Relevance of MUC1 mucin in gastric carcinogenesis

Relevância da mucina MUC1 na carcinogénese gástrica

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"Who can say for certain? Maybe you're still here
I feel you all around me, your memories so clear
Deep in the stillness, I can hear you speak
You're still an inspiration, can it be?
That you are my forever Love!
And you are watching over me from up above
Fly me up to where you are, beyond the distant star
I wish upon tonight to see you smile
If only for awhile, to know you're there
A breath away's not far to where you are
Are you gently sleeping, here inside my dream?
And isn't faith believing? All power can't be seen
As my heart holds you, just one beat away
I cherish all you gave me everyday
'Cause you are my forever Love!
Watching me from up above
And I believe that angels breathe
And that Love will live on and never leave
Fly me up to where you are, beyond the distant star
I wish upon tonight to see you smile
If only for awhile, to know you're there
A breath away's not far to where you are
I know you're there, a breath away's not far
To where you are!"

O Teu Amor é eterno
Brilhante como o luar
Cada momento é um sonho
Na esperança de te abraçar

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Abstract

Gastric cancer is the third most frequent cause of cancer death worldwide. Reduced knowledge about gastric carcinogenesis model contributes for this high mortality. Gastric carcinogenesis is assumed to result from the interplay of genetic and environmental factors at the mucosa level, determined in great extent by mucus layer mucins. MUC1 is a major component of the stomach mucus layer that modulates interactions between the epithelium and external factors and its loss of polarity, overexpression, abnormal glycosylation and phosphorylation have been reported in gastric cancer and several other tumor types, suggesting a relevant role in cell transformation. MUC1 polymorphisms have been associated with susceptibility for gastric cancer development. Among environmental factors, *Helicobacter pylori* infection is known to be the most relevant event for gastric cancer development. This bacterium binds to MUC1 Variable Number of Tandem Repeats region, among other ligands, and exerts its effects on cells due the virulence factors it carries, ultimately leading to gastric cancer.

Recent studies have shown that MUC1 can function as a cytokine receptor-like molecule and that its cytoplasmic domain participates in intracellular signaling pathways that lead to cellular growth control. This domain contains several residues that can be phosphorylated and also binding sites for molecules well known to participate in cell signaling cascades. Up to now there are no data about the relevance of MUC1-cytoplasmic domain phosphorylation status or MUC1-mediated signaling pathways in the pathophysiology of gastric cancer cells. This project will a) evaluate the impact of MUC1 Variable Number of Tandem Repeats length in *Helicobacter pylori* bacterium adhesion, b) evaluate the impact of MUC1 on gene expression, phenotype and tumorigenicity, c) identify MUC1-mediated oncogenic signaling pathways, d) evaluate MUC1 impact in the expression and phosphorylation of gastric oncogenesis-related proteins and e) evaluate the impact of *Helicobacter pylori* binding in MUC1-mediated oncogenic signaling pathways in gastric carcinoma cells.

Sumário

O cancro gástrico é a terceira causa mais frequente de morte por cancro no mundo. O conhecimento reduzido do modelo de carcinogénese gástrica contribui para esta mortalidade elevada. A carcinogénese gástrica é assumida como sendo o resultado da interacção de factores genéticos e ambientais ao nível da mucosa gástrica, determinado em grande parte pela sua composição. A mucina MUC1 é um dos principais componentes da camada de muco que reveste o estômago e modula interacções entre o epitélio e a mucosa gástrica. A sua perda de polaridade, sobre-expressão e glicosilação anormais têm sido reportados com frequência em cancro gástrico e outros tipos de cancro, sugerindo um papel relevante na transformação celular. Entre os factores ambientais, a infecção pela bactéria *Helicobacter pylori* é o factor mais relevante para o desenvolvimento de cancro gástrico. Esta bactéria liga-se à região com um número variável de Tandem Repeats de MUC1, entre outros ligandos, e exerce os seus efeitos nas células devido aos seus factores de virulência, que levam ao cancro gástrico.

Estudos recentes demonstraram que a mucina MUC1 pode funcionar como uma molécula do tipo receptor de citocinas e que o seu domínio citoplasmático participa em vias intracelulares de sinalização que controlam o crescimento celular. Este domínio contém diversos resíduos que podem ser fosforilados e também locais de ligação a moléculas que participam em cascatas de sinalização celular. Até à data, não existem dados acerca da relevância do estado de fosforilação de do domínio citoplasmático de MUC1 ou da sua participação em cascatas de sinalização na patofisiologia de células de carcinoma gástrico. Este projecto vai a) avaliar o impacto do comprimento do Número Variável de Tandem Repeats Tandem na adesão da bactéria *Helicobacter pylori*, b) avaliar o impacto da expressão de MUC1 na expressão de genes, fenótipo e tumorigenicidade, c) identificar vias de sinalização oncogénicas mediadas por MUC1, d) avaliar o impacto de MUC1 na expressão e fosforilação de proteínas relacionadas com a oncogénese gástrica e d) avaliar o impacto da ligação de *Helicobacter pylori* em vias de sinalização oncogénicas mediadas por MUC1 em células de carcinoma gástrico.

Abbreviations

AKT	Protein Kinase B
AP-1	Activator protein 1
ATCC	American Type Culture Collection
ATM	Ataxia telangiectasia mutated
BabA	Blood group antigen binding adhesin
BRAF	V-raf murine sarcoma viral oncogene homolog B1
BSA	Bovine Serum Albumin
B23	Nucleophosmin
CDK1/2	Cyclin dependent kinases 1 and 2
CD8	Cluster of differentiation 8
c-Abl	Abelson murine leukemia viral oncogene homolog 1
cDNA	Complementary DNA
c-Src	Cellular sarcoma (Schmidt-Ruppin A-2) protein
CagA	Protein encoded by the cytotoxin- associated gene A
CAML	Calcium-modulating cyclophilin ligand
CDC	Centers for Disease Control and Prevention
COX-2/PGE2	Cyclooxygenase-2/Prostaglandin E2
CO_2	Carbon dioxide
DAPI	Diamidino phenylindole

DNA	Deoxyribo nucleic acid
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylene diamine tetraacetic acid
EGFR/ErbB	Epidermal Growth Factor Receptor
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER-α	Estrogen receptor alpha
ERK	Extracellular signal-regulated kinase
FBS	Foetal Bovine Serum
FITC	Fluorescein isothiocyanate
GC	Gastric cancer
Grb2	Growth factor receptor-bound protein 2
GSK3- β	Glycogen synthase kinase 3 beta
HCl	Hydrochloric Acid
HEPES	Hydroxy ethyl piperazine ethane sulfonic acid
HER	Human epidermal growth factor receptor
H. pylori	*Helicobacter pylori*
HSP90	Heat shock protein 90
H_2O_2	Hydrogen peroxide
ICAM	Intercellular adhesion molecule

IL-1	Interleukin 1
IL-8	Interleukin 8
IL-10	Interleukin 10
KDa	Kilodalton(s)
Lck	Lymphocyte-specific protein tyrosine kinase
Le^b	Lewis b antigen
LGAL4	Gene that codes for the Galectin 4 protein
Lyn	Tyrosine-protein kinase Lyn
MAPK	Mitogen-activated protein kinase
MEK	MAP kinase kinase
MOI	Multiplicity of infection
Mops	Morpholino propane sulfonic acid
mRNA	Messenger RNA
MTT	Methyl thiazol tetrazolium
MUC1	Mucin 1 from human origin
MUC1-N	MUC1 N-terminal subunit
MUC1-C	MUC1 N-terminal subunit
MUC1-CD	MUC1 cytoplasmic domain
MUC1-CT2	Primary antibody against MUC1-CD
MUC1-VNTR	MUC1 Variable Number of Tandem Repeats Region
Muc1	Mucin 1 from non-human origin

MUC2	Mucin 2
MUC5AC	Mucin 5AC
MUC6	Mucin 6
NaCl	Sodium chloride
NFK-B	Nuclear factor kappa-light-chain-enhancer of activated B cells
O.D.	Optical density
O.N.	Overnight
Paraformaldheyde	PF
PAI	Pathogenicity island
PBS	Phosphate buffered saline
PI3K	Phosphoinositide 3 kinase
PLA	Proximity Ligation Assay
PKC-δ	Protein kinase C delta
PGRN	Progranulin
PMSF	Phenylmethylsulfonyl fluoride
PPARδ	Peroxisome proliferator-activated receptor delta
PVDF	Polyvinylidene fluoride
p38	Mammalian orthologue of the yeast Hog1p MAP
p53	Tumor protein p53
RAF	Rapidly Accelerated Fibrosarcoma
RNA	Ribonucleic acid

RNAi	Ribonucleic acid interference
RPMI	Roswell Park Memorial Institute
RT	Room Temperature
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SEA domain	Sea urchin sperm protein-enterokinase-agrin domain
ShRNA	Short-hairpin RNAs
S-Le^x	Sialyl Lewis x antigen
Sos	Son of sevenless
STAT1/3	Signal transducer and activator of transcription 1/3
TBS	Tris-Buffered Saline
TMB	Tetramethylbenzidine
TGF-β	Transforming growth factor beta
TNF-α	Tumor Necrosis factor alpha
TLR4	Toll-like receptor 4
TR	Tandem Repeat
Tris	Tris hydroxylmethyl amino methane
TUNEL	Terminal Transferase dUTP Nick End Labeling
VacA	Vacuolating cytotoxin A
Wnt	Hybrid of Int and Wg in *Drosophila*
ZAP70	Zeta-chain-associated protein kinase 70

Introduction

Introduction

1. Gastric cancer

1.1. Gastric cancer epidemiology

Gastric cancer (GC) is one of the most common and life-threatening cancers worldwide [1] and nowadays the third cause of cancer-related deaths all over the world and the fifth in Portugal [2] **(Figure 1)**. Although its incidence has been impressively falling in some world areas, there are still approximately 700,000 patients dying each year from GC [3]. The poor prognosis of this disease with a 5-year survival rate of 20% [4] reflects the reduced understanding of its etiological factors and pathogenesis, absence of specific symptoms and consequent late diagnosis and lack of effective treatments.

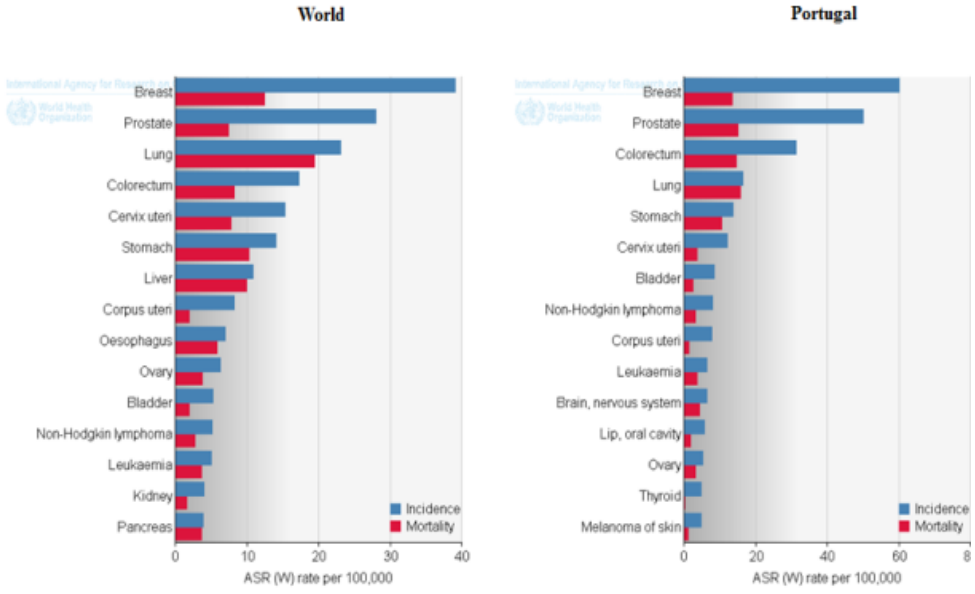


Figure 1 > Incidence and mortality estimates of the most common cancers in both sexes in the whole world (left) and in Portugal (right). ASR-age-standardized rate. [Adapted from GLOBOCAN 2008 [2]].

Although GC has been considered as a chemosensitive tumor for many years, no significant progress in its management has resulted within the last two decades.

Therefore, the identification of GC susceptibility markers, new tumorigenic markers and therapeutic targets assumes a significant priority, which will allow an early diagnosis and also the increase of survival and life quality of GC patients.

1.2. Gastric carcinogenesis

Gastric adenocarcinomas represent more than 95% of gastric neoplasms and are divided in two major types: diffuse and intestinal, with differences in morphologic, clinical and epidemiologic characteristics. In the intestinal type, there is preservation of the glandular structure and cell polarity, whereas in the diffuse type these characteristics are lost [5]. The intestinal type is more common and is associated with a sequence of pre-neoplastic lesions [6], triggered by *Helicobacter pylori* (*H. pylori*) infection. In fact, it is known that more than 80% of GC cases can be attributed to *H. pylori* chronic infection [7] and eradication of this bacterium in early stages of GC significantly decreases the incidence of this disease [8]. The symptoms of early stages of GC are often clinically silent, with patients frequently presenting advanced stage disease at the time of diagnosis.

The diffuse type carcinoma is characterized by a poorly differentiated and infiltrative pattern [9]. There are some few cases of GC with mixed histology, and *H. pylori* was also shown to be associated with diffuse type GC [10]. However, chronic inflammation is not a prerequisite for development of diffuse-type GC cancer, suggesting that different mechanisms are responsible for the ability of *H. pylori* to induce gastric malignancies.

Oncogenic transformation is a multistep process that arises from a complex interplay between genetic and environmental factors at the gastric mucosa level [6]. The gastric microenvironment is a crucial factor, determined to a large extent by the mucin and carbohydrate composition of the gastric mucus layer [11], which directly influences the impact of external factors on gastric mucosa. Several individual genetic polymorphisms have been shown to be related with susceptibility to GC development, such as polymorphisms in the gastric mucins Mucin 1 (MUC1) [12,13], Mucin 5AC (MUC5AC) [14] and Mucin 6 (MUC6) [15], Inflammation related proteins interleukin 1 (IL-1), Interleukin 8 (IL-8), Interleukin 10 (IL-10) (for review see [16]), Tumor necrosis factor alpha (TNF-α) [17] and pattern recognition systems such as Toll-like receptor 4 (TLR4) [18], among others.

