Counter (PerkinElmer). The inducible firefly luciferase activity was normalised relative to *Renilla*'s activity, and expressed as fold changes in stimulated samples in comparison to non-stimulated cells.

13. Statistical analysis

The statistical analysis of the results was performed using the Student's t-test. The data were expressed as mean fold change ± SEM of at least two independent experiments, with the value of the control normalised to 1. Differences were considered significant at P-values less than 0.05.

RESULTS

1. Analysis of the effect of *H. pylori* on MMP-10 and ETS-1 expression

This study started with the results obtained from a whole genome cDNA microarray, previously performed by our Grup (data not shown), in which the changes in gene expression profile of gastric cell line AGS in reponse to *H. pylori* strain 60190 infection were characterized, after 24 hours of infection. Two genes, namely MMP-10 and ETS-1 transcription factor, were found to be up-regulated by *H. pylori* (Table 4).

Table 4. List of relevant up-regulated genes after *H. pylori* infection.

Up-regulated genes	Fold increase
ETS-1	2.52
MMP-10	14.6

Firstly, we assessed whether *H. pylori* modulates the expression of MMP-10 and ETS-1. For that, AGS cells were co-cultured with two *H. pylori* strains, 26695 and 60190, for a 24 hours period. After the infection period, the expression levels of MMP-10 and ETS-1 were evaluated by qRT-PCR and compared with non-infected controls.

Concerning MMP-10, there was a significant increase on MMP-10 gene expression upon infection with both *H. pylori* strains (Figure 5A). However, this increase was more pronounced with *H. pylori* strain 26695 (30-fold increased compared with non-infected condition) (Figure 5A). Futhermore, in order to assess whether MMP-10 secretion levels followed the same expression pattern, the conditioned medium was collected and concentrated and the levels of secreted MMP-10 were assessed by western blot. The results showed an enhancement of MMP-10 protein levels upon infection with both *H. pylori* strains (Figure 5B). Taken together, the qRT-PCR and western blot obtained results were in accordance with the array results for MMP-10 (Table 4).

Regarding ETS-1, there was also a significant increase on the ETS-1 mRNA levels upon infection, being more pronounced with *H. pylori* strain 26695 (nearly 3 fold increased compared with non-infected condition) (Figure 5C). Moreover, the levels of ETS-1 present on cell lysates were also assessed. The results showed an increase

on ETS-1 protein levels upon infection with both *H. pylori* strains, being more pronounced with *H.pylori* strain 26695 (Figure 5D). Once again, the qRT-PCR and western blot obtained results were in accordance with the array results, showing an up-regulation of ETS-1 upon infection with *H. pylori* (Table 4).

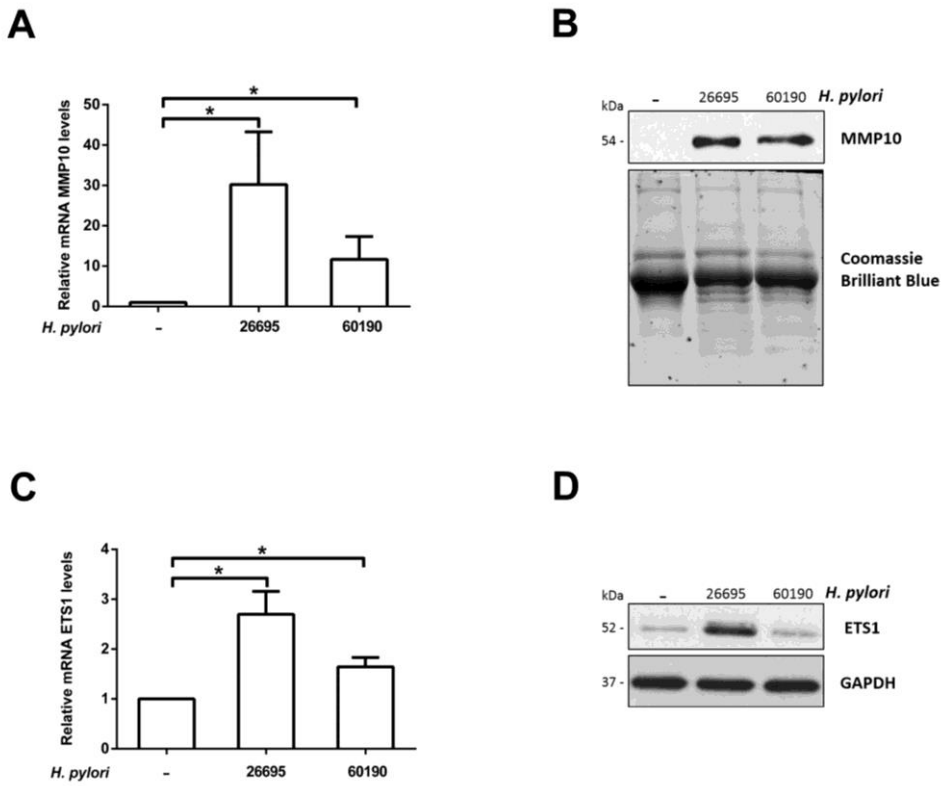

Figure 5. *H. pylori* strains 26695 and 60190 induce an increase in MMP-10 and ETS-1 expression at mRNA and protein levels in AGS cell line. AGS cells were co-culture with two *H. pylori* strains, 26695 and 6019, at a multiplicity of infection of 100, for a 24 hours period. Total RNA was isolated and the relative mRNA levels of **(A)** MMP-10 and **(C)** ETS-1 were analysed by qRT-PCR and normalized to GAPDH expression. The results are expressed as fold-difference relatively to non-infected controls, with mean ± SE; n = 3 independent experiments. **(B)** Conditioned medium was collected and concentrated and MMP-10 secretion was assessed by western blot. The coomassie brilliant blue was used to control protein loading. **(D)** The ETS-1 protein present on cell lysates was analysed by western blot. GAPDH was used as an internal control. The blots shown are representative of 3 independent experiments. * $p < 0.05$, relative to non-infected cells.

We next assessed how ETS-1 expression induced by *H. pylori* was modulated over the time of infection. To test that, five different time periods for *H. pylori* infections were established (4h, 8h, 12h, 16h, and 24h) and experiments were performed

using *H. pylori* strains 26695 and 60190. After each infection period, the expression levels of ETS-1were analysed by qRT- PCR and western blot, and compared with non-infected controls.

As shown on Figure 6 (A and B), despite being up-regulated throughout time, the ETS-1 expression levels tended to decrease with increasing time of infection. The peak of mRNA expression was reached in early infection periods, more precisely after 4 hours of infection (Figure 6A). However, concerning the levels of ETS-1 protein, due to the variability among the experiments, this peak varies between 4 and 8 hours of infection (Figure 6B).

In order to investigate if the levels of secreted MMP-10 followed the same expression pattern, the conditioned medium from each infection period was collected and concentrated. The levels of secreted MMP-10 were analysed by western blot and compared with non-infected controls. As shown on Figure 6C, despite being up-regulated throughout time, the levels of secreted protein tended to decrease with increasing times of infection. The maximum levels of MMP-10 secretion were observed after 4 hours of infection.

Overall, these results show that *H. pylori* infection leads to an up-regulation of MMP-10 and ETS-1.

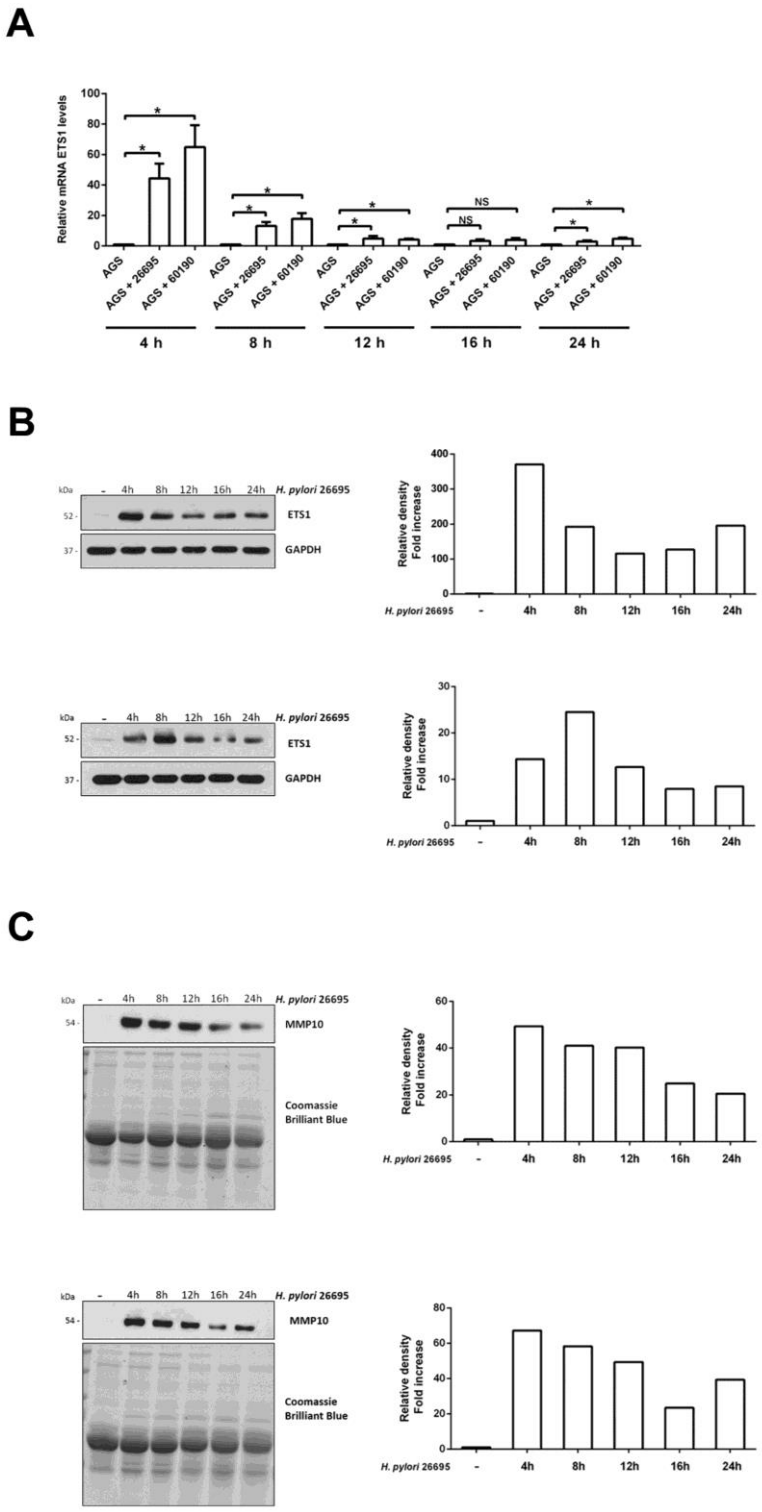

Figure 6. Effect of *H. pylori* infection on ETS-1 and MMP-10 expression in a time course of infection (4h, 8h, 12h, 16h and 24h). AGS cells were infected with *H. pylori* strains 26695 **(B, C)** and 60190, at a multiplicity of infection of 100, for the indicated periods of time. After each infection period, **(A)** total RNA was isolated and the relative mRNA levels were analysed by qRT-RT PCR and normalized to GAPDH expression. The results are expressed as fold-difference relatively to non-infected controls, with mean ±SE; n = 3 independent experiments. **(B)** ETS-1 protein present on the lysates was analysed by western blot and GAPDH was used as an internal control. **(C)** Conditioned medium was

collected and concentrated and MMP-10 secretion was assessed by western blot. The coomassie brilliant blue was used to control protein loading. * $p < 0.05$, relative to non-infected cells; NS non-satistically significant difference relative to non-infected cells.

2. Role of ETS-1 on *H. pylori*-mediated MMP-10 expression

Several ETS transcription factor family members are known to have the ability to bind and thus regulate the expression of various MMPs (74, 141), namely MMP-10 (142). Since previous results have shown an increase in expression of ETS-1 transcription factor and MMP-10 (Figures 5-6) in the presence of *H. pylori* and because the relationship between these two proteins has never been explored in the context of *H. pylori* infection, we investigated if ETS-1 played a role on the modulation of *H. pylori*-mediated MMP-10 expression. To test that, AGS cells were transiently transfected with a siRNA specific to ETS-1 or with a non-targeting siRNA, and performed a 4 hours (Figure 7A-B) or 24 hours (Figure 7C-D) infection with *H. pylori* strain 26695. After each infection period, the expression levels of ETS-1 and MMP-10 were evaluated by qRT- PCR and western blot, and compared with controls treated with a non-targeting siRNA.

The results showed that ETS-1 expression was completly abrogated after both infection periods (Figure 7A and C). After 4 hours of infection (Figure 7B), the abrogation of ETS-1 expression by a specific siRNA to ETS-1 resulted in a non-significant decrease in MMP-10 mRNA levels. Nevertheless, despite the variability among experiments, the abrogation of ETS-1 activity resulted on a pronounced reduction of *H. pylori*-induced MMP-10 (Figure 7B).

After 24 hours of infection, the abrogation of ETS-1 activity led to a significant decreased on MMP-10 mRNA levels (Figure 7D). However, concerning the secreted MMP-10, although ETS-1 activity was completely abrogated (Figure C), there was a non-significant reduction on MMP-10 secretion, in comparisson with the one registered after 4 hours of infection (Figure 7B and D).

Overall, these results suggest that ETS-1 is involved in *H. pylori*-mediated MMP-10 expression.

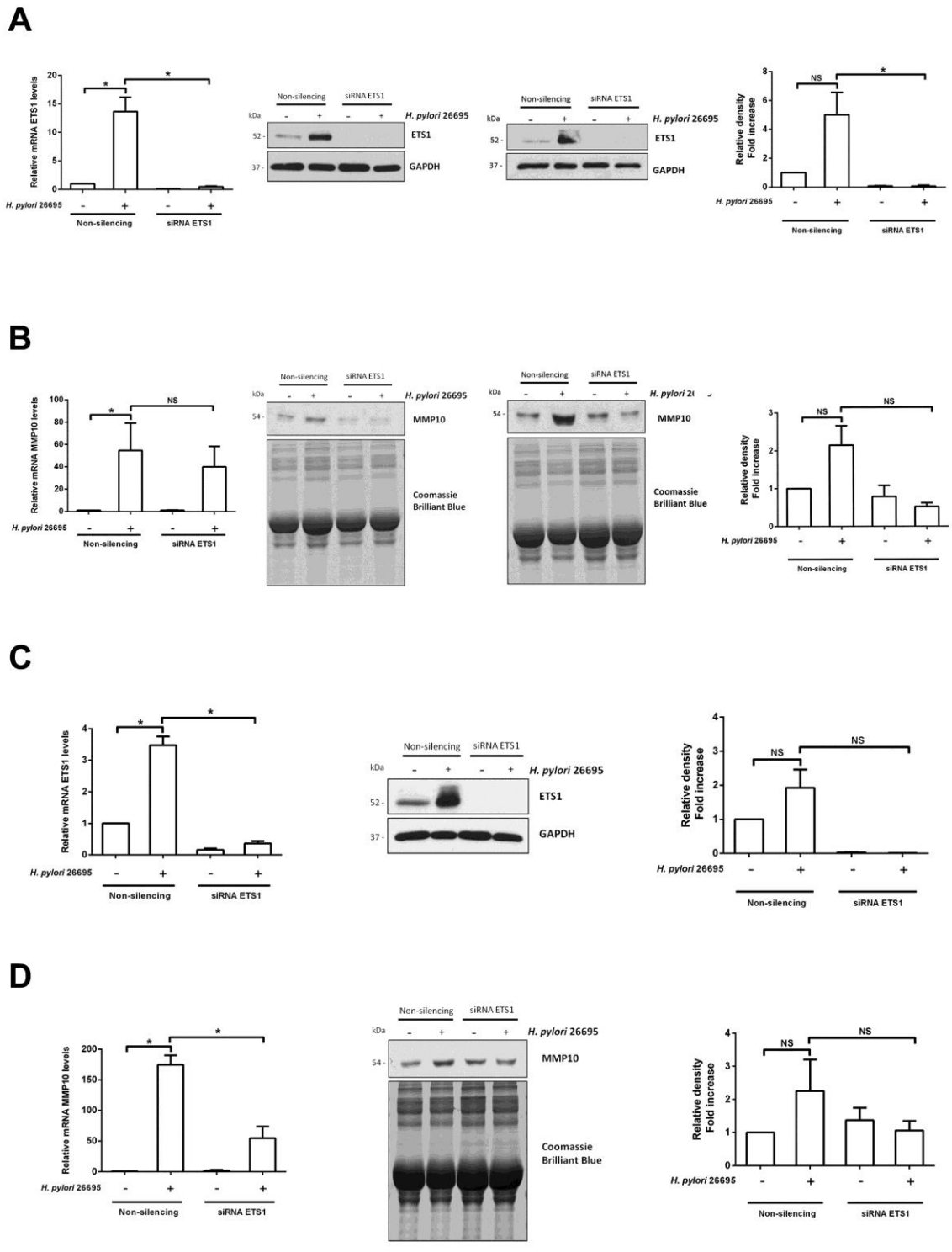

Figure 7. ETS-1 transcription factor is required for MMP-10 activation during *H. pylori* infection. siRNA-tranfected cells were infected with *H. pylori* strain 26695, at a multiplicity of infection of 100, for **(A-B)** 4h or **(C-D)** for 24h. Transient transfections were performed using a siRNA specific to ETS-1 or a non-targeting siRNA. **(A, C)** ETS-1 and **(B, D)** MMP-10 relative mRNA levels were analysed by

qRT-PCR and normalized to GAPDH expression. **(A, C)** ETS-1 protein present on cell lysates was evaluated by western blot and GAPDH was used as an internal control. **(B, D)** Conditioned medium was collected and concentrated and MMP-10 secretion was assessed by western blot. The coomassie brilliant blue was used to control protein loading. The results are expressed relatively to cells treated with a non-targeting siRNA, with mean ± SE; **(A-B)** n = 3 or **(C-D)** n =2 independent experiments. * $p < 0.05$, relative to non-silencing cells infected with *H. pylori*; NS non statistically significant differences relative to non-silencing cells infected with *H. pylori*.

3. Role of EGFR on *H. pylori*-mediated ETS-1 and MMP-10 expression

It has been reported the involvement of several growth factors receptors, such as EGFR, on the regulation of MMPs expression, but the precise mechanisms through which it occurs remain poorly described (139, 143, 144). *H. pylori* plays an important role on this process by triggering EGFR phosporylation (137, 145) which may induce MMPs up-regulation. Moreover, previous results from our group have shown that the increase on MMP-10 expression upon *H. pylori* infection occurred *via* EGFR (146). Several data have pointed that ETS transcription factor family members, namely ETS-1 (127), may also take part on the mediation of EGFR-dependent induction of MMPs (147, 148). Since a relationship between *H. pylori*-induced ETS-1 and MMP-10 expression was observed (Figure 7), we next addressed the role of EGFR on the modulation of *H. pylori*-mediated ETS-1 expression.

To test that, AGS cells were treated with a pharmacological inhibitor of EGFR (AG1478) or DMSO, and then infected for 4 hours with *H. pylori* strain 26695. After the infection period, the expression levels of ETS-1 and MMP-10 were evaluated by qRT-PCR and western blot, and compared with non-infected controls.

As shown on Figure 8A, the EGFR inhibition by AG1478 led to a significant decrease on ETS-1 mRNA and protein expression upon *H. pylori* infection. Concerning MMP-10, in accordance with previous results from our group, the inhibition of EGFR activity significantly reduced *H. pylori*-induced MMP-10 expression and secreted protein (Figure 8B).

These results suggest that the increase on *H. pylori*-induced MMP-10 expression is mediated by EGFR-induced ETS-1.