As for environmental factors, *H. pylori* is known to be the most important risk factor for the development of GC, as referred before, but other factors, such as high salt intake [19] , low vegetables and fruits consumption [20] and tobacco smoking [21] have also been associated with the development of this disease. GC is now known to be a result of a multiple and complex deregulation of several potentially oncogenic cell signaling cascades [22], either due to external stimuli or internal cellular factors, or most probably both.

1.3. Deregulation of signaling pathways in gastric carcinogenesis

One of the outcomes of the alteration of environmental factors and host genetic factors at the gastric mucosa level is the deregulation of the normal working of cell signaling cascades. There are several cell signaling pathways found to be deregulated in GC (for review see [23]). One of the most important is the Mitogen-activated Protein Kinase (MAPK) signaling pathway. This pathway is activated either by extracellular or intracellular ligands and regulates several processes, such as cell proliferation, differentiation and death. The extracellular signal-regulated kinase (ERK) MAPK pathway consists on a series of kinases (abbreviation of 'Rat sarcoma' (RAS), Rapidly accelerated fibrosarcoma (RAF), MAP kinase kinase (MEK), ERK) that are activated by phosphorylation and has often been found to be deregulated in several carcinomas, leading to cell's oncogenic properties (for review see [24]). RAS family members were found to be deregulated in GC [25,26]. RAS/MAPK activation was found to be associated with cell proliferation in GCs [27]. ERK1/2, the final effectors of this cascade, were also found to be activated in GC [28] and in *H. pylori*-related cancers [29,30].

The Epidermal growth factor receptor (EGFR) is a member of the Human epidermal growth factor receptor (HER) family and works as a cell surface receptor of extracellular ligands. Its activation initiates a series of intracellular signals, including the activation of the ERK-MAPK signaling pathway. This molecule modulates processes of cell proliferation, migration, adhesion and proliferation and it is known to provide tumor cell with growth and survival advantages (for review see [31]). EGFR expression was found to be deregulated in GC, and correlated with disease prognosis [32] and presence of lymph node metastasis [33]. Several other pathways apart from the MAPK

pathway have been found to be often deregulated in GC, such as Hybrid of Int and Wg in *Drosophila* (Wnt)/β-catenin pathway, Sonic-Hedgehog pathway, Notch pathway, Cyclooxygenase-2/Prostaglandin E2 (COX-2/PGE2) pathway, Nuclear factor kappa-light-chain-enhancer of activated B cells (NFK-B) pathway, Transforming growth factor beta (TGF-β) pathway, Bone morphogenetic proteins (BMPs) pathway (for review see [23]).

Oncogenic signaling pathways in GC have been recently chosen as elective targets for innovative therapeutic approaches. Inhibition of the EGFR molecule via monoclonal antibodies (i.e. cetuximab [34]) or tyrosine kinase inhibitors (i.e. gefitinib [35]), and inhibition of HER2 by trastuzumab monoclonal antibody [36] in combination with chemotherapy showed to date the most meaningful clinically survival improvement in patients with advanced GC that overexpress these receptors [37]. Also, the use of geldanamycin, an inhibitor of Heat shock protein 90 (HSP90) (a molecular chaperone increased in GC [38]), has been proven to reduce the activation of several potentially oncogenic signaling pathways [39].

2. Helicobacter pylori

The Gram-negative microaerophilic bacterium *H. pylori* is involved in the pathogenesis of several gastrointestinal diseases, ultimately leading to GC [6,40]. According to the Centers for Disease Control and Prevention (CDC), approximately two-thirds of the world's population carries this bacterium, with percentages of infection higher in developing countries than in developed ones. In Portugal, it is estimated that more than 80% of the adult population (15-70 years old) is infected by this bacterium [41]. The infection is generally acquired during childhood and can persist during the lifetime of the individual, if untreated [42]. The high prevalence of this bacterium is in part due to the inability of the host immune system to fight the infection [43]. Most infected individuals don’t develop any *H. pylori*-associated diseases, and only a very small percentage develops GC. The majority of *H.pylori* is present in the mucus layer of gastric mucosa, but some bacteria can be attached to gastric epithelial cells [44], providing a crucial step for the maintenance, spreading and severity of the infection. Adhesion of this bacterium to the mucosal surface helps the bacteria to be protected from the continuous mucus washing of the mucosal surface. Upon cell binding, *H. pylori* induces important morphological alterations [45] and also alterations in the

glycosylation patterns of gastric cells [46]. It also activates several signaling pathways and proteins, such as MAPK pathway, NFK-B pathway, activator protein 1 (AP-1) pathway, Wnt/β-catenin pathway, Phosphoinositide 3 kinase (PI3K) pathway and Signal transducer and activator of transcription 3 (STAT3) pathway (for review see [47]), Mammalian orthologue of the yeast Hog1p MAP kinase (p38), ERK1/2 and Progranulin (PGRN) [48], β-catenin, p120 catenin, and Peroxisome proliferator-activated receptor delta (PPARδ) [49] and TNF-α [50] proteins, among others. Other studies have shown that adherence of this bacterium induces cell proliferation and apoptosis during the early phase of chronic inflammation of the gastric mucosa [51] .

The susceptibility of developing GC upon *H. pylori* infection is not only dependent on host genetic characteristics but also on the different genotypes of bacterial virulence genes [52]. There are mainly three bacterial proteins that are crucial for the development of GC, which are: blood group antigen binding adhesin (BabA) [53], protein encoded by the cytotoxin- associated gene A (CagA) [54] and vacuolating cytotoxin A (VacA) [55].

BabA mediates bacterial adhesion to antigens present on the cell surface, whereas CagA and VacA are associated with deregulation of basic functions and signaling pathways and cell vacuolation, respectively. *H. pylori* has been shown to induce the nuclear accumulation and activation of β-catenin [56,57] and EGFR [58], therefore deregulating important cell signaling cascades.

Host genetic characteristics that influence the development of GC upon *H.pylori* infection include MUC1 Variable Number of Tandem Repeats (VNTR) variability. It has been previously shown that individuals homozygous for short VNTR domains have an increased risk for the development of pre-cancerous gastric lesions [12,13].

The mucin MUC1 has been recently identified as an *H. pylori* binding target [59,60]. However, it has been shown to limit *H. pylori* colonization [61,62,63]. Mice deficient in Muc1 were shown to be more susceptible to infection by *H. pylori* [63]. Furthermore, there is evidence that *H. pylori* blocks the expression of MUC1 on cell membranes and therefore a decrease in MUC1 synthesis might be a host defensive response against stomach colonization [64,65]. Extracellular MUC1 VNTR domain is highly glycosylated and under normal conditions presents Lewis b (Le^b) carbohydrate structures that are involved in the strong binding of *H. pylori* to gastric cells through its adhesin BabA [66]. Under conditions of inflammation, synthesis of sialylated

carbohydrate structures such as sialyl-Lewis x (sialyl -Le^x) occurs in the gastric mucosa, including in the VNTR region of MUC1. Another bacterial adhesin, SabA, binds to these antigens, promoting a weaker but closer adhesion of *H. pylori* to gastric epithelial cells [67] **(Figure 2)**. This weaker binding allows the bacteria a constant attachment/detachment, enabling an efficient escape from the immune system effectors.

The relevance of MUC1 VNTR variability for *H. pylori* adhesion to gastric cells and possible consequent infection remains to be clarified.

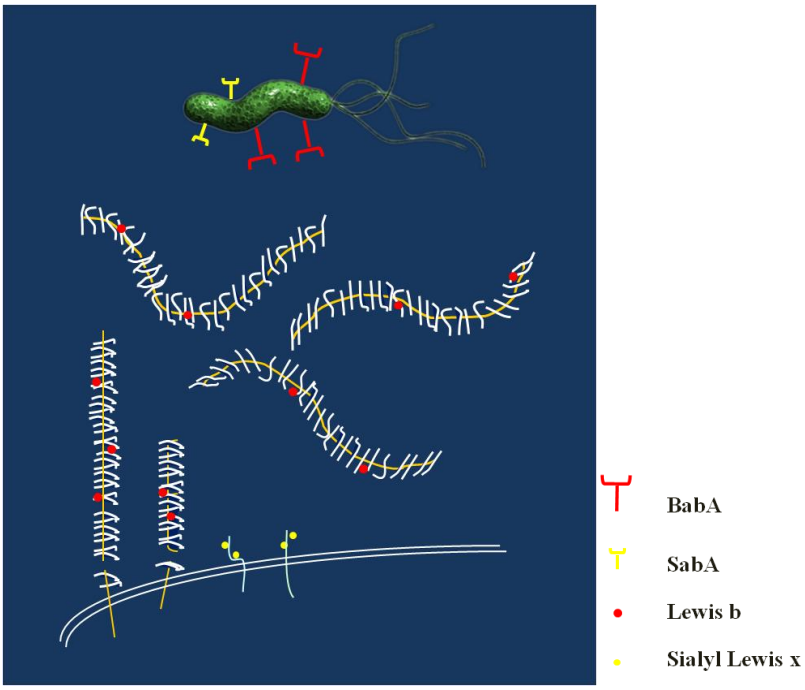


Figure 2 > *H. pylori* binding to Le^b and Sialyl-Le^x antigens in gastric cells secreted and membrane-associated mucins, through its adhesins BabA and SabA, respectively. In the first steps of infection, the BabA adhesin binds Le^b antigens present in healthy epithelia. After infection and inflammation, the expression of Sialyl-Le^x antigens allows the bacteria to bind closer to the cells.

The eradication of *H.pylori* has been proved as effective in the prevention of GC [68]. The best strategy to prevent co-morbitidies associated with *H. pylori* infection is the development of a prophylactic vaccine, since bacterial eradication strategies with antibiotics are becoming inefficient [69]. Another possible and in theory ideal strategy would be to stop the deregulation of signaling pathways triggered by bacterial infection, but this approach would need a deep and detailed knowledge about such pathways and

the development of peptides/molecules to prevent them from being deregulated, without interfering with their normal action.

3. Gastric mucins

Independently of the genetic predisposition for the development of GC, interaction of the gastric mucosa with the external environment is essential for the initiation of the neoplastic process. Therefore, understanding the biological mechanisms that occur at the interface between the epithelium and external environment is crucial to establish a carcinogenesis model and to develop new therapeutic strategies. The glycoproteins (mucins) present in the mucus layer that covers the gastric epithelium are an efficient barrier and thus constitute elective targets to study the gastric oncogenesis model.

The mucus layer lining the stomach is comprised of secreted and membrane-associated mucins which canonical function is to provide protection to vulnerable epithelia. Secreted mucins act mainly by lubricating and protecting the epithelia, constituting a barrier against microorganisms and chemical aggressions [70]. Membrane-associated mucins also share these features, with the addition of also participating in cell signaling cascades, immune responses, cell adhesion and differentiation and renewal of the epithelium [71].

Mucins are large, heavily O-glycosylated glycoproteins ubiquitously expressed in the surface of all epithelial cell. Mucins genes contain TR motifs that code for regions with a high amount of serine, threonine and proline aminoacids. The serine and threonine residues can be extensively *O*-glycosylated, and are responsible for most of the mucins molecular weight. The length and number of the TR region varies between mucins and also between individuals – VNTR polymorphism.

The gastric mucus of a normal stomach is composed of two secreted mucins, MUC5AC and MUC6, and one membrane-associated mucin, MUC1, that is also the most highly expressed gastric mucin [72,73,74] **(Figure 3)**.

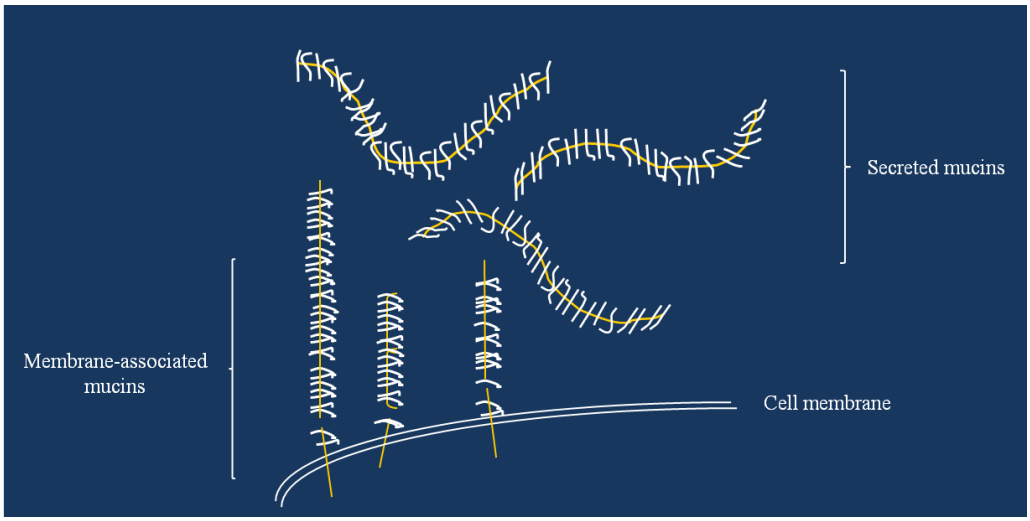


Figure 3 > Representation of the normal gastric mucus layer, composed of secreted (MUC5AC and MUC6) and membrane-associated (MUC1) mucins. These mucins form a cohesive net that protects the epithelial cells surface.

MUC1, MUC5AC and MUC6 polymorphisms have been associated with individual susceptibility to GC development [12,13,14,15]. During the development of intestinal type GC, there is a *de novo* expression of the intestinal Mucin 2 (MUC2) and it is also common to exist alterations in the expression of MUC1, MUC5AC and MUC6 [75]. In the diffuse GC, there is also a common significant alteration of the expression of all these mucins [76,77].

4. Relevance of MUC1 in carcinogenesis

Abnormal expression and/or glycosylation of mucins, namely MUC1, have been described in several cancer models, suggesting their involvement in cancer development [75,78,79,80,81]. MUC1 was the first mucin to be identified and encodes a transmembrane glycoprotein with structural features similar to receptors of cytokines and growth factors **(Figure 4)**.