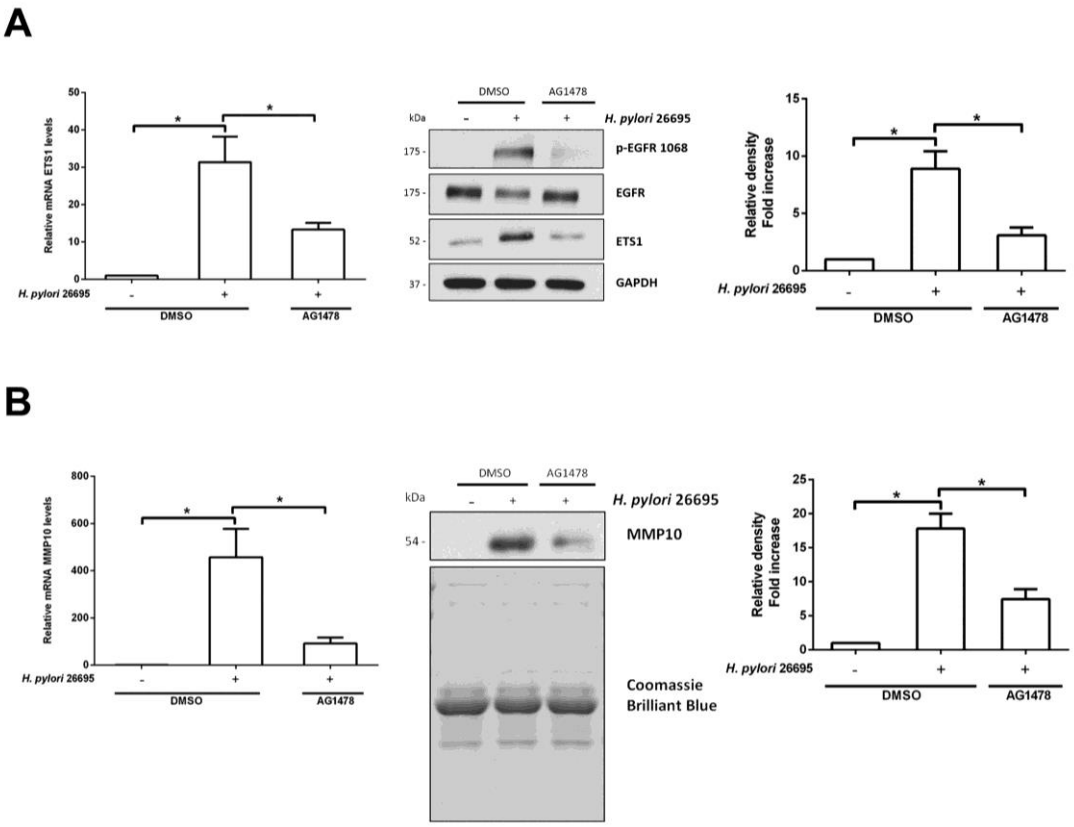

Figure 8. *H. pylori*-induced ETS-1 modulates MMP10 expression via EGFR pathways. AGS cells were treated with 5μM of the pharmacological inhibitor AG1478, or DMSO, for 1 hour and then infected with *H. pylori* strain 26695, at a multiplicity of infection of 100, for a 4 hours period. **(A)** ETS-1 and **(B)** MMP-10 relative mRNA levels were analysed by qRT-PCR and normalized to GAPDH expression. **(A)** Cells lysates were analyased by western blot using p-EFGR, EGFR and ETS-1 antibodies. p-EGFR was used as control of EGFR inhibition and GAPDH as a loading control. **(B)** Conditioned medium was collected and concentrated and MMP-10 secretion was assessed by western blot. The coomassie brilliant blue was used to control protein loading. The results are expressed relatively to non-infected cells treated with DMSO, with mean ± SE; n = 3 independent experiments.* $p < 0.05$, relative to non-infected cells treated with DMSO.

4. Involvement of ETS-1 on the regulation of MMP-10 expression during *H. pylori* infection

Based on the *in silico* analysis of potential ETS-1 transcription factor binding sites on MMP-10 promoter region, we determined whether the two ETS-1 predicted binding sites on MMP-10 promoter, obtained by crossing three online databases, were important for ETS-1-mediated MMP-10 expression.

For this purpose, three MMP-10 promoter deletion constructs were generated from the pre-define full-length MMP-10 promoter region (948 bp) into a promoterless

luciferase reporter vector pGL4.10. The constructs were transiently transfected into AGS cells and, after the transfection period, were infected, or not, with *H. pylori* strain 26695 for 4 hours (Figure 9A), or treated with EGF or DMSO for the same time period (Figure 9B). After 4 hours, the luciferase activity from each condition was measured.

As shown on Figure 9A, for all MMP-10 promoter constructs, *H. pylori* infection did not lead to a pronounced increase on the promotion of MMP-10 expression, since the relative luciferase activity remained very close to the one obtained with uninfected AGS cells.

Concerning the relative luciferase activity among constructs, no differences were registered between constructs 1, 2, and the Full-Length MMP-10 Promoter construct (Figure 9A). This result suggests that the upstream ETS-1 binding site may not play an important role on the promotion of MMP-10 expression. On the other hand, Construct 3, which lacked two ETS-1 binding sites, led to a significant decrease on the relative luciferase activity. Such result indicates that the absence of both ETS-1 binding sites clearly affects the promotion of MMP-10 expression.

Regarding the EGF treatment condition (Figure 9B), all four MMP-10 promoter constructs showed a pronounced increase on the relative luciferase activity, when compared with the untreated condition. Moreover, in this condition, the reduction in length within the MMP-10 promoter constructs was accompanied by a decrease on the relative luciferase activity (Figure 9B). Once again, the absence of the upstream ETS-1 binding site does not seem to have a major impact on the relative luciferase activity, whereas the abrogation of both ETS-1 binding sites (Construct 3) seems to be important for the promotion of MMP-10 expression (Figure 9A and B).

Overall, these results reinforce the importance of the ETS-1 transcription factor on the regulation of MMP-10 expression and the role played by EGFR on the induction of ETS-1 and MMP-10 expression.

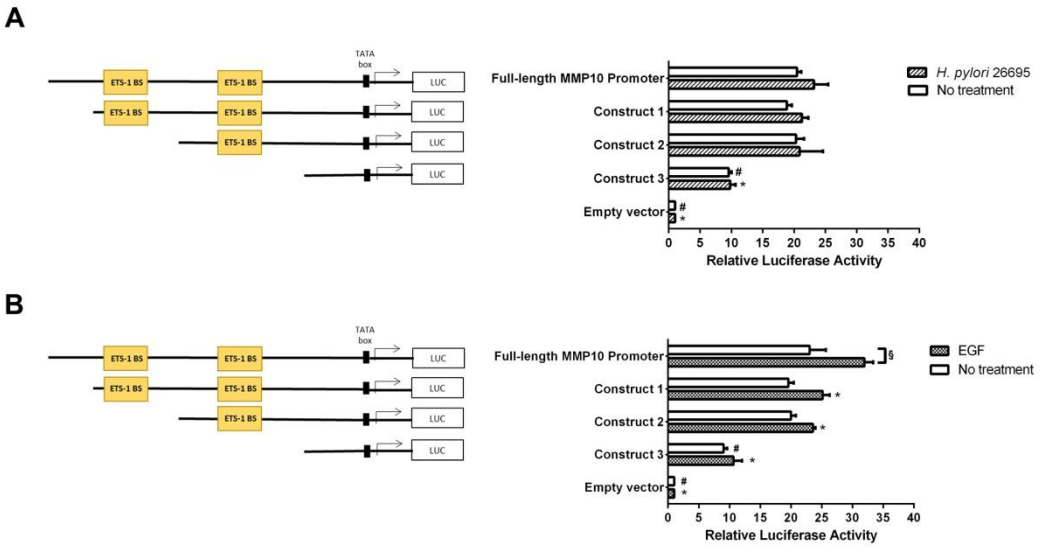

Figure 9. ETS-1 is required for MMP-10 promoter activation. MMP-10 promoter constructs (Full-length MMP-10 Promoter Construct, Construct 1, Construct 2 and Construct 3) were transiently transfected into AGS cells. Transiently transfected cells were **(A)** infected for 4 hours with *H. pylori* strain 26695 or **(B)** treated with EGF (50 ng/ml) for 4 hours, and a reporter gene assay was performed. The no treatment condition represents the basal MMP-10 promoter activity. The scheme on the left shows the ETS-1 transcription factor binding sites present on each MMP-10 promoter construct. For each construct, the individual effect of *H. pylori* and EGF on each MMP-10 promoter constructs was assessed by comparison with the respective no treatment conditions. Additionally, each of the constructs was compared with the Full-length MMP-10 Promoter Construct of their respective condition. The results are expressed as mean ± SE; n = 3 independent experiments. For each condition, statistically significant differences were obtained comparing each construct with the respective empty vector. Apart from Full-length MMP-10 Promoter Construct on the EGF condition, no statistically significant differences were obtained when comparing each construct with the respective no treatment condition. § $p < 0.05$ relative to the no treatment condition; * $p < 0.05$ relative to the *H. pylori* or EGF Full-length MMP10 Promoter Construct, respectively; # $p < 0.05$ relative to the no treatment Full-length MMP10 Promoter Construct. ETS-1 BS (yellow boxes) represents the ETS-1 transcription factor binding sites on MMP-10 promoter region; arrow stands for start codon (ATG) and LUC represents the luciferase reporter gene *luc2*.

Discussion and Conclusion

During cancer progression, the process of cellular invasion is of great importance. This process is extremely well regulated and involves degradation of basement membranes and stromal extracellular matrix. Increasing evidence have supported the role of MMPs in these processes, since its expression and activity are increased in almost every human cancers (80). In the context of gastric epithelial cells, the role played by *H. pylori* on the induction of MMPs expression has already been described, and is strongly associated with the promotion of gastric tissue damage and cell invasion (83, 85, 86, 92, 93, 130, 149). MMPs can be regulated, at the transcription level (primarily), in response to growth factors and cytokines, or by other environmental factors (77, 80). However, concerning *H. pylori* infection, the exact mechanisms whereby *H. pylori* induces MMPs expression are not fully explored.

Several ETS transcription factor family members, namely ETS-1(116, 124, 125, 150, 151), have been claimed to be involved on the modulation of MMPs' gene expression (107) but, so far, in the context of *H. pylori* infection, the relationship between ETS factors and the modulation of MMPs has never been explored.

The ETS-1 transcription factor gene and the MMP-10 gene, were found to be up-regulated by *H. pylori* in a cDNA microarray performed by our group. Concerning MMP-10, our Group has previously observed a correlation between *H. pylori* infection and MMP-10 expression (146). However, the exact mechanism underlying MMP-10 activation upon infection is still largely unknown.