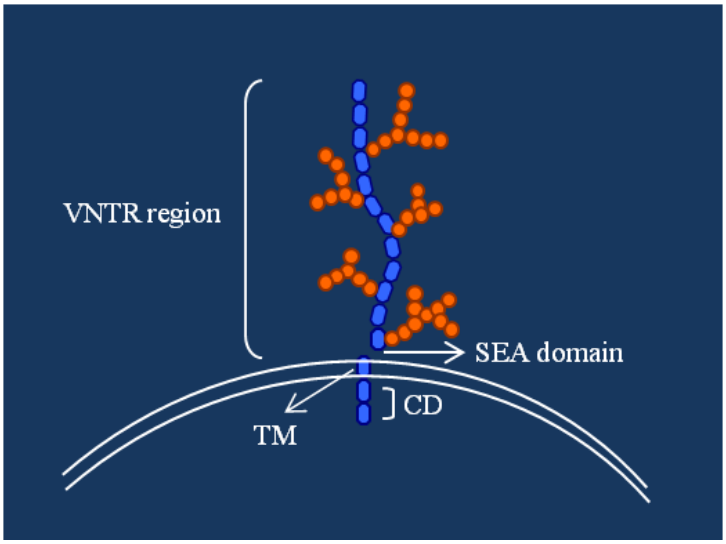


Figure 4 > Schematic representation of MUC1, including an N-terminal domain with a VNTR region, a SEA domain and a C-terminal domain with a transmembrane (TM) region and a cytoplasmic domain (CD).

MUC1 is an heterodimer composed of two distinct subunits, formed by autocatalytic cleavage of the Sea urchin sperm protein-enterokinase-agrin (SEA) domain and bound by non-covalent forces [82]. The N-terminal subunit (MUC1-N) can protrude 200 nm to 500 nm from the cell surface [83] and therefore is the first barrier encountered by microorganisms / other cells. MUC1 extracellular domain can be released from the epithelial surface, thereby acting as a releasable decoy [61]. This domain includes the VNTR region, ranging from 20 to 120 TR of 20 aminoacids - "short" or "large" alleles) [84], that are extensively modified by O-linked glycans [85,86] . Glycosylation of this extracellular domain, together with the variability of its length, can provide tumor cells an enormous range of potential interactions with other ligands and/or receptors at the cell surface [70], namely with Intercellular Adhesion Molecule 1 (ICAM-1) [87] and galectin-3 [88] , mediating processes of cell adhesion [88], motility and migration [89,90], metastasis [91] and cell-cell aggregation [92]. Another study has shown that *Pseudomonas aeruginosa* or its flagellin protein can serve as activators of MUC1-mediated signaling by binding MUC1 extracellular domain and promoting activation of the MAPK pathway [93]. MUC1 abnormal expression, glycosylation and loss of apical expression have been observed in several cancer cells [75,78,94] **(Figure 5)**.

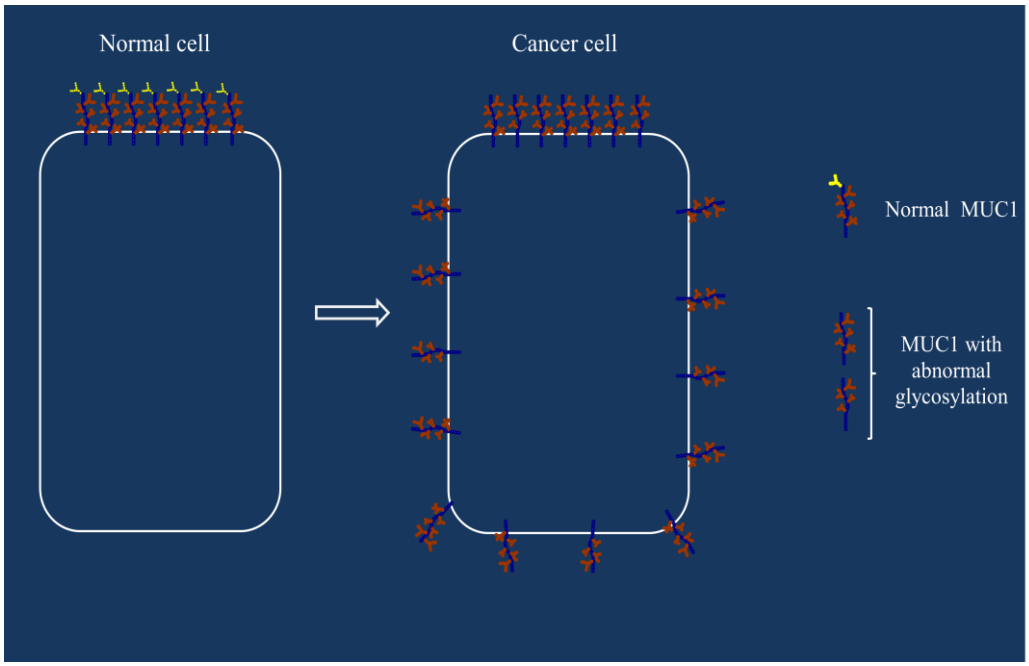


Figure 5 > MUC1 expression, glycosylation and membrane localization in normal and cancer cells.

Furthermore, it has been shown that in *H.pylori* infected individuals, the highly glycosylated extracellular domain of MUC1 is shed from the gastric surface [59], suggesting that this bacterium interferes with many processes mediated by the MUC1 extracellular domain.

MUC1 C-terminal sub-unit (MUC1-C) encompasses an extracellular, a transmembrane region and a cytoplasmic domain. MUC1-C extracellular domain interacts with the molecule galectin-3, forming complexes with EGFR [95]. MUC1-CD contains seven highly conserved tyrosines and several serine/threonine phosphorylation sites [96] and interacts with several molecules involved in intracellular signaling pathways such as β-catenin, Cellular sarcoma (Schmidt-Ruppin A-2) protein (c-Src), Growth factor receptor-bound protein 2/Son of sevenless (Grb2/Sos), tumor protein 53 (p53), Glycogen synthase kinase 3 beta (GSK-3β), EGFR and protein kinase C delta (PKC-δ) [97], Tyrosine-protein kinase Lyn (Lyn) [98] , Lymphocyte-specific protein tyrosine kinase **(**Lck) and Zeta-chain-associated protein kinase 70 (Zap 70) [99], Estrogen receptor alpha (ER-α) [100] and NFK-B [101].

5. MUC1 in oncogenic signaling pathways

MUC1 is predicted to function as an "oncogene", whereby overexpression correlates with tumor formation, tumor progression and poor survival of cancer patients [102]. Although both MUC1 extracellular domain and MUC1-CD were shown to be essential for tumor formation [103], much of the ability of MUC1 to foster tumorigenesis and tumor progression likely originates from the interaction of its cytoplasmic domain with proteins involved in oncogenic signaling.

Zrihan–Licht *et al* were the first to demonstrate that MUC1-CD could be tyrosine phosphorylated and that it contains a putative binding motif for the signaling molecule Grb2 in human breast cancer cells [104]. Studies with MCF-7 breast carcinoma cells have also shown that MUC1/Grb2 complex associates with the protein Sos and with Ras at the cell membrane, which further supports a role for MUC1 in intracellular signaling [105]. Meerzaman *et al* demonstrated, using a Cluster of differentiation 8 (CD8)/MUC1 chimeric receptor, that tyrosine phosphorylation of MUC1-CD led to the activation of the RAS-RAF-MEK-ERK2 MAPK pathway [106]. *In vitro* and *in vivo* approaches have shown that MUC1-CD could be phosphorylated by c-Src tyrosine kinase and PKC-δ, increasing the binding to β-catenin, while its phosphorylation by GSK3β kinase decreased the binding to β-catenin [107,108,109]. Direct binding of MUC1 with β-catenin and δ-catenin (proteins involved in cell adhesion with E-cadherin and in transcriptional complexes) demonstrates a connection between MUC1 and signaling networks between the cell membrane and the nuclear compartment [108,109,110].

MUC1 physically associates with all four EGFR family receptors in a MUC1 transgenic mouse model and this interaction increases ERK1/2 activation [111]. MUC1 overexpression is not only associated with its accumulation in the cytoplasm, but also in the cell nucleus [112] Chromatin immunoprecipitation and reciprocal coimmunoprecipitation assays have shown that at the nucleus compartment MUC1-CD associates with β-catenin, δ-catenin, p53 and ER-α, supporting the hypothesis that MUC1 contributes to transcriptional regulation, as been also shown for other molecules [101,113,114,115,116,117,118,119,120].

Currently, data about potential docking sites for Grb2/Sos, AP-2, p53, ER-α and β-catenin and phosphorylation sites to GSK-3β, c-Src, EGFR, Lyn, Lck, PKC-δ and

Zap70 provide significant evidence to support MUC1-CD involvement in signal transduction [94,105,107,108,110,111,120,121]. MUC1 has been also shown to interact with other molecules such as Abelson murine leukemia viral oncogene homolog 1 (c-Abl) [122], Ataxia telangiectasia mutated (ATM) [123] and Calcium-modulating cyclophilin ligand (CAML) [124].

MUC1-CD overexpression has been shown to activate β-catenin [117], NFK-B [101], STAT1/STAT3 [125,126], Protein Kinase B (also known as Akt) and ERK [127,128] signaling. Although a role for MUC1-CD as a scaffolding protein has not yet been clearly established, its interaction with these proteins that represent several different signaling cascades suggests a role for MUC1-CD as an integrator of signaling networks with oncogenic potential and therefore a promising target for the development of new therapeutic strategies against GC (reviewed in [129]).

Most studies examining MUC1 signaling activity have focused on breast cancer cells, where MUC1 is overexpressed, aberrantly glycosylated, and constitutively phosphorylated. Up to now there is little information about MUC1 participation in signaling pathways of GC cells, which reinforces the relevance of a comprehensive study of the MUC1-receptor model in these cells. It has been found that ERK1/2 molecules, the final effectors of the MAPK signaling pathway, were deregulated in *H.pylori*-related GCs [29]. It is known that MUC1 overexpression is correlated with the progression and prognosis of GCs [76,130] and therefore this molecule is likely to play a relevant role in oncogenic signaling pathways of GC cells.

5.1. MUC1 – cancer biomarker *vs* therapy target

Since MUC1 has been shown to play an important role in the tumorigenesis of several tumor types, there is a major drive to explore its potential either as a cancer biomarker as well as a therapy target. Circulating MUC1-N, result of cleavage by the action of proteases [131] has been used as a prognostic and predictive cancer biomarker, mainly in breast carcinoma [132]. However, recent studies have shown that the significance of circulating MUC1 is not that relevant, since its levels greatly vary in healthy individuals. Furthermore, although very sensitive, these biomarkers will be influenced by individual polymorphisms, multiple epitopes or glycosylation differences. It is thought that MUC1-C terminus will be a better prognostic and predictive marker in carcinomas that overexpress MUC1 [133].

Immunotherapy against this molecule has been explored for the treatment of several cancers. However, there has been a general failure in MUC1 immunotherapy treatments to overcome the pre-clinical phases, which can be explained by several factors. MUC1 immunogen used for many of these studies is the TR domain, which glycosylation varies significantly between normal and cancer cells and even between cells of the same tumor. The TR contains a repeating unit of 20 aminoacids and therefore is only capable of generating a weak immune response [134]. Furthermore, the TR region is known to interact with several other extracellular molecules and this interaction may reduce the efficacy of the immunological surveillance. Strategies have been developed in order to overcome this problem [135], opening exciting paths for MUC1 immunotherapy.

Recently, MUC1 peptides and small molecule inhibitors have been proven to be effective in inhibiting cancer progression, both *in vitro* and *in vivo* [136,137,138]. However, all MUC1-based therapies that are under development are still relatively recent and not yet fully tested or even proven to be efficient. Therapies that were successful in mice have not progressed to clinic [139] and therefore it is urgent to develop new and more efficient therapies.

Considering that existing chemo and radiotherapies have been proven not to be effective in GC treatment, the detailed characterization of MUC1 impact in gastric carcinogenesis assumes a significant relevance for the development of future MUC1-based therapies.

Aims

General Aims

With this project, we plan to evaluate the relevance of MUC1 in gastric carcinogenesis, namely its impact in *H.pylori* adhesion and its participation in oncogenic signaling pathways.

Specific Aims

1. **Evaluation of the impact MUC1 VNTR length in *H.pylori* adhesion to GC cells. Paper I.**

In the first part of the work, we tested the hypothesis that MUC1 VNTR polymorphism affects the adhesion of *H. pylori* to GC cells and thus plays an important role in the colonization of gastric mucosa. We used *H. pylori* strains with different pathogenicities (pathogenic strain HP26695 and non-pathogenic strain HPTx30a), co-cultured with two different GC-derived cell lines GP202 and MKN45, and GP202 clones expressing recombinant MUC1 with different VNTR lengths. Adhesion was evaluated by an ELISA-based adhesion assay [140], that was optimized in our laboratory.

2. Evaluation of MUC1 impact on gene expression, phenotype and tumorigenicity of GC cells. Paper II.

In the second part of the work, we tested the hypothesis that MUC1 is a relevant molecule for GC biology. We used retrovirus-mediated transfection of short-hairpin RNAs (shRNA) to induce a stable downregulation of MUC1 in the GC-derived cell lines MKN45 and GP202. Four MUC1 downregulated clones (MKN45-C1 and MKN45-C2, GP202-C1 and GP202-C2) and two scrambled controls (MKN45-SC and GP202-SC) were successfully isolated and expanded. The impact of MUC1 downregulation on global gene expression in the GC cells MKN45 and GP202 was evaluated by microarray analysis. The effects of MUC1 downregulation in MKN45 and GP202 cells phenotype were studied *in vitro* with respect to cell proliferation, apoptosis, migration, invasion and cell-cell aggregation. *In vivo* assays were also performed in mice with the MKN45 GC cell line, in order to study the tumorigenicity of cells with and without MUC1 downregulation.

3. Identification of MUC1-mediated oncogenic signaling pathways in GC cells. Paper III.

In the third part of the work, we have identified MUC1 signaling partners in MKN45 GC cells. We used immunoprecipitations and Proximity Ligation Assays (PLAs) to identify interactions between MUC1-CD and oncogenic signaling molecules. The interactions of MUC1-CD with oncogenic signaling partners were also studied in GC and normal gastric tissue samples, in order to evaluate their relevance for gastric carcinogenesis.

4. Evaluation of MUC1 impact in the expression and phosphorylation of gastric oncogenesis-related proteins in GC cells. Paper III.

In the fourth part of the work, we evaluated the impact of MUC1 downregulation in the MKN45 GC cell line on gastric oncogenesis-related proteins expression and phosphorylation by immunoblots, RT-PCR and Kinexus phospho-antibody screening.

5. Evaluation of the impact of *H. pylori* binding in MUC1-mediated oncogenic signaling pathways in GC cells.

In the fifth part of the work, we tested the hypothesis that *H. pylori* triggers MUC1-dependent oncogenic signaling pathways in GC cells. We evaluated the phosphorylation of ERK1/2, using co-cultures of *H. pylori* pathogenic strain HP26695 and MKN45 GC cells.