In this study, we report that infection of gastric epithelial cells with *H. pylori* strains 26695 and 60190, stimulates the expression of ETS-1 transcription factor and MMP-10 throughout time. As expected, the ETS-1 mRNA and protein levels were highly expressed in early infection periods (4 hours), but a decreased in expression with increasing time of infection was also registered. Interestingly, significantly stimulation of secreted MMP-10 protein was observed after 4 hours, following the same expression pattern of ETS-1. Given the fact that MMP secretion and activity are usually observed a few hours after cellular stimulation (72), one would expect that MMP-10 secretion would have been more pronounced in later time periods, giving time to early response genes, such as ETS-1, to bind to MMP-10 promoter and activate its response. Since the earliest time point established was 4 hours of infection and the peak of secreted MMP-10 was reached after this infection period, this may be an indicator that ETS-1 might have a more pronounced expression in shorter infection periods (less than 4 hours). Further studies should be performed

with shorter infection periods, in order to evaluate the effect that it might have on ETS-1 and MMP-10 expression.

Several studies in different tumour models have shown that ETS-1 is able to trigger the activation of several MMPs, namely MMP -1,-2, -9 and -13 (124, 125, 127, 138, 151, 152). Concerning MMP-10, one of the lesser studied MMPs, in human umbilical vein endothelial cells (HUVECs) the transcription of MMP-10 is modulated by ETS-1 upon VEGF stimulation (142).

In our study, and for the first time, the role of ETS-1 transcription factor on the modulation of *H. pylori*-mediated MMP-10 expression was explored.

We demonstrated that the abrogation of *H. pylori*-induced ETS-1 expression by a siRNA led to a significant decrease in MMP-10 mRNA levels and protein secretion. The results also showed that, as expected, this decrease was more pronounced after 4 hours of infection. After 24 hours of infection, although ETS-1 expression was completely abrogated, MMP-10 was still being secreted. Due to the long time point used, one might expect that in addition to ETS-1 other unknown factors may contribute to MMP-10 up-regulation. One may hypothesize that in early infection periods ETS-1 alone is able to modulate the MMP-10 expression. However, since ETS-1 expression tends to decrease with increasing time of infection, in later infection periods it might cooperate with other factors to promote MMP-10 expression. Functional cooperation among ETS family members such as that between ETS-1 (153) and AP-1 (154) has been considered critical for the control of the expression of MMPs (108, 141, 155). Moreover, since AP-1 binding sites were found to be present on MMP-10 promoter (near ETS binding sites) (99) and because *H. pylori* infection also leads to an up-regulation of AP-1 (147, 156), one might suspect that AP-1—ETS-1 cooperation might be responsible for the modulation of MMP-10 at later time periods. Whether this interaction is important for *H. pylori*-induced MMP-10 expression will be an interesting topic to address in future studies. These could include protein-protein interaction studies such as, co-immunoprecipitation assays or pull-down assays, in order to address if the two transcription factors interact with each other upon *H. pylori* infection. Furthermore, studies could also include protein-DNA interaction assays, such as EMSA (electrophoretic mobility shift assay) or CZEMSA (capillary zone electrophoretic mobility shift assay) to assess whether this interaction complex is responsible for *H.pylori*-mediated MMP-10 activation.

The role of EGFR on the modulation of *H. pylori*-mediated ETS-1 expression was also evaluated in this study. Previous results from our Group have shown that EGFR

was involved in *H. pylori*-mediated MMP-10 expression (146). Added to that, other studies showed that ETS family members, namely ETS-1(127), can mediate MMPs up-regulation in an EGFR-dependent manner. (147, 148). We demonstrated, for the first time, that the abrogation of EGFR by a pharmacological inhibitor (AG178) led to a significantly decrease on stimulated ETS-1 mRNA and protein expression upon *H. pylori* infection. Since a relationship between ETS-1 and MMP-10 expression in the context of *H. pylori* infection, and MMP-10 mRNA and protein secretion were significantly decreased upon EGFR inhibition, it may be concluded that the increase on *H. pylori*-induced MMP-10 expression is mediated by EGFR-induced ETS-1.

It was previously reported that *H. pylori*-induced EGFR phosporylation can lead to the activation of the RAS signalling cascade, which results in ERK1/2 phosporylation (145, 146). ETS-1 contains a RAS-responsive phosporylation site near the Pointed domain (at threonine-28) that once phosporylated by ERKs, increases the transcriptional activity of ETS-1 (110, 116, 117, 157). Since our Group has shown that *H. pylori*-induced MMP-10 occurs *via* ERK and JNK pathways, it will be interesting in future studies to address the role of ERKs, and other downstream EGFR targets in *H. pylori*-induced ETS-1 activation. These studies could be performed by first inhibiting EGFR with a pharmacological inhibitor and evaluating the effect on ERK and JNK; studies could also involve the inhibition of ERK and JNK with siRNAs or pharmacological inhibitors, and the effect of this inhibition on *H. pylori*-induced ETS-1 expression.

Finally, in order to establish a stronger association between the involvement of ETS-1 transcription factor in *H. pylori*-mediated MMP-10 expression, and thus validate the main aim of this study, luciferase gene reporter assays were performed. *In silico* analysis predicted two potencial ETS-1 transcription factor binding sites in the pre-defined MMP-10 promoter region. To address the relevance of these two ETS-1 transcription factor binding sites on MMP-10 regulation, three MMP-10 promoter deletion constructs were generated from the full-length MMP-10 promoter region. As expected, in all tested conditions, the reduction in length of the MMP-10 promoter led to a decrease on the relative luciferase activity.

Concerning the relative luciferase activity under control of MMP-10 promoter upon *H. pylori* infection, contrarily to what was expected, it remained very close to the one obtained in uninfected cells. Given the fact that both ETS-1 and MMP-10 genes showed high expression in short infection periods, an increase in the relative

luciferase activity in this condition was expected. New experiments with shorter or longer times of infection or with different multiplicities of infection should be performed in order to better clarify the results obtained.

Concerning the relative luciferase activity under control of MMP-10 promoter upon EGF stimulation, in each construct the EGF stimulation led to an increase in the relative luciferase activity, relatively to the non-treated condition. In this condition, as stated before, the reduction in length of the MMP-10 promoter led to a decrease on the relative luciferase activity. However, no differences were registered on the relative luciferase activity between Constructs 1 and 2. This result suggests that the first ETS-1 transcription factor binding site might not be biologically relevant for the induction of MMP-10 expression, since the lack of this site (Construct 1 to Construct 2) did not have an effect on the relative luciferase activity. Further studies to test the biological relevance of this site, should include a new construct that comprises the first ETS-1 binding site but lacks the second site.

On the other hand, Construct 3, which lacked the two ETS-1 binding sites, led to a significant decrease on the relative luciferase activity in all tested conditions, suggesting that the two predicted ETS-1 transcription factor binding sites might be required for the modulation of MMP-10 expression by ETS-1.

Although a decrease on the relative luciferase activity when the two ETS-1 binding sites were not present was observed, which might indicate the involvement of ETS-1 on MMP-10 regulation, one cannot be sure whether this effect was exclusively mediated by this transcription factor. Similarly, the increased relative luciferase activity in cells treated with EGF might have also been mediated by other transcription factors, such AP-1(147), whose expression is also mediated by EGFR (147), or even by interaction of those transcription factors with ETS-1 (154, 158).

In order to complement the results obtained in the framework of this thesis, it would be interesting to test the importance of these two predicted ETS-1 binding sites by other techniques. For example, different site-directed mutagenesis constructs could be generated in order to ensure that the obtained results are more likely to be due to the effet of ETS-1. Futhermore, protein-DNA binding studies, such as ChiP (Chromatin Immunoprecipitation) or EMSA assays could also be performed, in order to better elucidate the levels of interaction between ETS-1 and the MMP-10 promoter leading to increase MMP-10 expression.

Taken together, in shorter infection periods, the expression of *H. pylori*-induced MMP-10 is mediated by ETS-1 transcription factor, and involves the activation of EGFR signalling pathways.

On the basis of the results presented in this thesis, and taking into account previous results from our Group and from the literature, a model is proposed for *H. pylori*-mediated MMP-10 expression, in shorter infection periods (Figure 10). According to the model, through the induction of EGFR phosphorylation, *H. pylori* is able to mediate the activation of RAS signalling cascade, leading to ERK phosphorylation (110, 116, 145, 146). Activated ERKs are then able to bind to the RAS responsive element near the ETS-1 Pointed domain, which increases ETS-1 transcription factor activity (117). Once up-regulated, ETS-1 binds to ETS binding sites on MMP-10 promoter, leading to an increase in MMP-10 mRNA levels and consequently MMP-10 protein secretion.

In longer infection periods, since ETS-1 expression tends to decrease, one can speculate that ETS-1 might cooperate with other transcription factors, such as AP-1, whose expression is also mediated through EGFR signalling pathway, leading to the activation of *H. pylori*-induced MMP-10 expression. The study of these interactions could be an interesting aim for future studies.

Tumour invasion develops through a series of steps in which MMPs (97, 103, 159), but also ETS transcription factors (160, 161) play important roles. Since ETS-1 has been claimed to be strongly associated with cell invasiveness and migration (162), and unpublished results from our Group have shown that MMP-10 plays a role in *H. pylori*-mediated cell invasion, it would also be interesting, in future studies, to abrogate ETS-1 expression and perform invasion assays, in order to see how it would affect the invasion phenotype.