Materials and Methods

Materials and Methods

Cell culture

We have used two GC cell lines: GP202, previously established in our laboratory [141] from a signet ring cell GC that constitutively expresses MUC1 and MKN45, derived from a poorly differentiated adenocarcinoma, Japan Health Sciences Foundation [142], both derived from diffuse type GCs. MKN45 and GP202 cell lines were grown in Roswell Park Memorial Institute (RPMI) 1640 containing Glutamax^{TM}I and 25 mM Hydroxy ethyl piperazine ethane sulfonic acid (HEPES), supplemented with 10% FBS and 50 µg/ml gentamicin. The GC intestinal-type cell line AGS was obtained from ATCC (CRL-1739) and grown under the same conditions described.

GP202 clones expressing recombinant MUC1 with different VNTR lengths were previously established by stable transfection with an eukaryotic expression vector pHb-APr1-neo containing subcloned epitope-tagged MUC1 (FLAG-MUC1) complementary DNAs (cDNAs) with different number of TR units (0, 3, 9 and 42 repeats, respectively GP202- ΔTR, GP202- 3TR, GP202- 9TR and GP202- 42TR) [85]. GP202-Neo was obtained by transfection with the empty vector. Recombinant MUC1 transfectants were cultured at 37ºC in a humidified 5% carbon dioxide (CO_2) incubator and maintained in RPMI 1640 medium (with Glutamax and 25 mmol/L Hepes) supplemented with 10% foetal bovine serum (FBS) and 50 µg/mL gentamicin.

The packaging cell line PhoenixGP [143] was maintained in Dulbecco's Modified Eagle Medium (DMEM) containing Glutamax^{TM}I, 4,500 mg/l D-Glucose and Sodium Pyruvate, supplemented with 10% FBS and 1% (v/v) penicillin/streptomycin.

Stable MUC1 downregulated clones were grown in standard growth medium supplemented with 5µg/ml puromycin. After evaluation of MUC1 levels at different time points in culture, all the assays were performed considering the time of cell culture in which the downregulation was higher, at 96 h of cell culture for MKN45 and 48 h for GP202 cells. Cells were grown at 37ºC with 5% CO_2 in humidified atmosphere.

Cell culture reagents were obtained from Invitrogen.

H. pylori strains

Two *H. pylori* strains were used in this study: the pathogenic strain HP26695 (*vacA* s1/m1, *cag* pathogenicity island (PAI)+, American Type Culture Collection (ATCC) 700392) and the non-pathogenic strain HPTx30a (*vacA* s2/m2, *cag* PAI-, ATCC 51932). Bacteria were grown on Trypticase soy agar with 5% sheep blood (BioMérieux) at 37ºC in microaerobic conditions.

Clinical Samples

Normal gastric tissues were obtained from the University of Nebraska Medical Center's Tissue Bank through the Rapid Autopsy Pancreatic program in compliance with IRB 091-01. To ensure minimal degradation of tissue, organs are harvested within three hours post mortem and the specimens placed in formalin for immediate fixation. Sections were cut from paraffin blocks into 4 micron thick sections and mounted on charged slides. For GC tissue samples, we have used paraffin embebbed slides from the antrum part of the stomach treated in a similar way as the normal samples and obtained from the Medical Faculty of Porto from gastric biopsies.

Co-cultures and Enzyme-linked immunosorbent assay (ELISA) assays

Quantitative evaluation of *H. pylori* adhesion to gastric cells was performed by ELISA, as previously described [140], with some modifications. Briefly, cells were cultured in 96 well plates and allowed to form confluent monolayers. Cells were washed and *H. pylori* suspension was added in a 200:1 Multiplicity of infection (MOI) and incubated for 60 min. Cells were washed and fixed at 4ºC with 8% paraformaldehyde (PF) for 60 min. Endogenous peroxidase was inactivated by addition of 1% Hydrogen peroxide (H_2O_2) in methanol. After washing with PBS solution, anti-*H. pylori* monoclonal antibody MAB922 (Chemicon) was added overnight (O.N.), at 4ºC, followed by the addition of peroxidase-conjugated goat anti-mouse immunoglobulins (Santa Cruz Biotechnology) for 30 min, RT. Tetramethylbenzidine (TMB) (Sigma) was added and reaction stopped with 1M Hydrochloric Acid (HCl). Plates were read in a 680 ELISA microplate reader (Bio-Rad) at 450 nm. Optical density (O.D.) values were used as the index of the number of *H. pylori* adhering to cells. Two sets of triplicates were made for each assay. Statistical analysis was performed using the Mann-Whitney test - StatView

Software version 5.0 (SAS Institute, Cary, NC). A *P* value of less than 0.05 was accepted as statistically significant.

Co-cultures with MKN45 GC cell line and HP26695 bacteria

MKN45-SC cells were cultured until subconfluency in 24-well plates. HP26695 bacteria were added to each plate in a MOI of 100:1 and incubation was performed for 1 h or 6 h. Washes were performed with Tris-buffered saline (TBS) to remove non adherent bacteria. Protein extraction was performed in the remaining plates, as will be hereafter described for the Kinexus assays.

MUC1 downregulation strategy

MUC1 downregulated cells were produced using a retroviral expression system with short hairpin Ribonucleic acid (RNA)s. Briefly, a 21-nucleotide sequence of the MUC1 gene, with no homology to other Deoxyribo nucleic acid (DNA) sequences detected in a BLAST search, was chosen according to standard Ribonucleic acid interference (RNAi) rules [144]. The scrambled control was designed and tested for homology in a BLAST search as well. Sense and antisense oligos (Proligo) were ligated and inserted in the pSUPER.retro.puro vector (Oligoengine). The oligos used were the following: MUC1 Exon 2 (*sense*: GATCCCCACCTCCAGTTTAATTCCTC TTCAAGAGAGAGGAATTAAACTGGAGGTTTTTTA; *antisense*: AGCTTAAAAA ACCTCCAGTTTAATTCCTCTCTCTTGAAGAGGAATTAAACTGGAGGTGGG;the MUC1-cDNA target region is underlined) and scrambled control (*sense*: GATCCCCATCACCTTCGTACTCCTTATTCAAGAGATAAGGAGTACGAAGGTG ATTTTTTA, *antisense*: AGCTTAAAAAATCACCTTCGTACTCCTTATCTCTTGAA TAAGGAGTACGAAGGTGATGGG; the "unpaired"-cDNA target region is underlined). The MUC1 specific target or the scrambled control constructs were transfected into PhoenixGP packaging cell line by calcium-phosphate mediated transfection and transfected cells were selected using puromycin. Stable transfectants were seeded in a 6-well plate ($1x10^6$cells/well) and incubated for 24 h at 32ºC. The media containing the virus was collected, filtered through a 0.45 µm filter to remove remnant cells, and used to infect MKN45 and GP202 cells, during 24 h at 37ºC. The viral supernatant was then replaced by the standard growth medium and cells were

incubated 48 h at 37ºC. Efficiently transduced cells were selected and grown in standard media supplemented with puromycin. Two independent MUC1 downregulated clones (C1 and C2) were isolated and expanded for three times, using cloning rings.

Immunofluorescence and antibodies

MKN45 and GP202 cells in culture were harvested, seeded in 12-well slides (Cell Line) and air-dried O.N. at RT. Cells were then fixed in ice cold acetone for 5 min, washed twice with PBS and blocked with normal rabbit serum (DAKO) diluted 1:5 in 10% bovine serum albumin (BSA) for 30 min. Serum was then replaced by the MUC1 monoclonal antibody HMFG1 (NovoCastra) diluted 1:50 in 5% BSA, and incubated O.N. at 4ºC. After three washes with PBS, cells were incubated with a rabbit anti-mouse Fluorescein isothiocyanate (FITC) labeled antibody (DAKO) diluted 1:70 in 5% BSA for 30 min in the dark at RT. Cells were washed 3 times with PBS and mounted in vectashield (Vectorlabs). Images were acquired in a Leica DMIRE2 fluorescence microscope. Results are representative of three independent experiments.

Confocal Microscopy

MKN45-SC and MKN45-C2 cells were grown until confluency in round slides, washed with PBS and fixed with 4% PF/120mM sucrose for 15 min. 0.1M glycine was added after fixative removal for 15 min. Cells were washed with PBS 1% BSA twice and permeabilized with 0.15% Triton-X100/1%BSA in PBS for 15 min. After washing with PBS 1% BSA, the primary antibodies were added in PBS 1% BSA : MUC1-CT2 1:300 (ThermoScientific) and calreticulin 1:50 (Cell Signaling) and incubated O.N. at 4ºC. After washing with PBS, the secondary antibodies were added (Alexa Dylight-488 anti-hamster 1:500 and donkey anti-rabbit-649 1:500) for 1 h at RT. After PBS washing, nuclei were stained with DAPI (1:10,000 in PBS for 2 min), cells were washed again with PBS and mounted in Vectashield. Images were acquired in a Zeiss Confocal Microscope LSM410.

Protein extraction, Western blot and Immunoprecipitations

MKN45 and GP202 cells were cultured in 60-mm dishes to 80-90%-confluence. After washing twice with PBS, lysis buffer (10 mM Tris hydroxylmethyl amino methane (Tris) pH 7.4, 150 mM Sodium chloride (NaCl), 0.1% (p/v) Sodium dodecyl sulfate (SDS), 1 mM Phenylmethylsulfonyl fluoride (PMSF), 1% (v/v) Triton X-100) was added and cells were scraped. Lysates were incubated on ice for 15 min and centrifuged for 30 min at 4ºC to collect the supernatants. Protein content was assessed by the bicinchoninic acid method (Pierce), as described in the manufacturer's instruction manual. Protein extracts were analysed by a 4-10% Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) (Invitrogen), transferred to Polyvinylidene fluoride (PVDF) membranes (Amersham Biosciences), blocked with 5% nonfat dry milk in PBS containing 0.01% Tween 20 and blotted O.N. at 4ºC with anti MUC1-CT2 monoclonal antibody 1:300 (ThermoScientific), anti-β-actin polyclonal antibody 1:5,000 (Sigma), anti ERK1/2 1:1,000 (Cell Signaling), anti-EGFR 1:200 (Santacruz Biotec.), anti-B-RAF 1:200 (Santacruz Biotec.) and anti-Grb2 1:200 (Santacruz Biotec.) in 5% non-fat milk in PBS-0.1% Tween 20 (Sigma). Membranes were washed 3 times with PBS-0.1% Tween 20 and the primary antibodies were revealed using goat anti-mouse/rabbit/hamster peroxidase-conjugated antibodies (1:2,000, DAKO) in 5% non-fat milk in PBS-0.1% Tween 20, followed by ECL detection kit (BioRad).

For co-culture experiments (MKN45 cells and HP26695 bacteria), 20 µg of protein extracts were obtained as will be hereafter described for Kinexus assays and using TBS instead of PBS buffer and were analyzed using phospho-ERK1/2 antibody 1:1,000 (Cell Signaling). When β-actin values were not similar among samples, films were scanned in GS-800 Calibrated Densitometer (BioRad) and optical density of each specific P-ERK1/2 band was analysed using the Quantity One software (BioRad) and normalized to the β-actin density. Results are representative of three independent experiments.

For immunoprecipitations, 750 µg of protein lysate were incubated for 3 h at 4ºC with anti-MUC1-CT2 1:300 (ThermoScientific) and normal armenian hamster IgG (EBioscience), previously precipitated with Protein G Sepharose (Sigma) for 1 h at 4ºC. After washing with non-denaturing lysis buffer, the immunoprecipitates and lysates

were separated in 12% Tris-Glycine Gels (Invitrogen). The rest of the immunoblot procedure was performed as mentioned before.

RNA extraction and Real-Time PCR

Total RNA was isolated from MKN45 and GP202 cells in culture using TriReagent™ (Sigma), according to the manufacturer's instructions. 5 µg of RNA were primed with random hexamers (Invitrogen) and reverse transcribed with Superscript II (Invitrogen) in a final volume of 20 µl. 2 µl of a 1:10 dilution of cDNA were amplified with 300 nM of each primer and SYBRGreen (Applyed BioSystems) in a final volume of 20µl, using the fluorescence reader ABI Prism 7000. Each sample was run in triplicate. The primers used were the following: MUC1 (*sense*: CTCCTTTCTTCCTGCTGCTG, *antisense*: CTGGAGAGTACGCTGCTGGT); 18S (*sense*: CGCCGCTAGAGGTGAAATTC, *antisense*: CATTCTTGGCAAATGC TTTCG); TBP (*sense*: GCACAGGAGCCAAGAGTGAA, *antisense*: TCACAGC TCCCCACCATATT), EGFR (*sense*: GAGCGACTGCCTGGTCTGCC, *antisense*: CACGCAGGTGGCACCAAAGC), ERK2 (*sense*: GACACAACACCTCAGCAAT GACCA, *antisense*: GGCTTGAGGTCACGGTGCAGA) and BRAF (*sense*: TTAGT GAGCCAGGTAATGAGGCA; *antisense*: ATCAATTTGGGCAACGAGACCGA) and their specificity was confirmed using the software BLASTn on-line and by melt curve analysis. For each sample, the level of 18S/TBP RNA was measured and used for normalization of target genes abundance. Relative Messenger RNA (mRNA) levels were then calculated using the comparative C_t method [145]. Data are expressed as a ratio of the results obtained with each clone and the scrambled control, from three independent experiments. Statistical analysis was performed using the Mann-Whitney test - StatView Software version 5.0 (SAS Institute, Cary, NC).

Gene expression analysis

The expression of 12,135 genes in MKN45 and GP202-MUC1 downregulated clones and the respective scrambled control was evaluated following the same protocol as in [146]. Briefly, following RNA extraction (as described previously), cDNA was obtained by reverse transcription, during which labeled nucleotides were incorporated: MKN45-C1, MKN45-C2, GP202-C1 and GP202-C2 cDNAs were labeled with Cy3

(green emission) and MKN45-SC and GP202-SC controls with Cy5 (red emission). After hybridization, the mixture was hybridized with the array O.N. and then the array was digitalized with the ScanArray4000 (Perkin-Elmer) system and fluorescence analysed by the QuantArray software package (Perkin-Elmer).

Normalization and background subtraction were performed and ratios downregulated clones /SC control and SC control/downregulated clones were calculated using Microsoft Excel software. Gene expression with a ratio higher than 2 was considered statistically significant.

All data are MIAME compliant and the raw from the microarray experiments were uploaded onto the Gene Expression Omnibus Database http://www.ncbi.nlm.nih.gov/geo (Geo accession numbers: GSM717858 and GSM717859).

Methyl thiazol tetrazolium (MTT) proliferation assay

MKN45 and GP202 cells were plated in triplicate in 96-well plates at 5,000 cells per well and incubated at normal conditions. At each time point, the medium was removed and cells incubated with 20 µl of MTT solution (5mg/ml, Sigma) for 3 h at normal conditions. MTT was removed and 200µl of Dimethyl sulfoxide (DMSO) were added to each well to dissolve formazan. Finally, formazan O.D. was measured using a microplate reader at a wavelength of 540nm. The relative growth was defined as the following formula: Relative Growth = (A_{540nm} at Tn / A_{540nm} at $T0_{24h}$). Data are expressed as a ratio of the results obtained with each clone and the scrambled control, from three independent experiments. Statistical analysis was performed using the Mann-Whitney test - StatView Software version 5.0 (SAS Institute, Cary, NC).

TUNEL assay

Post-confluent MKN45 and GP202 cells were harvested and fixed with 4% PF in PBS for 15 min. Fixated cells were seeded in 12-well slides (Cell Line) and air-dried O.N. at RT. Following washing with PBS, cells were permeabilized with ice-cold freshly-made PBSTrCit solution (PBS + 0.1%TritonX + 0.1% Sodium Citrate) for 2 min on ice. Cells were washed again twice, and incubated with TUNEL reaction mix (enzyme solution, label solution and dilution buffer, 1:9:10, In Situ Death Detection Kit, Fluorescein, Roche) for 1 h at 37ºC. Two additional washing steps were performed and

slides were mounted in Vectashield with Diamidino phenylindole (DAPI) (Vectorlabs). Results were analysed under a Leica DMIRE2 fluorescence microscope and data are expressed as a ratio of the results obtained with each clone and the scrambled control, from three independent experiments. Statistical analysis was performed using the Mann-Whitney test - StatView Software version 5.0 (SAS Institute, Cary, NC).

Migration assay

MKN45 and GP202 cells were cultured in 60-mm dishes until full confluency. The epithelial cells monolayer was then washed with PBS and wounded with a 10µl micropipette tip. Non-adherent cells were removed by washing twice with PBS. Images of cells at the edge of the wound were acquired automatically at 20x magnification in a Leica DMIRE2 fluorescence microscope with a Leica DFC Twain camera for 144 frames at 10 min intervals (with a total time corresponding to 24 h) controlled by Leica FW4000 software. Frames from 0, 6, 12, 18 and 24 h were used to quantify the percentage of migration: a grid of 50x30 squares was used to fulfill the wound space and the percentage of migration was calculated by the number of squares occupied by cells at each time point. Data are expressed as a ratio of the results obtained with each clone and the scrambled control, from three independent experiments. Statistical analysis was performed using the Mann-Whitney test - StatView Software version 5.0 (SAS Institute, Cary, NC).

Matrigel invasion assay

Cell invasion was studied by using BD Biocoat™ Matrigel™ invasion chambers with 8-µm size pores (BD Biosciences), according to the manufacturer's instructions. MKN45 and GP202 cells in culture were harvested and seeded in duplicate at 250,000 cells per insert (sized for 24-well plates) in 1% FBS containing medium, and 20% FBS containing medium was added to the bottom of the growth well, as an attractant. Cells were allowed to invade for 22 h (37ºC, 5% CO_2 atmosphere). The non-invading cells were then swabbed from the top of the inserts and the invading cells on the lower surface were fixed with 100% methanol and stained with DAPI for 15 min in the dark. The membranes were removed and cells were counted under a Leica DMIRE2 fluorescence microscope. Data are expressed as a ratio of the results obtained with each

clone and the scrambled control, from three independent experiments. Statistical analysis was performed using the Mann-Whitney test - StatView Software version 5.0 (SAS Institute, Cary, NC).

Cell-cell Aggregation assay

MKN45 and GP202 cells in culture were harvested and seeded in duplicate at 250,000 cells per well in 24-well plates. Plates were placed at 37ºC with constant stirring (150rpm) for 1 and 2 h. Cells were fixed with 100 µl of 25% glutaraldehyde at time zero and at the end of the incubation. Aggregates were photographed under a light microscope and isolated cells were counted (cells in duplicates were counted as isolated cells). The aggregation index was defined taking into account the number of isolated cells at Tn and number of isolated cells at T0. Data are expressed as a ratio of the results obtained with each clone and the scrambled control, from three independent experiments. Statistical analysis was performed using the Mann-Whitney test - StatView Software version 5.0 (SAS Institute, Cary, NC).

***In Vivo* mice assays**

Six-week-old female N:NIH(s)II:nu/nu nude mice were obtained previously from the Medical School, University of Cape Town in 1991 and then reproduced, maintained and housed at IPATIMUP Animal House at the Medical Faculty of the University of Porto, in a pathogen-free environment under controlled conditions of light and humidity. Males and females, aged 6-8 weeks, were used for *in vivo* experiments. Animal experiments were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals, directive 86/609/EEC. Mice were subcutaneously injected in the dorsal flanks using a 25-gauge needle with $1x10^5$ of MKN45-SC (2 male and 2 female mice) or MKN45-C2 cells (3 male and 3 female mice). Mice were weighed, and tumor width and length were measured with calipers every week. Mice were euthanized 21 days after cell injection, time when there was the need of euthanizing the first mouse. For statistical analysis, the Mann Whitney test - StatView Software version 5.0 (SAS Institute, Cary, NC) was used. A *P* value of less than 0.05 was considered as statistically significant.

Phospho-antibody screening (Kinexus)

MKN45 and GP202 pre-confluent cells were lysed by the addition of lysis buffer (20 mM Morpholino propane sulfonic acid (Mops), pH 7.0, 2 mM Ethylene glycol tetraacetic acid (EGTA), 5 mM Ethylene diamine tetraacetic acid (EDTA), 30 mM sodium fluoride, 60 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM PMSF, 3 mM benzamidine, 5 μM pepstatin,10 μM leupeptin, and 0.5% Triton X-100; final pH 7.0). Cells sonication was performed twice for 15 s and the homogenate was subjected to ultracentrifugation for 30 min at 50,000 rpm.

Protein concentration was measured by the Bio-Rad assay and phospho-kinase screening was performed by Kinexus (Vancouver, BC; KPSS-10.1).

Proximity Ligation Assays

MUC1-CD antibody (MUC1-CT2, ThermoScientific) is produced in armenian hamster. Since there are no probes against hamster antibodies commercialized by O-link Biosciences, we have first labeled the antibody with a kit, the Duolink II Probemarker Plus (OLink Bioscience), according to the manufacturer's instructions.

MKN45-SC and MKN45-C2 clones were grown until subconfluency in round slides, fixed with PF 4% 120mM sucrose and permeabilized with Triton-X at RT and washed with PBS. We have followed the instructions of the kit DII anti-rabbit minus or the DII anti-mouse minus (both OLink Bioscience), followed by the detection kit DII Det. Reag.Orange (OLink Bioscience). Primary antibodies used were anti-MUC1-CT2 1:300 (labelled in the previous step), anti-ERK1/2 1:100 (Cell Signaling), anti-EGFR 1:50 (Santacruz Biotec.), anti-B-RAF 1:50 (Santacruz Biotec.), anti-Grb2 (1:50, Santacruz Biotec), anti CDK1/2 1:50 (Santacruz Biotec.), anti-B23 1:50 (Santacruz Biotec.), anti-E-cadherin 1:500 (BD Transduction Laboratories) and anti β-catenin 1:100 (BD Transduction Laboratories). Fluorescence spots were observed under a DMIRE2 fluorescence microscope and quantified using the Blobfinder software [147]. Negative controls (MKN45-SC (-)) correspond to the assay with the MUC1-CD labeled antibody only. The AGS cell line was also used to study the interaction between MUC1-CD and ERK1/2, using the same conditions described before.

PLA assays were also performed in both normal and GC tissue slides, using the same conditions described, after deparaffinization and rehydratation with xylene and ethanol. Antigen retrieval was performed by the method of Sodium Citrate Buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0). A positive control of the PLA assay in tissues was used with a GC slide and the hybridoma supernatants PMH1 and TKH2, for MUC2 and sialyl-Tn, a well known interaction in this tissue.

Results

Results

1. *Evaluation of the impact of MUC1 expression and variability (VNTR polymorphism) in H. pylori adhesion to GC cells.*

Evaluation of *H. pylori* adhesion shows that the pathogenic strain HP26695 (positive for the virulence factors *cagA* and *vacA*) has significantly ($P < 0.05$) higher adhesion values for both GP202 and MKN45 cell lines (1.97±0.10 and 1.47±0.06) when compared with the non-pathogenic strain HPTx30a (negative for the virulence factors *cag*A and *vac*A) (1.40±0.15 and 0.85±0.15) **(Figure 6)**.

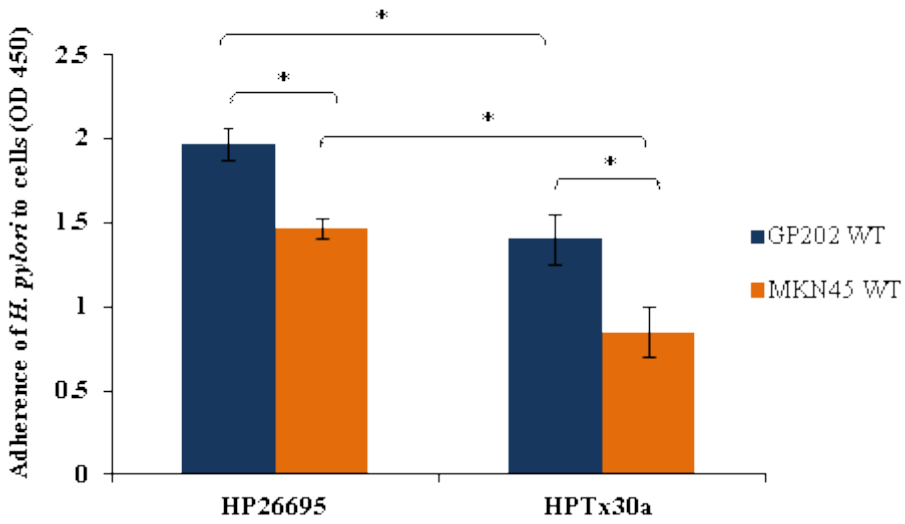


Figure 6 > Adhesion of HP26695 and HPTx30a *H. pylori* strains to GP202 and MKN45GC cell lines.* $P < 0.05$ (Mann-Whitney test).

Furthermore, GP202 cell line shows higher adhesion levels than MKN45 cell line for both bacterial strains (HP26695 strain 1.97 ± 0.10 *vs* 1.47 ± 0.06; HPTx30a strain 1.40 ± 0.15 and 0.85 ± 0.15) **(Figure 6)**.

This statistically significant association between pathogenicity and higher adhesion (strain HP26695 *vs* HPTx30a) is also observed for the GP202 MUC1 recombinant clones (GP202-Neo 1.1 ± 0.10 *vs* 0.72 ± 0.06; GP202-ΔTR 1.32 ± 0.09 *vs* 1.0 ± 0.10; GP202-3TR 1.45 ± 0.08 *vs* 1.18 ± 0.05; GP202-9TR 2.2 ± 0.12 *vs* 1.96 ± 0.12; and GP202-42TR 2.3 ± 0.07 *vs* 1.89 ± 0.11) **(Figure 7)**.

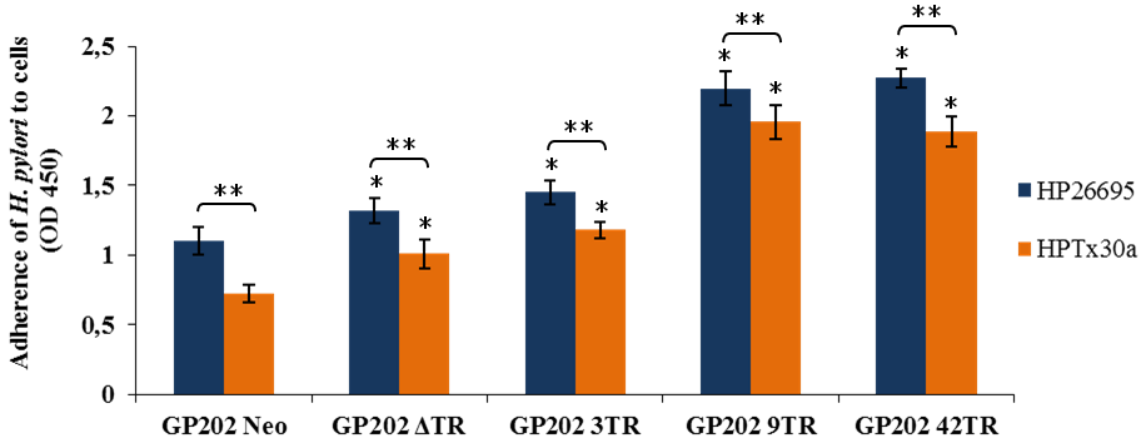


Figure 7 > Adhesion of HP26695 and HPTx30a *H. pylori* strains to GP202 transfectants GP202-Neo, GP202-ΔTR, GP202-3TR, GP202-9TR and GP202-42TR. * $P < 0.05$, compared to the control (GP202 Neo) and ** $P < 0.05$ (Mann-Whitney test).

Adhesion of both *H. pylori* strains (HP26695 and HPTx30a) is significantly higher in all the GP202-MUC1 transfectants overexpressing MUC1 (GP202-ΔTR 1.32 ± 0.09 and 1.0 ± 0.10; GP202-3TR 1.45 ± 0.08 and 1.18 ± 0.05; GP202-9TR 2.2 ± 0.12 and 1.96 ± 0.12; GP202-42TR 2.3 ± 0.07 and 1.89 ± 0.11) when compared with the control, GP202-Neo (1.1 ± 0.10 and 0.72 ± 0.06) **(Figure 7)**. There is also an association between the increased number of TR and increased adhesion, for both strains **(Figure 7)**.

2. *Evaluation of MUC1 impact on gene expression, phenotype and tumorigenicity of GC cells.*

For the MKN45 GC cell line, we established by shRNA two independent MUC1 downregulated clones, MKN45-C1 and MKN45-C2, and one scrambled control, MKN45-SC. MUC1 downregulation was verified by immunofluorescence **(Figure 8A)**, confocal microscopy **(Figure 8B)**, Western blot **(Figure 8C)** and Real-Time PCR **(Figure 8D)**.