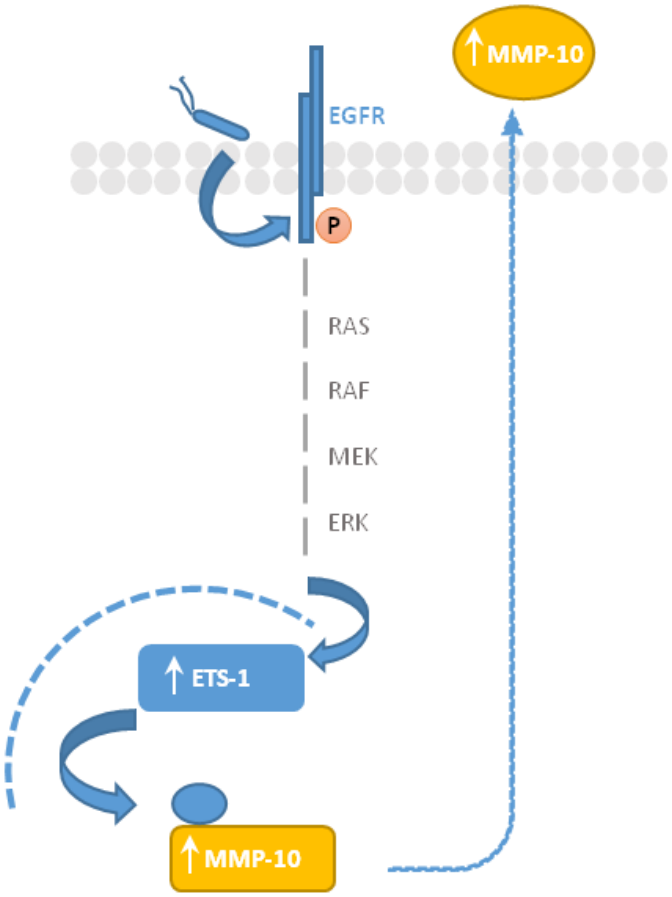

Figure 10. EGFR-induced ETS-1 transcription factor up-regulates MMP-10 expression during *H. pylori* infection. *H. pylori*-mediated EGFR phosphrylation leads to the activation of RAS signalling cascade, which results in ERK1/2 phosporylation. ETS-1 phosporylation by ERKs lead to an increase on its transcriptional activity. Once up-regulated, ETS-1 is able to bind to ETS binding sites on the MMP-10 promoter region, leading to an increase in MMP-10 expression and secreted protein. Grey colour represents previous results obtained by our Group and in the literature (110, 117, 145, 146).Yellow and blue colours represent the obtained results in this thesis; the boxes (yellow and blue) represent mRNA levels and the circles (yellow and blue) represent ETS-1 and MMP-10 proteins, respectively.

REFERENCE LIST

1. Marshall BJ, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet. 1(8390); 1984; p:1311-5.

2. Atherton JC, Blaser MJ. Coadaptation of Helicobacter pylori and humans: ancient history, modern implications. J Clin Invest. 119(9); 2009; p:2475-87.

3. Linz B, Balloux F, Moodley Y, Manica A, Liu H, Roumagnac P, et al. An African origin for the intimate association between humans and Helicobacter pylori. Nature. 445(7130); 2007; p:915-8.

4. Azevedo NF, Guimaraes, N., Figueiredo, C., Keevil, C.M., Vieira, M.J. A New Model for the Transmission of Helicobacter pylori: Role of Environmental Reservoirs as Gene Pools to Increase Strain Diversity. Crit Rev Microbiol. 33; 2007; p:157-69.

5. Salama NR, Hartung ML, Müller A. Life in the human stomach: persistence strategies of the bacterial pathogen Helicobacter pylori. Nature Rev Microbiol. 11(6); 2013; p:385-99.

6. Israel DA, Salama N, Krishna U, Rieger UM, Atherton JC, Falkow S, et al. Helicobacter pylori genetic diversity within the gastric niche of a single human host. Proc Natl Acad Sci U S A. 98(25); 2001; p:14625-30.

7. Patra R, Chattopadhyay S, De R, Ghosh P, Ganguly M, Chowdhury A, et al. Multiple infection and microdiversity among Helicobacter pylori isolates in a single host in India. PLoS One. 7(8); 2012; p:e43370.

8. Salama NR, Guillemin K, McDaniel TK, Sherlock G, Tompkins L, Falkow S. A whole-genome microarray reveals genetic diversity among Helicobacter pylori strains. Proc Natl Acad Sci U S A. 97(26); 2000; p:14668-73.

9. Dunne C, Dolan B, Clyne M. Factors that mediate colonization of the human stomach by Helicobacter pylori. World J Gastroenterol. 20(19); 2014; p:5610-24.

10. Kaji T, Ishihara S, Ashizawa N, Hamamoto N, Endo H, Fukuda R, et al. Adherence of Helicobacter pylori to gastric epithelial cells and mucosal inflammation. J Lab Clin Med. 139(4); 2002; p:244-50.

11. Tan S, Tompkins LS, Amieva MR. Helicobacter pylori usurps cell polarity to turn the cell surface into a replicative niche. PLoS Pathog. 5(5); 2009; p:e1000407.

12. Necchi V, Candusso ME, Tava F, Luinetti O, Ventura U, Fiocca R, et al. Intracellular, intercellular, and stromal invasion of gastric mucosa, preneoplastic lesions, and cancer by Helicobacter pylori. Gastroenterology. 132(3); 2007; p:1009-23.

13. Warren JR. Gastric pathology associated with Helicobacter pylori. Gastroenterol Clin North Am. 29(3); 2000; p:705-51.

14. Peek RM, Jr., Crabtree JE. Helicobacter infection and gastric neoplasia. J Pathol. 208(2); 2006; p:233-48.

15. Figueiredo C, Machado JC, Pharoah P, Seruca R, Sousa S, Carvalho R, et al. Helicobacter pylori and interleukin 1 genotyping: an opportunity to identify high-risk individuals for gastric carcinoma. J Natl Cancer Inst. 94(22); 2002; p:1680-7.

16. Peek RM, Jr., Blaser MJ. Helicobacter pylori and gastrointestinal tract adenocarcinomas. Nat Rev Cancer. 2(1); 2002; p:28-37.

17. Harris PR, Mobley HL, Perez-Perez GI, Blaser MJ, Smith PD. Helicobacter pylori urease is a potent stimulus of mononuclear phagocyte activation and inflammatory cytokine production. Gastroenterology. 111(2); 1996; p:419-25.

18. Montecucco C, Rappuoli R. Living dangerously: how Helicobacter pylori survives in the human stomach. Nat Rev Mol Cell Biol. 2(6); 2001; p:457-66.

19. Wroblewski LE, Peek RM. Helicobacter pylori in gastric carcinogenesis: mechanisms. Gastroenterol Clin North Am. 42(2); 2013; p:285-98.

20. Celli JP, Turner BS, Afdhal NH, Keates S, Ghiran I, Kelly CP, et al. Helicobacter pylori moves through mucus by reducing mucin viscoelasticity. Proc Natl Acad Sci U S A. 106(34); 2009; p:14321-6.

21. Atherton JC, Cao P, Peek RM, Jr., Tummuru MK, Blaser MJ, Cover TL. Mosaicism in vacuolating cytotoxin alleles of Helicobacter pylori. Association of specific vacA types with cytotoxin production and peptic ulceration. J Biol Chem. 270(30); 1995; p:17771-7.

22. Leunk RD, Johnson PT, David BC, Kraft WG, Morgan DR. Cytotoxic activity in broth-culture filtrates of Campylobacter pylori. J Med Microbiol. 26(2); 1988; p:93-9.

23. Cover TL, Blanke SR. Helicobacter pylori VacA, a paradigm for toxin multifunctionality. Nature Rev Microbiol. 3(4); 2005; p:320-32.

24. Gebert B, Fischer W, Weiss E, Hoffmann R, Haas R. Helicobacter pylori vacuolating cytotoxin inhibits T lymphocyte activation. Science. 301(5636); 2003; p:1099-102.

25. Papini E, Satin B, Norais N, de Bernard M, Telford JL, Rappuoli R, et al. Selective increase of the permeability of polarized epithelial cell monolayers by Helicobacter pylori vacuolating toxin. J Clin Invest. 102(4); 1998; p:813-20.

26. Polk DB, Peek RM. Helicobacter pylori: gastric cancer and beyond. Nat Rev Cancer. 10(6); 2010; p:403-14.

27. Torres VJ, VanCompernolle SE, Sundrud MS, Unutmaz D, Cover TL. Helicobacter pylori vacuolating cytotoxin inhibits activation-induced proliferation of human T and B lymphocyte subsets. J Immunol. 179(8); 2007; p:5433-40.

28. Rhead JL, Letley DP, Mohammadi M, Hussein N, Mohagheghi MA, Eshagh Hosseini M, et al. A new Helicobacter pylori vacuolating cytotoxin determinant, the intermediate region, is associated with gastric cancer. Gastroenterology. 133(3); 2007; p:926-36.

29. Ferreira RM, Machado JC, Letley D, Atherton JC, Pardo ML, Gonzalez CA, et al. A novel method for genotyping the Helicobacter pylori vacA intermediate region directly in gastric biopsy specimens. J Clin Microbiol. 50(12); 2012; p:3983-9.

30. Censini S, Lange C, Xiang Z, Crabtree JE, Ghiara P, Borodovsky M, et al. cag, a pathogenicity island of Helicobacter pylori, encodes type I-specific and disease-associated virulence factors. Proc Natl Acad Sci U S A. 93(25); 1996; p:14648-53.

31. Noto JM, Peek RM. The Helicobacter pylori cag Pathogenicity Island. Methods Mol Biol. 921; 2012; p:41-50.

32. Blaser MJ, Perez-Perez GI, Kleanthous H, Cover TL, Peek RM, Chyou PH, et al. Infection with Helicobacter pylori strains possessing cagA is associated with an increased risk of developing adenocarcinoma of the stomach. Cancer Res. 55(10); 1995; p:2111-5.

33. Kuipers EJ, Perez-Perez GI, Meuwissen SG, Blaser MJ. Helicobacter pylori and atrophic gastritis: importance of the cagA status. J Natl Cancer Inst. 87(23); 1995; p:1777-80.

34. Tegtmeyer N, Wessler S, Backert S. Role of the cag-pathogenicity island encoded type IV secretion system in Helicobacter pylori pathogenesis. The FEBS journal. 278(8); 2011; p:1190-202.

35. Viala J, Chaput C, Boneca IG, Cardona A, Girardin SE, Moran AP, et al. Nod1 responds to peptidoglycan delivered by the Helicobacter pylori cag pathogenicity island. Nat Immunol. 5(11); 2004; p:1166-74.

36. Allison CC, Kufer TA, Kremmer E, Kaparakis M, Ferrero RL. Helicobacter pylori induces MAPK phosphorylation and AP-1 activation via a NOD1-dependent mechanism. J Immunol. 183(12); 2009; p:8099-109.

37. Watanabe T, Asano N, Fichtner-Feigl S, Gorelick PL, Tsuji Y, Matsumoto Y, et al. NOD1 contributes to mouse host defense against Helicobacter pylori via induction of type I IFN and activation of the ISGF3 signaling pathway. J Clin Invest. 120(5); 2010; p:1645-62.

38. Nagy TA, Frey MR, Yan F, Israel DA, Polk DB, Peek RM, Jr. Helicobacter pylori regulates cellular migration and apoptosis by activation of phosphatidylinositol 3-kinase signaling. J Infect Dis. 199(5); 2009; p:641-51.

39. Backert S, Tegtmeyer N, Selbach M. The versatility of Helicobacter pylori CagA effector protein functions: The master key hypothesis. Helicobacter. 15(3); 2010; p:163-76.

40. Hatakeyama M. Oncogenic mechanisms of the Helicobacter pylori CagA protein. Nat Rev Cancer. 4(9); 2004; p:688-94.

41. Mueller D, Tegtmeyer N, Brandt S, Yamaoka Y, De Poire E, Sgouras D, et al. c-Src and c-Abl kinases control hierarchic phosphorylation and function of the CagA effector protein in Western and East Asian Helicobacter pylori strains. J Clin Invest. 122(4); 2012; p:1553-66.

42. Segal ED, Cha J, Lo J, Falkow S, Tompkins LS. Altered states: involvement of phosphorylated CagA in the induction of host cellular growth changes by Helicobacter pylori. Proc Natl Acad Sci U S A. 96(25); 1999; p:14559-64.

43. Tegtmeyer N, Backert S. Role of Abl and Src family kinases in actin-cytoskeletal rearrangements induced by the Helicobacter pylori CagA protein. Eur J Cell Biol. 90(11); 2011; p:880-90.

44. Higashi H, Tsutsumi R, Muto S, Sugiyama T, Azuma T, Asaka M, et al. SHP-2 tyrosine phosphatase as an intracellular target of Helicobacter pylori CagA protein. Science. 295(5555); 2002; p:683-6.

45. Mimuro H, Suzuki T, Tanaka J, Asahi M, Haas R, Sasakawa C. Grb2 is a key mediator of helicobacter pylori CagA protein activities. Mol Cell. 10(4); 2002; p:745-55.

46. Selbach M, Paul FE, Brandt S, Guye P, Daumke O, Backert S, et al. Host cell interactome of tyrosine-phosphorylated bacterial proteins. Cell Host Microbe. 5(4); 2009; p:397-403.

47. Saadat I, Higashi H, Obuse C, Umeda M, Murata-Kamiya N, Saito Y, et al. Helicobacter pylori CagA targets PAR1/MARK kinase to disrupt epithelial cell polarity. Nature. 447(7142); 2007; p:330-3.

48. Murata-Kamiya N, Kurashima Y, Teishikata Y, Yamahashi Y, Saito Y, Higashi H, et al. Helicobacter pylori CagA interacts with E-cadherin and deregulates the beta-catenin signal that promotes intestinal transdifferentiation in gastric epithelial cells. Oncogene. 26(32); 2007; p:4617-26.

49. Churin Y, Al-Ghoul L, Kepp O, Meyer TF, Birchmeier W, Naumann M. Helicobacter pylori CagA protein targets the c-Met receptor and enhances the motogenic response. J Cell Biol. 161(2); 2003; p:249-55.

50. Amieva MR, Vogelmann R, Covacci A, Tompkins LS, Nelson WJ, Falkow S. Disruption of the epithelial apical-junctional complex by Helicobacter pylori CagA. Science. 300(5624); 2003; p:1430-4.

51. Oldani A, Cormont M, Hofman V, Chiozzi V, Oregioni O, Canonici A, et al. Helicobacter pylori counteracts the apoptotic action of its VacA toxin by injecting the CagA protein into gastric epithelial cells. PLoS Pathog. 5(10); 2009; p:e1000603.

52. Yokoyama K, Higashi H, Ishikawa S, Fujii Y, Kondo S, Kato H, et al. Functional antagonism between Helicobacter pylori CagA and vacuolating toxin VacA in control of the NFAT signaling pathway in gastric epithelial cells. Proc Natl Acad Sci USA. 102(27); 2005; p:9661-6.

53. Peek RM, Jr., Fiske C, Wilson KT. Role of innate immunity in Helicobacter pylori-induced gastric malignancy. Physiol Rev. 90(3); 2010; p:831-58.

54. Portal-Celhay C, Perez-Perez GI. Immune responses to Helicobacter pylori colonization: mechanisms and clinical outcomes. Clin Sci (Lond). 110(3); 2006; p:305-14.

55. Wroblewski LE, Peek RM, Jr., Wilson KT. Helicobacter pylori and gastric cancer: factors that modulate disease risk. Clin Microbiol Rev. 23(4); 2010; p:713-39.

56. Ferlay J SI, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray, F. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide Lyon, France: International Agency for Research on Cancer; 2013 [Accessed 10/03/2015]. Available from: http://globocan.iarc.fr.

57. IARC. Schistosomes, liver flukes and Helicobacter pylori. IARC monographs on the evaluation of carcinogenic risks to humans. 7-14 June 1994 ed. Lyon: World Health Organization International Agency for Research on Cancer; 1994.

58. Correa P. Human gastric carcinogenesis: a multistep and multifactorial process--First American Cancer Society Award Lecture on Cancer Epidemiology and Prevention. Cancer Res. 52(24); 1992; p:6735-40.

59. Kusters JG, van Vliet AH, Kuipers EJ. Pathogenesis of Helicobacter pylori infection. Clin Microbiol Rev. 19(3); 2006; p:449-90.

60. Watanabe T, Tada M, Nagai H, Sasaki S, Nakao M. Helicobacter pylori infection induces gastric cancer in mongolian gerbils. Gastroenterology. 115(3); 1998; p:642-8.

61. Mera R, Fontham ET, Bravo LE, Bravo JC, Piazuelo MB, Camargo MC, et al. Long term follow up of patients treated for Helicobacter pylori infection. Gut. 54(11); 2005; p:1536-40.

62. Wong BC, Lam SK, Wong WM, Chen JS, Zheng TT, Feng RE, et al. Helicobacter pylori eradication to prevent gastric cancer in a high-risk region of China: a randomized controlled trial. JAMA. 291(2); 2004; p:187-94.

63. El-Omar EM, Rabkin CS, Gammon MD, Vaughan TL, Risch HA, Schoenberg JB, et al. Increased risk of noncardia gastric cancer associated with proinflammatory cytokine gene polymorphisms. Gastroenterology. 124(5); 2003; p:1193-201.

64. Macarthur M, Hold GL, El-Omar EM. Inflammation and Cancer II. Role of chronic inflammation and cytokine gene polymorphisms in the pathogenesis of gastrointestinal malignancy. Am J Physiol Gastrointest Liver Physiol. 286(4); 2004; p:G515-20.

65. Machado JC, Figueiredo C, Canedo P, Pharoah P, Carvalho R, Nabais S, et al. A proinflammatory genetic profile increases the risk for chronic atrophic gastritis and gastric carcinoma. Gastroenterology. 125(2); 2003; p:364-71.

66. Chao A, Thun MJ, Henley SJ, Jacobs EJ, McCullough ML, Calle EE. Cigarette smoking, use of other tobacco products and stomach cancer mortality in US adults: The Cancer Prevention Study II. Int J Cancer. 101(4); 2002; p:380-9.

67. Joossens JV, Hill MJ, Elliott P, Stamler R, Lesaffre E, Dyer A, et al. Dietary salt, nitrate and stomach cancer mortality in 24 countries. European Cancer Prevention (ECP) and the INTERSALT Cooperative Research Group. Int J Epidemiol. 25(3); 1996; p:494-504.

68. Gonzalez CA, Jakszyn P, Pera G, Agudo A, Bingham S, Palli D, et al. Meat intake and risk of stomach and esophageal adenocarcinoma within the European Prospective Investigation Into Cancer and Nutrition (EPIC). J Natl Cancer Inst. 98(5); 2006; p:345-54.

69. Kim JJ, Tao H, Carloni E, Leung WK, Graham DY, Sepulveda AR. Helicobacter pylori impairs DNA mismatch repair in gastric epithelial cells. Gastroenterology. 123(2); 2002; p:542-53.

70. Machado AM, Desler C, Boggild S, Strickertsson JA, Friis-Hansen L, Figueiredo C, et al. Helicobacter pylori infection affects mitochondrial function and DNA repair, thus, mediating genetic instability in gastric cells. Mech Ageing Dev. 134(10); 2013; p:460-6.

71. Park DI, Park SH, Kim SH, Kim JW, Cho YK, Kim HJ, et al. Effect of Helicobacter pylori infection on the expression of DNA mismatch repair protein. Helicobacter. 10(3); 2005; p:179-84.

72. Piperi C, Papavassiliou AG. Molecular mechanisms regulating matrix metalloproteinases. Curr Top Med Chem. 12(10); 2012; p:1095-112.

73. Cauwe B, Van den Steen PE, Opdenakker G. The biochemical, biological, and pathological kaleidoscope of cell surface substrates processed by matrix metalloproteinases. Crit Rev Biochem Mol Biol. 42(3); 2007; p:113-85.

74. Chakraborti S, Mandal M, Das S, Mandal A, Chakraborti T. Regulation of matrix metalloproteinases: an overview. Mol Cell Biochem. 253(1-2); 2003; p:269-85.

75. Parks WC, Wilson CL, Lopez-Boado YS. Matrix metalloproteinases as modulators of inflammation and innate immunity. Nat Rev Immunol. 4(8); 2004; p:617-29.

76. Liotta LA, Tryggvason K, Garbisa S, Hart I, Foltz CM, Shafie S. Metastatic potential correlates with enzymatic degradation of basement membrane collagen. Nature. 284(5751); 1980; p:67-8.

77. Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer. 2(3); 2002; p:161-74.

78. Sternlicht MD, Werb Z. How matrix metalloproteinases regulate cell behavior. Annu Rev Cell Dev Biol. 17; 2001; p:463-516.

79. Kessenbrock K, Plaks V, Werb Z. Matrix metalloproteinases: regulators of the tumor microenvironment. Cell. 141(1); 2010; p:52-67.

80. Westermarck J, Kähäri VM. Regulation of matrix metalloproteinase expression in tumor invasion. FASEB J. 13(8); 1999; p:781-92.