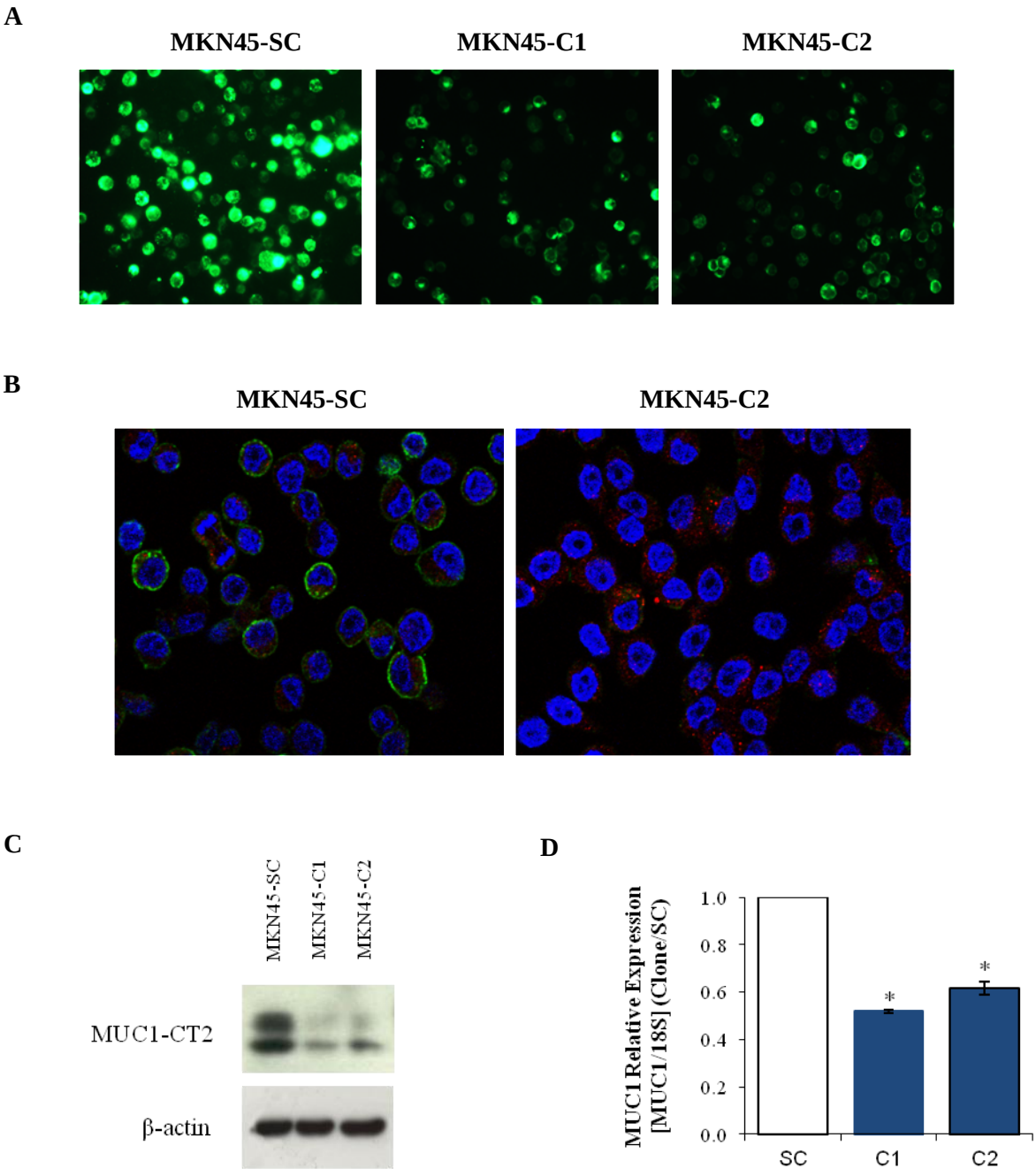


Figure 8 > MUC1 downregulation by shRNA in MKN45 GC cell line at 96 h in culture.

(A) MUC1-VNTR detection in green by immunofluorescence with HMFG-1 antibody in MKN45-C1 and MKN45-C2 and MKN45-SC control; **(B)** MUC1-CD protein detection by confocal microscopy (MUC1-CD in green, the endoplasmic reticulum in red and the nuclei in blue); **(C)** MUC1-CD protein detection by western blot with MUC1-CT2 and β-actin antibodies of total protein extracts from MKN45-C1, MKN45-C2 and MKN45-SC control; **(D)** Quantification of MUC1 RNA in MKN45-C1, MKN45-C2 and MKN45-SC control by Real-Time PCR. MUC1 expression was corrected to the house-keeping gene 18S and normalized to the data obtained with the scrambled control. *P < 0.01 (Mann-Whitney test).

For the GP202 GC cell line, two independent MUC1 downregulated clones, GP202-C1 and GP202-C2, and one scrambled control, GP202-SC were also established and downregulation verified by immunofluorescence **(Figure 9A)**, Western blot **(Figure 9B)** and Real-Time PCR **(Figure 9C)**.

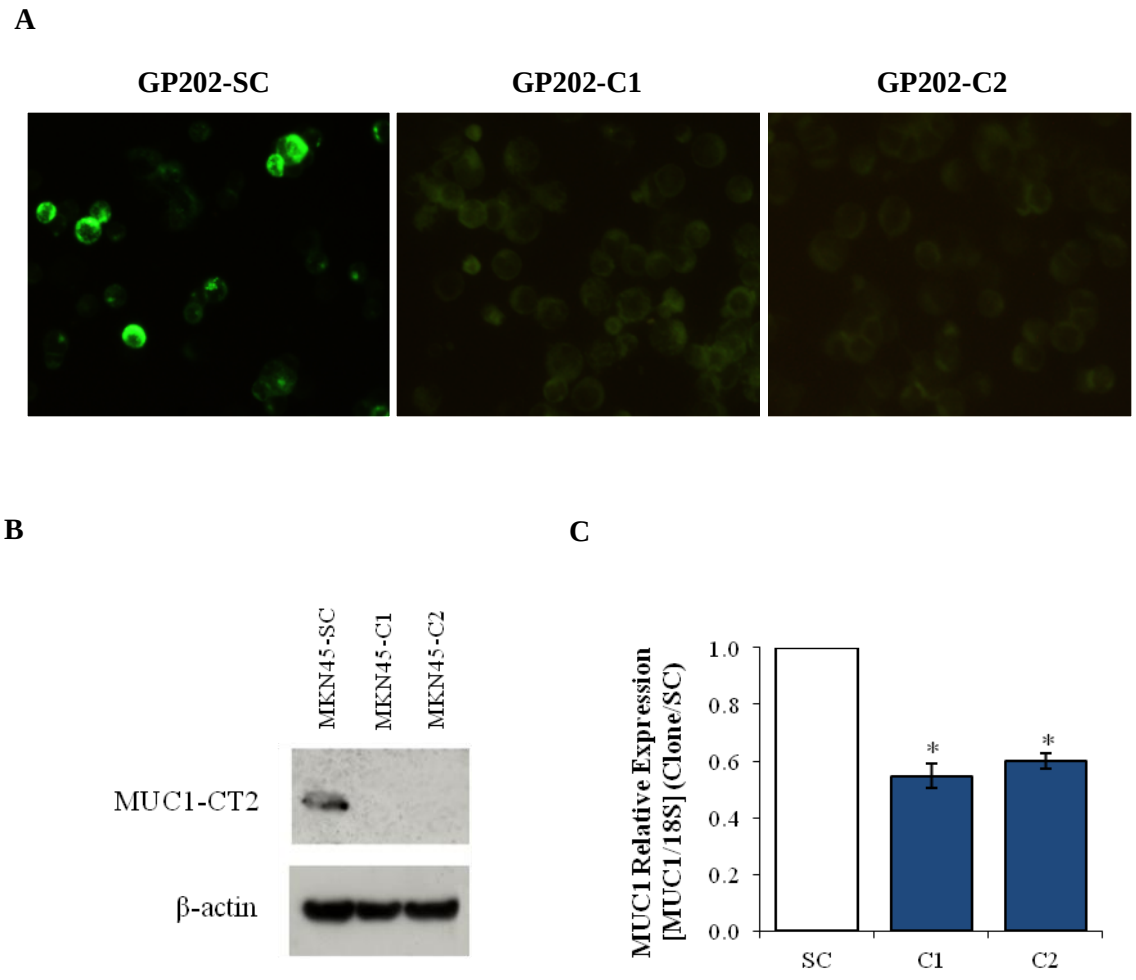


Figure 9 > MUC1 downregulation by shRNA in GP202 GC cell line at 48 h in culture.

(A) MUC1-VNTR detection in green by immunofluorescence with HMFG-1 antibody in GP202-C1 and GP202-C2 and GP202-SC control; **(B)** MUC1-CD protein detection by western blot with MUC1-CT2 and β-actin antibodies of total protein extracts from GP202-C1, GP2025-C2 and GP202-SC control; **(C)** Quantification of MUC1 RNA in GP202-C1, GP202-C2 and GP202-SC control by Real-Time PCR. MUC1 expression was corrected to the house-keeping gene 18S and normalized to the data obtained with the scrambled control. *$P < 0.01$ (Mann-Whitney test).

There was a significant downregulation of MUC1 expression in MKN45-C1, MKN45-C2, GP202-C1 and GP202-C2 clones when compared to the respective MKN45-SC and GP202 controls. The expression of MUC1 at the protein level was detected with two different antibodies, one that binds the VNTR extracellular domain (HMFG-1, **Figure 8A and 9A**) and other that recognizes a 14-28 KDa sequence in MUC1-CD (MUC1-CT2, **Figure 8C and 9B**). Both showed a significant reduction of the amount of MUC1 protein in MUC1 downregulated clones when compared to the scrambled controls. Real-Time PCR results indicate that the MUC1 downregulation was 48% (MKN45-C1) and 38% (MKN45-C2) **(Figure 8D)** and 48% (GP202-C1) and 42% (GP202-C2) **(Figure 9C)**. MUC1 RNA levels were evaluated at 48, 72 and 96h of cell culture and the highest downregulation has occurred at 96h for MKN45 cells and at 48h for GP202 cells (results not shown). All the further assays were performed with cells at these time points for each cell line.

2.1. Effects of MUC1 downregulation on MKN45 and GP202 cells global gene expression

We evaluated the overall effects of downregulating MUC1 in the MKN45 and GP202 GC cell lines by performing a global analysis of gene expression by oligonucleotide microarrays **(Tables 1 and 2**, respectively**)**. Only differences that were observed for both clones were considered and are shown. The results revealed that a number of genes that influence proliferation, migration, invasion and motility were differentially expressed in MKN45-C1 and MKN45-C2 clones and the MKN45-SC control. The most significant differences were found for TCN1, KLK6 and ADAM29 genes (>10 fold upregulated between the MKN45-C1 and MKN45-C2 clones and the MKN45-SC control) and LGALS4, TSPAN8 and SHPS-1 (> 3.5 fold downregulated between the MKN45-C1 and MKN45-C2 clones and the MKN45-SC control).

Table 1. Oligonucleotide microarray results by comparison between MKN45-C1/MKN45-C2 and MKN45-SC control cells, by order of magnitude.

Genes upregulated > 2 fold in MKN45-C1 and MKN45-C2 downregulated clones	Gene function
Transcobalamin 1 (TCN1)	Vitamin B12 transport to cells [148]
Kallikrein-related peptidase 6 (KLK6)	Serine protease [149]
Desintegrin and metalloproteinase 29 (ADAM29)	Cell-cell and cell-matrix interactions [150]
Keratoepithelin **(**TGFBI)	Anti adhesion cell molecule [151]
MRP family of ATP transport member 2 (ABCC2)	Multi drug resistance molecule [152]
Amyloid beta precursor-like protein 2 (APLP2)	Synaptic transmission [153]
Mitochondrial ATP synthase (ATP5I)	ATP synthesis [154]
Sulfide dehydrogenase like protein (SQRDL)	Involved in metabolic syndrome [155]
Sarcoglycan, epsilon (SGCE)	Cell-matrix interactions [156]
Hypothetical protein (FLJ20323)	Unknown
Galectin 1 (LGALS1)	Cell-cell and cell-matrix interactions [157]
Proline-histidine rich protein (PHLDA1)	Anti-apoptotic effect [158]
Trypsin 2 (PRSS2)	Serine protease [159]
Mesotrypsin (PRSS3)	Serine protease [160]
SP2 transcription factor (SP2)	Regulation of chorionic gonadotropin hormone expression [161]
Ubiquitin-conjugating enzyme (UBE2L6)	Ubiquitinilation of proteins [162]
Vitellogenic-like carboxypeptidase (CPVL)	Carboxypeptidase activity [163]
Genes downregulated > 2 fold in MKN45-C1 and MKN45-C2 downregulated clones	**Gene function**
Galectin 4 (LGALS4)	Cell-cell and cell-matrix interactions [164]
Tetraspanin 8 (TSPAN8)	Cell motility [165]
Tyrosine phosphatase SHP substrate (SHPS-1)	Regulation Insuline growth factor1 [166]
Polymerase (DNA-directed), delta 4 (POLD4)	Polymerase activity [167]
H2B histone family, member J (HIST1H2BH)	Nucleossome Structuration
H2B histone family, member T (HIST1H2Bk)	Nucleossome Structuration
Carcinoembryonic molecule 5 (CEACAM5)	Cell adhesion [168]
Annexin IV (ANXA4)	Cellular dissemination [169]
Intercellular adhesion molecule 4 (ICAM4)	Cell extravasation [170]
Polypeptide 39 (DDX39)	RNA helicase [171]
Apolypoprotein B (APOBEC2)	Citidine-deaminase activity [172]
Clusterin (CLU)	Apoptosis [173]
GDP-mannose 4,6-dehydratase (GMDS)	Fucose synthesis [174]
Serine/threonine kinase 38 like (STK38L)	Serine/threonine kinase [175]
CD55 (CD55)	Complement system activation [176]
Apolipoprotein 3C (APOBEC3C)	Citidine-deaminase activity [177]
Cell adhesion related-molecule (CDON)	Cell-cell interaction and differentiation [178]
Villin-1 (VIL1)	Migration and actin organization [179]

In the case of GP202 cells, only three genes were differentially expressed between MUC1 downregulated clones and the control. There is no direct association between the genes with altered expression levels and migration, invasion and motility upon MUC1 downregulation, as found for MKN45 GC cell line.