81. Zhang M, Zhu GY, Gao HY, Zhao SP, Xue Y. Expression of tissue levels of matrix metalloproteinases and tissue inhibitors of metalloproteinases in gastric adenocarcinoma. J Surg Oncol. 103(3); 2011; p:243-7.

82. Sampieri CL, de la Pena S, Ochoa-Lara M, Zenteno-Cuevas R, Leon-Cordoba K. Expression of matrix metalloproteinases 2 and 9 in human gastric cancer and superficial gastritis. World J Gastroenterol. 16(12); 2010; p:1500-5.

83. Crawford HC, Krishna US, Israel DA, Matrisian LM, Washington MK, Peek RM, Jr. Helicobacter pylori strain-selective induction of matrix metalloproteinase-7 in vitro and within gastric mucosa. Gastroenterology. 125(4); 2003; p:1125-36.

84. Gooz M, Gooz P, Smolka AJ. Epithelial and bacterial metalloproteinases and their inhibitors in H. pylori infection of human gastric cells. Am J Physiol Gastrointest Liver Physiol. 281(3); 2001; p:G823-32.

85. Pillinger MH, Marjanovic N, Kim SY, Lee YC, Scher JU, Roper J, et al. Helicobacter pylori stimulates gastric epithelial cell MMP-1 secretion via CagA-dependent and -independent ERK activation. J Biol Chem. 282(26); 2007; p:18722-31.

86. Krueger S, Hundertmark T, Kalinski T, Peitz U, Wex T, Malfertheiner P, et al. Helicobacter pylori encoding the pathogenicity island activates matrix metalloproteinase 1 in gastric epithelial cells via JNK and ERK. J Biol Chem. 281(5); 2006; p:2868-75.

87. Wu JY, Lu H, Sun Y, Graham DY, Cheung HS, Yamaoka Y. Balance between polyoma enhancing activator 3 and activator protein 1 regulates Helicobacter pylori-stimulated matrix metalloproteinase 1 expression. Cancer Res. 66(10); 2006; p:5111-20.

88. Sokolova O, Vieth M, Naumann M. Protein kinase C isozymes regulate matrix metalloproteinase-1 expression and cell invasion in Helicobacter pylori infection. Gut. 62(3); 2013; p:358-67.

89. Bebb JR, Letley DP, Thomas RJ, Aviles F, Collins HM, Watson SA, et al. Helicobacter pylori upregulates matrilysin (MMP-7) in epithelial cells in vivo and in vitro in a Cag dependent manner. Gut. 52(10); 2003; p:1408-13.

90. Ogden SR, Noto JM, Allen SS, Patel DA, Romero-Gallo J, Washington MK, et al. Matrix metalloproteinase-7 and premalignant host responses in Helicobacter pylori-infected mice. Cancer Res. 70(1); 2010; p:30-5.

91. Ogden SR, Wroblewski LE, Weydig C, Romero-Gallo J, O'Brien DP, Israel DA, et al. p120 and Kaiso regulate Helicobacter pylori-induced expression of matrix metalloproteinase-7. Mol Biol Cell. 19(10); 2008; p:4110-21.

92. Wroblewski LE, Noble PJ, Pagliocca A, Pritchard DM, Hart CA, Campbell F, et al. Stimulation of MMP-7 (matrilysin) by Helicobacter pylori in human gastric epithelial cells: role in epithelial cell migration. J Cell Sci. 116(Pt 14); 2003; p:3017-26.

93. Bergin PJ, Anders E, Sicheng W, Erik J, Jennie A, Hans L, et al. Increased production of matrix metalloproteinases in Helicobacter pylori-associated human gastritis. Helicobacter. 9(3); 2004; p:201-10.

94. Caruso R, Fina D, Peluso I, Fantini MC, Tosti C, Del Vecchio Blanco G, et al. IL-21 is highly produced in Helicobacter pylori-infected gastric mucosa and promotes gelatinases synthesis. J Immunol. 178(9); 2007; p:5957-65.

95. Kundu P, Mukhopadhyay AK, Patra R, Banerjee A, Berg DE, Swarnakar S. Cag pathogenicity island-independent up-regulation of matrix metalloproteinases-9 and -2 secretion and expression in mice by Helicobacter pylori infection. J Biol Chem. 281(45); 2006; p:34651-62.

96. Mori N, Sato H, Hayashibara T, Senba M, Geleziunas R, Wada A, et al. Helicobacter pylori induces matrix metalloproteinase-9 through activation of nuclear factor kappaB. Gastroenterology. 124(4); 2003; p:983-92.

97. Oliveira MJ, Costa AC, Costa AM, Henriques L, Suriano G, Atherton JC, et al. Helicobacter pylori induces gastric epithelial cell invasion in a c-Met and type IV secretion system-dependent manner. J Biol Chem. 281(46); 2006; p:34888-96.

98. Nam YH, Ryu E, Lee D, Shim HJ, Lee YC, Lee ST. CagA phosphorylation-dependent MMP-9 expression in gastric epithelial cells. Helicobacter. 16(4); 2011; p:276-83.

99. Yan C, Boyd DD. Regulation of matrix metalloproteinase gene expression. J Cell Physiol. 211(1); 2007; p:19-26.

100. Clark IM, Swingler TE, Sampieri CL, Edwards DR. The regulation of matrix metalloproteinases and their inhibitors. Int J Biochem Cell Biol. 40(6-7); 2008; p:1362-78.

101. Wang X, Bi Z, Chu W, Wan Y. IL-1 receptor antagonist attenuates MAP kinase/AP-1 activation and MMP1 expression in UVA-irradiated human fibroblasts induced by culture medium from UVB-irradiated human skin keratinocytes. Int J Mol Med. 16(6); 2005; p:1117-24.

102. Benbow U, Brinckerhoff CE. The AP-1 site and MMP gene regulation: what is all the fuss about? Matrix Biol. 15(8-9); 1997; p:519-26.

103. Westermarck J, Kahari VM. Regulation of matrix metalloproteinase expression in tumor invasion. FASEB J. 13(8); 1999; p:781-92.

104. Leprince D, Gegonne A, Coll J, de Taisne C, Schneeberger A, Lagrou C, et al. A putative second cell-derived oncogene of the avian leukaemia retrovirus E26. Nature. 306(5941); 1983; p:395-7.

105. Oikawa T, Yamada T. Molecular biology of the Ets family of transcription factors. Gene. 303; 2003; p:11-34.

106. Seth A, Watson DK. ETS transcription factors and their emerging roles in human cancer. Eur J Cancer. 41(16); 2005; p:2462-78.

107. Findlay VJ, LaRue AC, Turner DP, Watson PM, Watson DK. Understanding the role of ETS-mediated gene regulation in complex biological processes. Adv Cancer Res. 119; 2013; p:1-61.

108. Li R, Pei H, Watson DK. Regulation of Ets function by protein - protein interactions. Oncogene. 19(55); 2000; p:6514-23.

109. Sementchenko VI, Watson DK. Ets target genes: past, present and future. Oncogene. 19(55); 2000; p:6533-48.

110. Charlot C, Dubois-Pot H, Serchov T, Tourrette Y, Wasylyk B. A review of post-translational modifications and subcellular localization of Ets transcription factors: possible connection with cancer and involvement in the hypoxic response. Methods Mol Biol. 647; 2010; p:3-30.

111. Hsu T, Trojanowska M, Watson DK. Ets proteins in biological control and cancer. J Cell Biochem. 91(5); 2004; p:896-903.

112. Morishita A, Gong J, Masaki T. Targeting receptor tyrosine kinases in gastric cancer. World J Gastroenterol. 20(16); 2014; p:4536-45.

113. Westermarck J, Seth A, Kahari VM. Differential regulation of interstitial collagenase (MMP-1) gene expression by ETS transcription factors. Oncogene. 14(22); 1997; p:2651-60.

114. Hahne JC, Okuducu AF, Sahin A, Fafeur V, Kiriakidis S, Wernert N. The transcription factor ETS-1: its role in tumour development and strategies for its inhibition. Mini Rev Med Chem. 8(11); 2008; p:1095-105.

115. Lincoln DW, Bove K. The transcription factor Ets-1 in breast cancer. Front Biosci. 10; 2005; p:506-11.

116. Trojanowska M. Ets factors and regulation of the extracellular matrix. Oncogene. 19(55); 2000; p:6464-71.

117. Dittmer J. The biology of the Ets1 proto-oncogene. Mol Cancer. 2; 2003; p:29.

118. Lelievre E, Lionneton F, Soncin F, Vandenbunder B. The Ets family contains transcriptional activators and repressors involved in angiogenesis. Int J Biochem Cell Biol. 33(4); 2001; p:391-407.

119. Calmels TP, Mattot V, Wernert N, Vandenbunder B, Stehelin D. Invasive tumors induce c-ets1 transcription factor expression in adjacent stroma. Biol Cell. 84(1-2); 1995; p:53-61.

120. Behrens P, Rothe M, Wellmann A, Krischler J, Wernert N. The Ets-1 transcription factor is up-regulated together with MMP 1 and MMP 9 in the stroma of pre-invasive breast cancer. J Pathol. 194(1); 2001; p:43-50.

121. Behrens P, Rothe M, Florin A, Wellmann A, Wernert N. Invasive properties of serous human epithelial ovarian tumors are related to Ets-1, MMP-1 and MMP-9 expression. Int J Mol Med. 8(2); 2001; p:149-54.

122. Takanami I, Takeuchi K, Karuke M. Expression of ETS-1 is correlated with urokinase-type plasminogen activator and poor prognosis in pulmonary adenocarcinoma. Tumour Biol. 22(4); 2001; p:205-10.

123. Nakada M, Yamashita J, Okada Y, Sato H. Ets-1 positively regulates expression of urokinase-type plasminogen activator (uPA) and invasiveness of astrocytic tumors. J Neuropathol Exp Neurol. 58(4); 1999; p:329-34.

124. Ghosh S, Basu M, Roy SS. ETS-1 protein regulates vascular endothelial growth factor-induced matrix metalloproteinase-9 and matrix metalloproteinase-13 expression in human ovarian carcinoma cell line SKOV-3. J Biol Chem. 287(18); 2012; p:15001-15.