Table 2. Oligonucleotide microarray results by comparison between GP202-C1/GP202-C2 and GP202-SC control cells, by order of magnitude.

Genes downregulated > 2 fold in GP202-C1 and GP202-C2 downregulated clones	Gene function
Calcium P-protein (S100P)	Differentiation, Cell cycle progression [180]
α-protein interferon- induced (ISG15)	Ubiquitin-proteossome regulation [181]
β-tubulin 2A (TUBB2A)	Cytoskeleton organization [182]

2.2. Effects of MUC1 downregulation on MKN45 and GP202 cells phenotype

In the previous immunofluorescence assays **(Figures 8A and 9A)** it has been shown that MKN45-SC cells express a higher amount of MUC1 when compared to GP202-SC cells. Furthermore, downregulation levels differ between cell lines in terms of total MUC1 molecules present upon downregulation. Thus, it is expectable that MUC1 downregulation has different effects in these cells phenotypic characteristics. Levels of cell proliferation, apoptosis, aggregation, migration and invasion were measured for both GC cell lines.

2.2.1. Cell proliferation

MKN45-C1 and MKN45-C2 cells showed significantly increased proliferation rates ($P < 0.01$) when compared to the MKN45-SC control (2.29 and 2.48 *vs* 1), when evaluated by MTT assay **(Figure 10A)**. In the case of GP202 cells, the results were not consistent since one of the clones showed decreased proliferation and the other showed increased proliferation ($P < 0.01$, 0.30 and 2.14 *vs* 1) **(Figure 10 B)**.

A **B**

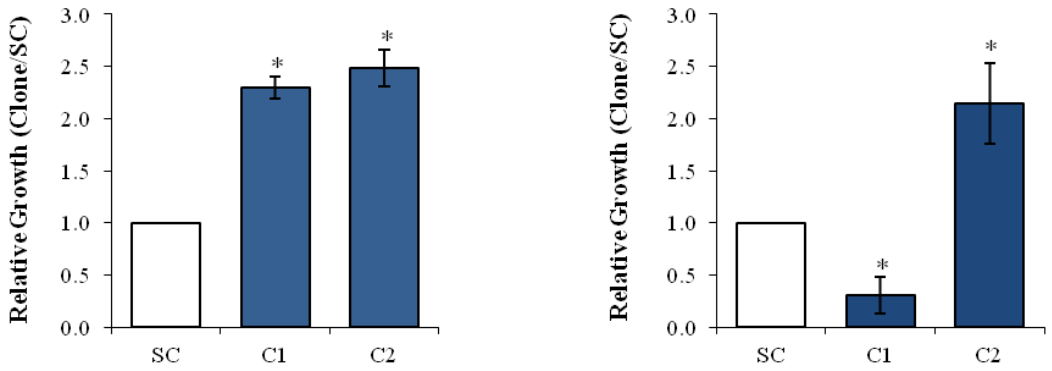


Figure 10 > Quantification of cell proliferation by MTT assay**.**

(A) Quantification of metabolically active cells by MTT assay in MKN45-C1 and MKN45-C2 clones and MKN45-SC control at 96h in culture and **(B)** quantification of metabolically active cells by MTT assay in GP202-C1 and GP202-C2 clones and GP202-SC control at 48h in culture. Data from 24 h were used to set time zero and results were normalized to the data obtained with the scrambled control.*$P < 0.01$ (Mann-Whitney test).

2.2.2. Cell apoptosis

MKN45-C1 and MKN45-C2 cells showed significantly increased levels of apoptosis ($P < 0.01$) when compared to the MKN45-SC control (3.32 and 2.41 *vs* 1), when evaluated by a TUNEL assay **(Figure 11A)**. In the case of GP202 cells, the apoptosis levels of GP202-C1 and GP202-C2 results were not significantly different when compared to the control, GP202-SC (1.28 and 1.62 *vs* 1) **(Figure 11 B)**.

A **B**

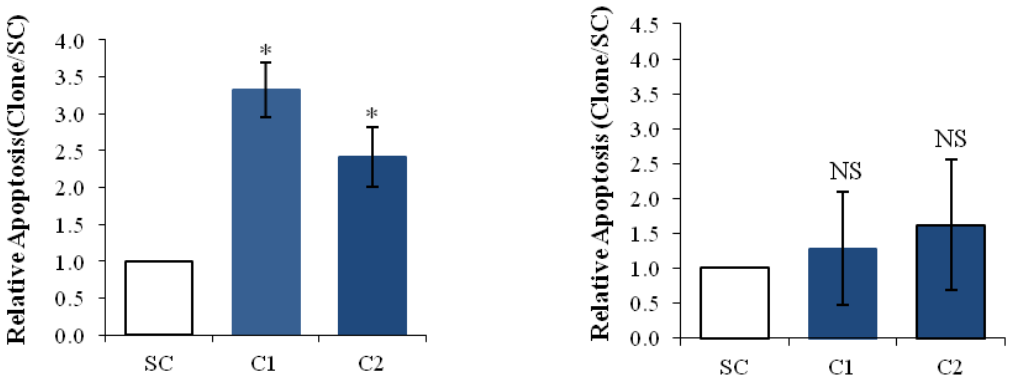


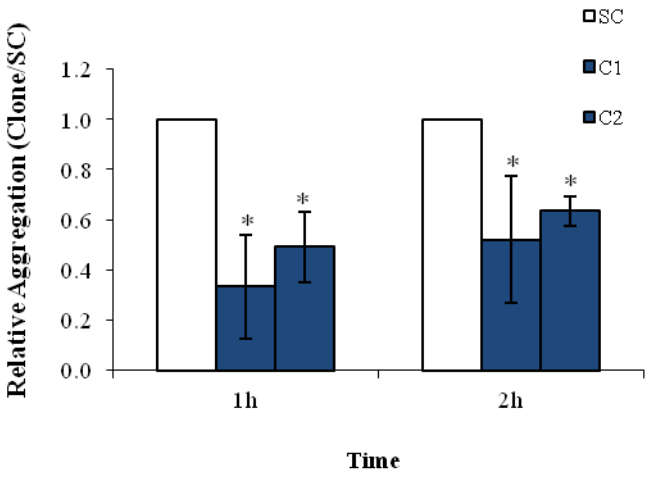
Figure 11 > Quantification of apoptotic cells by TUNEL assay.

(A) Apoptosis of MKN45-C1 and MKN45-C2 clones and MKN45-SC control was evaluated at 96h in culture by a TUNEL assay. Results were normalized to the data obtained with the scrambled control; **(B)** Apoptosis of GP202-C1 and GP202-C2 clones and GP202-SC control were also evaluated at 48h in culture. Results were normalized to the data obtained with the scrambled control. *$P < 0.01$, NS- not significant (Mann-Whitney test).

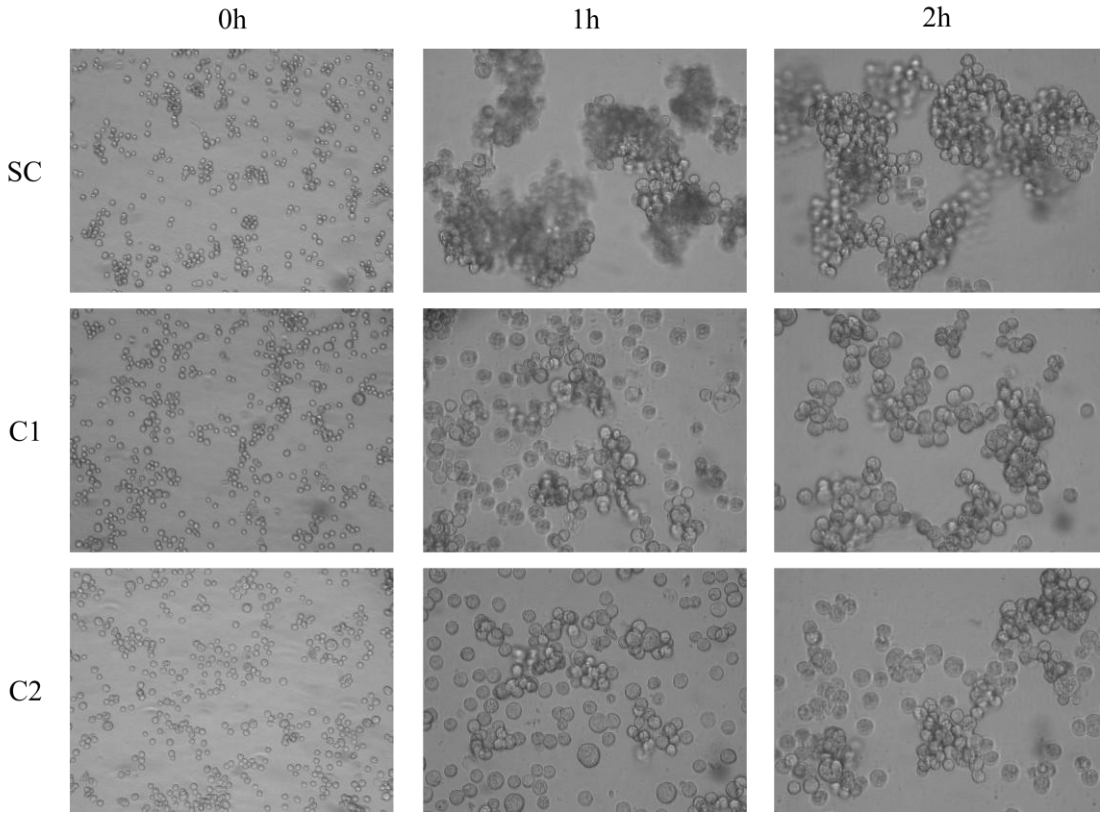
2.2.3. Cell-cell aggregation

MKN45-C1 and MKN45-C2 cells showed significantly decreased cell-cell aggregation levels ($P < 0.01$), when compared to the MKN45-SC control (0.34 and 0.49 *vs* 1 at 1h; 0.52 and 0.64 *vs* 1 at 2h), when evaluated by a cell aggregation assay **(Figures 12A1)**. In the case of GP202 cells, GP202-C1 and GP202-C2 cells showed significantly decreased cell-cell aggregation levels ($P < 0.01$), when compared to the GP202-SC control but only after 2 h of stirring (0.64 and 0.66 *vs* 1 at 2h) **(Figure 12B1)**.

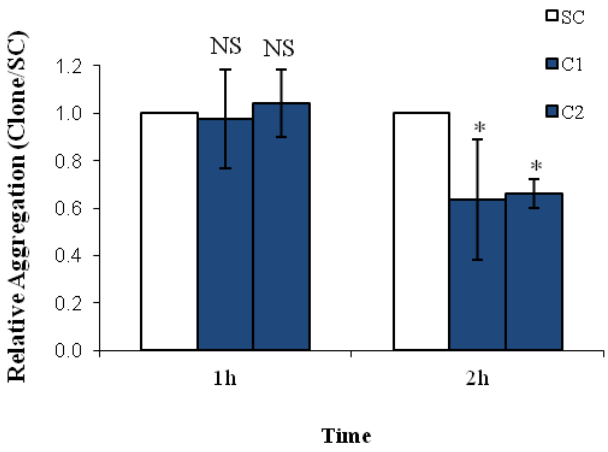
A1


SC
C1
C2
Relative Aggregation (Clone/SC)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
*
1h
2h
Time

A2


0h
1h
2h
SC
C1
C2

B1



B2

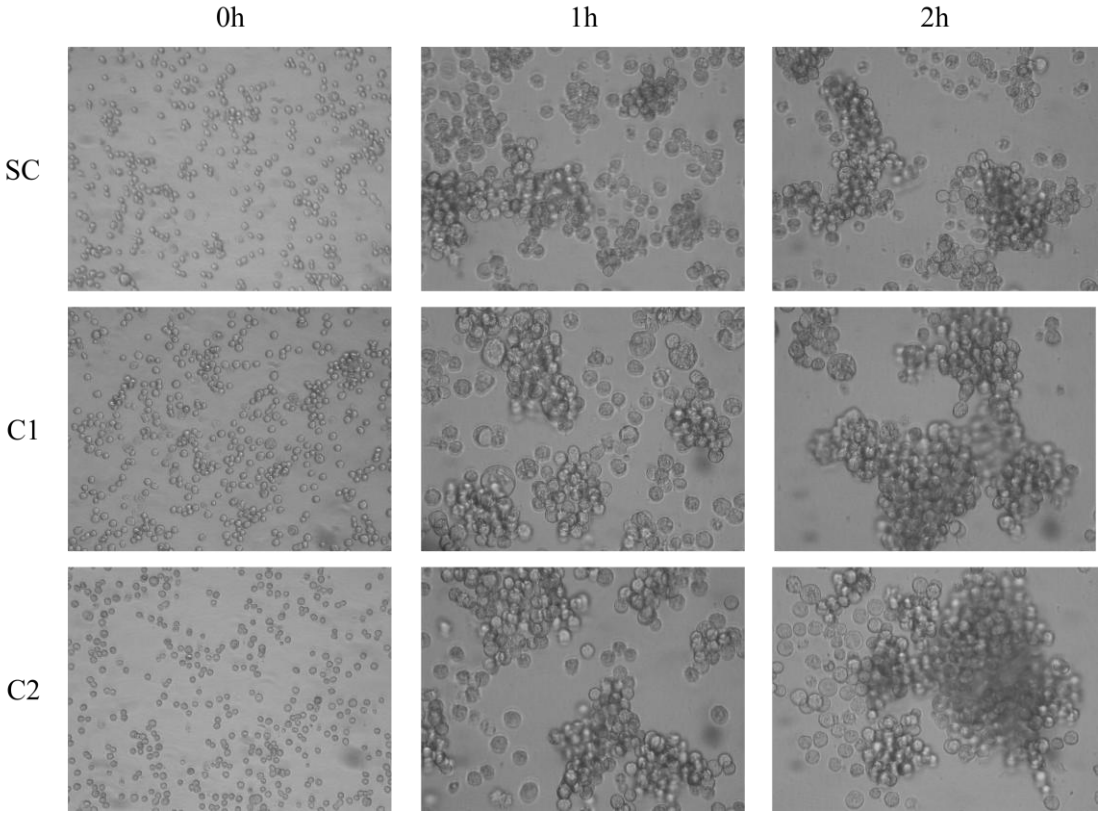


Figure 12 > Quantification of cell-cell aggregation by a cell aggregation assay.