125. Okuducu AF, Zils U, Michaelis SA, Michaelides S, von Deimling A. Ets-1 is up-regulated together with its target gene products matrix metalloproteinase-2 and matrix metalloproteinase-9 in atypical and anaplastic meningiomas. Histopathology. 48(7); 2006; p:836-45.

126. Rutter JL, Mitchell TI, Butticè G, Meyers J, Gusella JF, Ozelius LJ, et al. A single nucleotide polymorphism in the matrix metalloproteinase-1 promoter creates an Ets binding site and augments transcription. Cancer Res. 58(23); 1998; p:5321-5.

127. Park YH, Jung HH, Ahn JS, Im YH. Ets-1 upregulates HER2-induced MMP-1 expression in breast cancer cells. Biochem Biophys Res Commun. 377(2); 2008; p:389-94.

128. Hahne JC, Kummer S, Heukamp LC, Fuchs T, Gun M, Langer B, et al. Regulation of protein tyrosine kinases in tumour cells by the transcription factor Ets-1. Int J Oncol. 35(5); 2009; p:989-96.

129. Jiang H, Zhou Y, Liao Q, Ouyang H. infection promotes the invasion and metastasis of gastric cancer through increasing the expression of matrix metalloproteinase-1 and matrix metalloproteinase-10. Exp Ther Med. 8(3); 2014; p:769-74.

130. Rautelin HI, Oksanen AM, Veijola LI, Sipponen PI, Tervahartiala TI, Sorsa TA, et al. Enhanced systemic matrix metalloproteinase response in Helicobacter pylori gastritis. Ann Med. 41(3); 2009; p:208-15.

131. Yeh YC, Sheu BS, Cheng HC, Wang YL, Yang HB, Wu JJ. Elevated serum matrix metalloproteinase-3 and -7 in H. pylori-related gastric cancer can be biomarkers correlating with a poor survival. Dig Dis Sci. 55(6); 2010; p:1649-57.

132. Aung PP, Oue N, Mitani Y, Nakayama H, Yoshida K, Noguchi T, et al. Systematic search for gastric cancer-specific genes based on SAGE data: melanoma inhibitory activity and matrix metalloproteinase-10 are novel prognostic factors in patients with gastric cancer. Oncogene. 25(17); 2006; p:2546-57.

133. Seargent JM, Loadman PM, Martin SW, Naylor B, Bibby MC, Gill JH. Expression of matrix metalloproteinase-10 in human bladder transitional cell carcinoma. Urology. 65(4); 2005; p:815-20.

134. Mathew R, Khanna R, Kumar R, Mathur M, Shukla NK, Ralhan R. Stromelysin-2 overexpression in human esophageal squamous cell carcinoma: potential clinical implications. Cancer Detect Prev. 26(3); 2002; p:222-8.

135. Zhang G, Miyake M, Lawton A, Goodison S, Rosser CJ. Matrix metalloproteinase-10 promotes tumor progression through regulation of angiogenic and apoptotic pathways in cervical tumors. BMC Cancer. 14; 2014; p:310.

136. Liu H, Qin YR, Bi J, Guo A, Fu L, Guan XY. Overexpression of matrix metalloproteinase 10 is associated with poor survival in patients with early stage of esophageal squamous cell carcinoma. Dis Esophagus. 25(7); 2012; p:656-63.

137. Wallasch C, Crabtree JE, Bevec D, Robinson PA, Wagner H, Ullrich A. Helicobacter pylori-stimulated EGF receptor transactivation requires metalloprotease cleavage of HB-EGF. Biochem Biophys Res Commun. 295(3); 2002; p:695-701.

138. Watabe T, Yoshida K, Shindoh M, Kaya M, Fujikawa K, Sato H, et al. The Ets-1 and Ets-2 transcription factors activate the promoters for invasion-associated urokinase and collagenase genes in response to epidermal growth factor. Int J Cancer. 77(1); 1998; p:128-37.

139. Anand M, Van Meter TE, Fillmore HL. Epidermal growth factor induces matrix metalloproteinase-1 (MMP-1) expression and invasion in glioma cell lines via the MAPK pathway. J Neurooncol. 104(3); 2011; p:679-87.

140. O'Hagan RC, Hassell JA. The PEA3 Ets transcription factor is a downstream target of the HER2/Neu receptor tyrosine kinase. Oncogene. 16(3); 1998; p:301-10.

141. Crawford HC, Matrisian LM. Mechanisms controlling the transcription of matrix metalloproteinase genes in normal and neoplastic cells. Enzyme Protein. 49(1-3); 1996; p:20-37.

142. Heo SH, Choi YJ, Ryoo HM, Cho JY. Expression profiling of ETS and MMP factors in VEGF-activated endothelial cells: role of MMP-10 in VEGF-induced angiogenesis. J Cell Physiol. 224(3); 2010; p:734-42.

143. Wilkins-Port CE, Higgins PJ. Regulation of extracellular matrix remodeling following transforming growth factor-beta1/epidermal growth factor-stimulated epithelial-mesenchymal transition in human premalignant keratinocytes. Cells Tissues Organs. 185(1-3); 2007; p:116-22.

144. Herr MJ, Mabry SE, Jameson JF, Jennings LK. Pro-MMP-9 upregulation in HT1080 cells expressing CD9 is regulated by epidermal growth factor receptor. Biochem Biophys Res Commun. 442(1-2); 2013; p:99-104.

145. Keates S, Sougioultzis S, Keates AC, Zhao D, Peek RM, Jr., Shaw LM, et al. cag+ Helicobacter pylori induce transactivation of the epidermal growth factor receptor in AGS gastric epithelial cells. J Biol Chem. 276(51); 2001; p:48127-34.

146. Costa AM, Ferreira R. M. , Carneiro F. , Leite M. , . FC. Helicobacter pylori CagA-positive strains activate matrix metalloproteinase-10 in gastric cells via ERK and JNK pathways. 25th International Workshop on Helicobacter and Related Bacteria in Chronic Digestive Inflammation and Gastric Cance. 17; 2012; p:1.

147. Ashktorab H, Daremipouran M, Wilson M, Siddiqi S, Lee EL, Rakhshani N, et al. Transactivation of the EGFR by AP-1 is induced by Helicobacter pylori in gastric cancer. Am J Gastroenterol. 102(10); 2007; p:2135-46.

148. Chaturvedi R, Asim M, Piazuelo MB, Yan F, Barry DP, Sierra JC, et al. Activation of EGFR and ERBB2 by Helicobacter pylori results in survival of gastric epithelial cells with DNA damage. Gastroenterology. 146(7); 2014; p:1739-51.e14.

149. Lukaszewicz-Zajac M, Mroczko B, Szmitkowski M. Gastric cancer - The role of matrix metalloproteinases in tumor progression. Clin Chim Acta. 412(19-20); 2011; p:1725-30.

150. Wernert N, Gilles F, Fafeur V, Bouali F, Raes MB, Pyke C, et al. Stromal expression of c-Ets1 transcription factor correlates with tumor invasion. Cancer Res. 54(21); 1994; p:5683-8.

151. Naito S, Shimizu S, Matsuu M, Nakashima M, Nakayama T, Yamashita S, et al. Ets-1 upregulates matrix metalloproteinase-1 expression through extracellular matrix adhesion in vascular endothelial cells. Biochem Biophys Res Commun. 291(1); 2002; p:130-8.

152. Jinnin M, Ihn H, Mimura Y, Asano Y, Yamane K, Tamaki K. Matrix metalloproteinase-1 up-regulation by hepatocyte growth factor in human dermal fibroblasts via ERK signaling pathway involves Ets1 and Fli1. Nucleic Acids Res. 33(11); 2005; p:3540-9.

153. Logan SK, Garabedian MJ, Campbell CE, Werb Z. Synergistic transcriptional activation of the tissue inhibitor of metalloproteinases-1 promoter via functional interaction of AP-1 and Ets-1 transcription factors. J Biol Chem. 271(2); 1996; p:774-82.

154. Basuyaux JP, Ferreira E, Stehelin D, Buttice G. The Ets transcription factors interact with each other and with the c-Fos/c-Jun complex via distinct protein domains in a DNA-dependent and -independent manner. J Biol Chem. 272(42); 1997; p:26188-95.

155. Gutman A, Wasylyk B. The collagenase gene promoter contains a TPA and oncogene-responsive unit encompassing the PEA3 and AP-1 binding sites. EMBO J. 9(7); 1990; p:2241-6.

156. Kong Y, Ma LQ, Bai PS, Da R, Sun H, Qi XG, et al. Helicobacter pylori promotes invasion and metastasis of gastric cancer cells through activation of AP-1 and up-regulation of CACUL1. Int J Biochem Cell Biol. 45(11); 2013; p:2666-78.

157. Yang BS, Hauser CA, Henkel G, Colman MS, Van Beveren C, Stacey KJ, et al. Ras-mediated phosphorylation of a conserved threonine residue enhances the transactivation activities of c-Ets1 and c-Ets2. Mol Cell Biol. 16(2); 1996; p:538-47.

158. Jayaraman G, Srinivas R, Duggan C, Ferreira E, Swaminathan S, Somasundaram K, et al. p300/cAMP-responsive element-binding protein interactions with ets-1 and ets-2 in the transcriptional activation of the human stromelysin promoter. J Biol Chem. 274(24); 1999; p:17342-52.

159. Willis AL, Sabeh F, Li XY, Weiss SJ. Extracellular matrix determinants and the regulation of cancer cell invasion stratagems. J Microsc. 251(3); 2013; p:250–60.

160. Scheiber MN, Watson PM, Rumboldt T, Stanley C, Wilson RC, Findlay VJ, et al. FLI1 expression is correlated with breast cancer cellular growth, migration, and invasion and altered gene expression. Neoplasia (New York, NY). 16(10); 2014; p:801–13.

161. Nakayama T, Ito M, Ohtsuru A, Naito S, Nakashima M, Fagin JA, et al. Expression of the Ets-1 proto-oncogene in human gastric carcinoma: correlation with tumor invasion. Am J Pathol. 149(6); 1996; p:1931–9.

162. Hahne JC, Okuducu AF, Kaminski A, Florin A, Soncin F, Wernert N. Ets-1 expression promotes epithelial cell transformation by inducing migration, invasion and anchorage-independent growth. Oncogene. 24(34); 2005; p:5384–8.