(A1 and A2) Quantification of the cell-cell aggregation index in MKN45-C1, MKN45-C2, GP202-C1 and GP202-C2 clones and MKN45-SC and GP202-SC controls. The cell-cell aggregation index was assessed by scoring the number of isolated cells over time, and normalized to the data obtained with the scrambled control. *P < 0.01 , NS- not significant (Mann-Whitney test); **(B1 and B2)** Images of the aggregates formed after 1 and 2 h of constant stirring. First column shows isolated cells at time 0h (20x magnification) and second and third columns show aggregates formed after 1h and 2h of stirring (40x magnification), in MKN45-C1,MKN45-C2, GP202-C1 and GP202-C2 clones and MKN45-SC and GP202-SC controls.

2.2.4. Cell invasion and migration

There were no significant differences in cell invasion of MKN45-C1 and MKN45-C2 clones when compared to the MKN45-SC control, when evaluated by invasion assays (results not shown). However, differences were observed concerning the GP202 cell line. GP202-C1 and GP202-C2 showed significantly increased invasion levels ($P < 0.01$) when compared to GP202-SC control (3.29 and 4.14 *vs* 1) **(Figure 13)**.

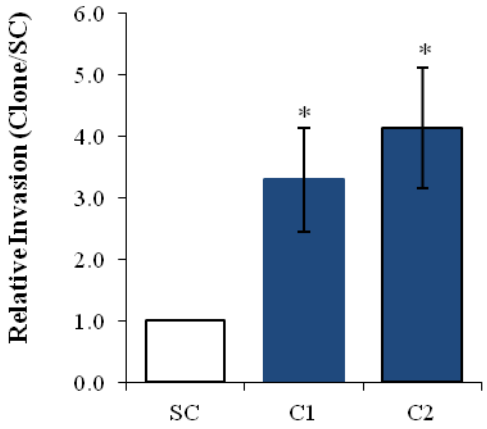


Figure 13 > Quantification of cell invasion by cell invasion assay.
Quantification of the cell invasion in GP202-C1 and GP202-C2 clones and GP202-SC control. *$P < 0.01$ (Mann-Whitney test).

In terms of cell migration, GP202-C1 and GP202-C2 cells showed significantly increased migration levels ($P < 0.01$) when compared to GP202-SC control (2.61 and 2.13 *vs* 1) **(Figure 14A)**, whereas no significant differences were found for MKN45-C1 and MKN45-C2 when compared to MKN45-SC control (1.23 and 0.66 *vs* 1) **(Figure 14B)**.

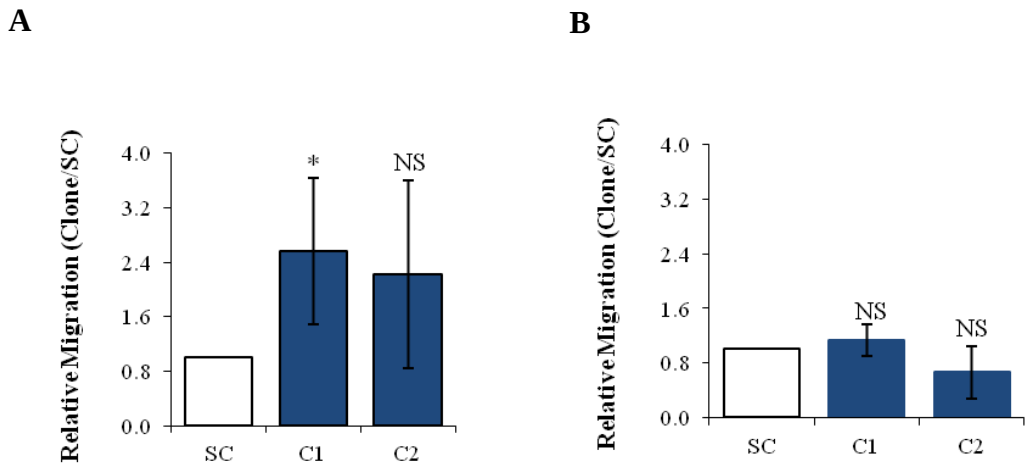


Figure 14 > Quantification of cell migration by wound-healing assay.

(A) Quantification of the cell migration in GP202-C1 and GP202-C2 clones and GP202-SC control and **(B)** MKN45-C1 and MKN45-C2 clones and MKN45-SC control by a wound-healing assay. *$P < 0.01$, NS- not significant (Mann-Whitney test).

2.3. Effects of MUC1 downregulation in MKN45 GC cells tumorigenicity

Our next step was to study the effects of MUC1 suppression in the tumorigenic potential of MKN45 GC cells *in vivo*. Mice injected with MKN45-C2 MUC1 downregulated clones have shown smaller and slower-growing tumors when compared to mice injected with the MKN45-SC control cells **(Figure 15)**.

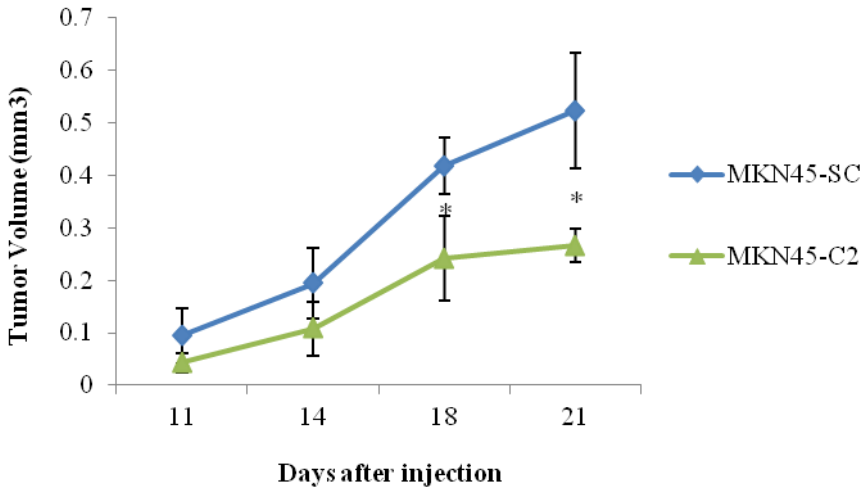


Figure 15 > Tumor growth curves. 1 x 10^5 cells were subcutaneously injected in mice at day 0. The curves show tumor growth until day 21, the day on which all mice were sacrificed.* $P < 0.05$ (Mann-Whitney test), when compared to the MKN45-SC control cell line.

3. Identification of MUC1-mediated oncogenic signaling pathways in GC cells.

The phenotypic modifications observed in MUC1 downregulated clones were likely due in part to alterations in signal transduction pathways mediated by MUC1-CD, since overexpression of MUC1 has been shown to modulate gene expression through reprograming transcription of multiple genes [101,113,114,115,116,117,118,119].

3.1. Interaction between MUC1-CD and ERK1/2 signaling effectors in MKN45 GC cells - Immunoprecipitation assays

We started by studying the molecule ERK1/2, a member of the MAPK signaling pathway often found to be deregulated in GC, as mentioned before. MUC1 was shown to interact with ERK1/2 in MKN45 control and downregulated cells, as shown by immunoprecipitation assays **(Figure 16)**.

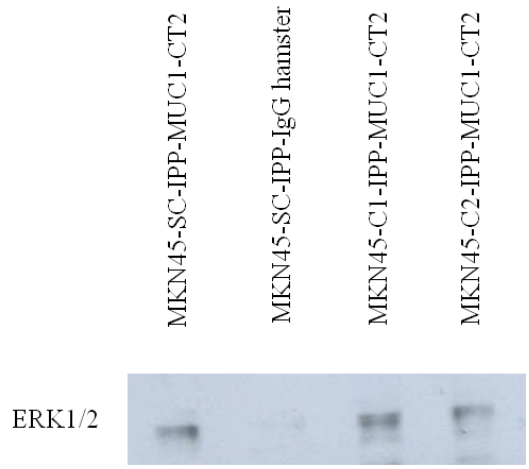


Figure 16 > In the figure above, MKN45-SC, C1 and C2 lysates were immunoprecipitated (IPP) with anti-MUC1-CT2 and with an hamster control IgG and blotted with anti-ERK1/2 antibody.

3.2. Interaction between MUC1-CD and MAPK signaling effectors - PLA assays

Due to higher sensitivity of PLA technique, PLA assays were performed to confirm previously found MUC1-CD-ERK1/2 interaction and also in an attempt to find new ones. We have looked for molecules related to ERK1/2 proteins and MAPK signaling pathway (such as EGFR, B-RAF and Grb2).

The interaction between MUC1-CD and ERK1/2 was confirmed **(Figure 17)** and we have also found interactions between MUC1-CD and EGFR, B-RAF and Grb2 **(Figures 18, 19 and 20).**

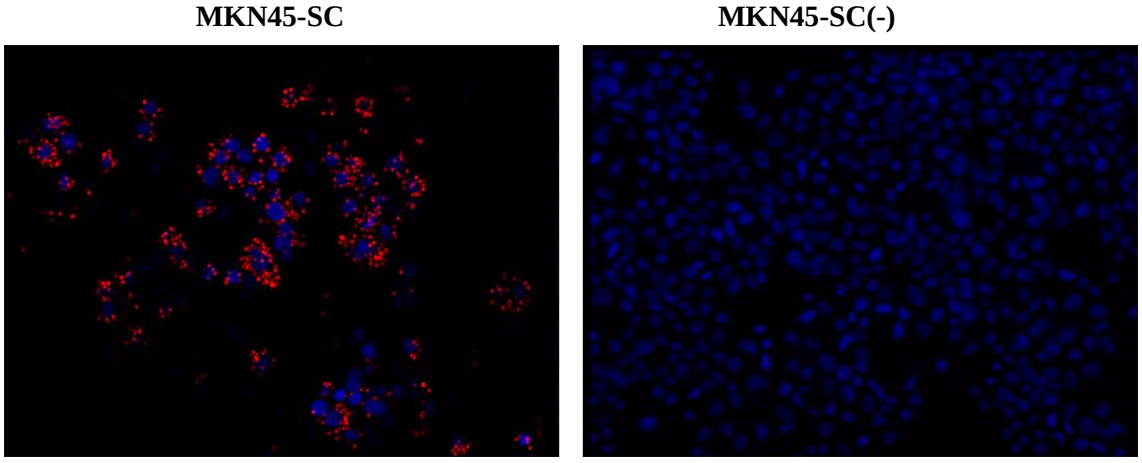


Figure 17 > *In situ* PLA assay. PLA assay was performed using MKN45-SC cells and the antibodies MUC1-CT2 and ERK1/2 (signal from interaction of both molecules is in red). MKN45-SC(-) – negative control.

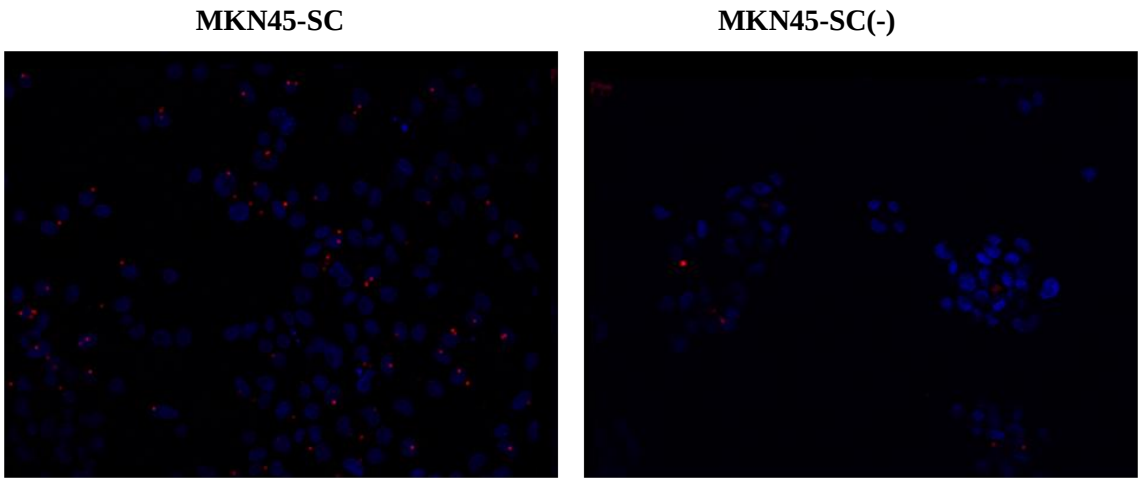


Figure 18 > *In situ* PLA assay. PLA assay was performed using MKN45-SC cells and the antibodies MUC1-CT2 and EGFR (signal from interaction of both molecules is in red). MKN45-SC(-) – negative control.

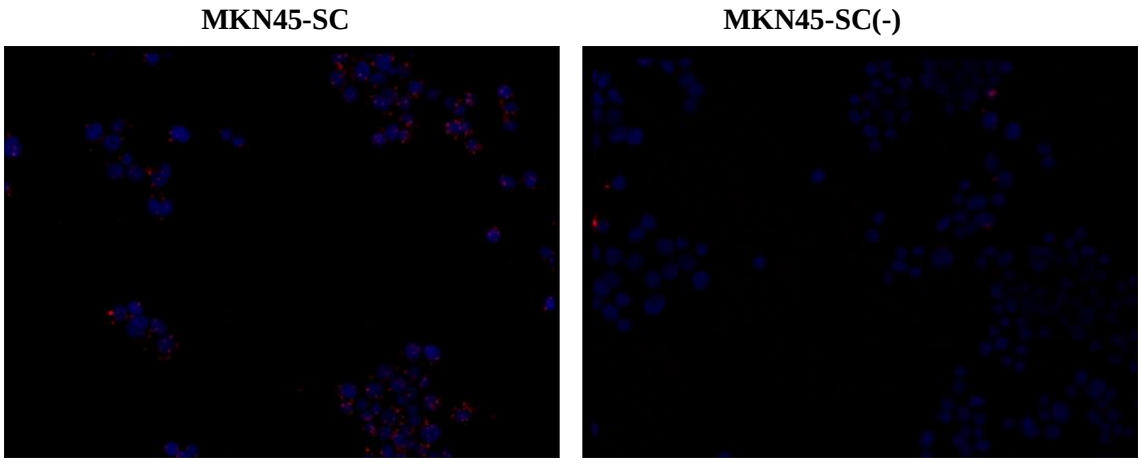


Figure 19 > *In situ* PLA assay. PLA assay was performed using MKN45-SC cells and the antibodies MUC1-CT2 and B-RAF (signal from interaction of both molecules is in red). MKN45-SC(-) – negative control.

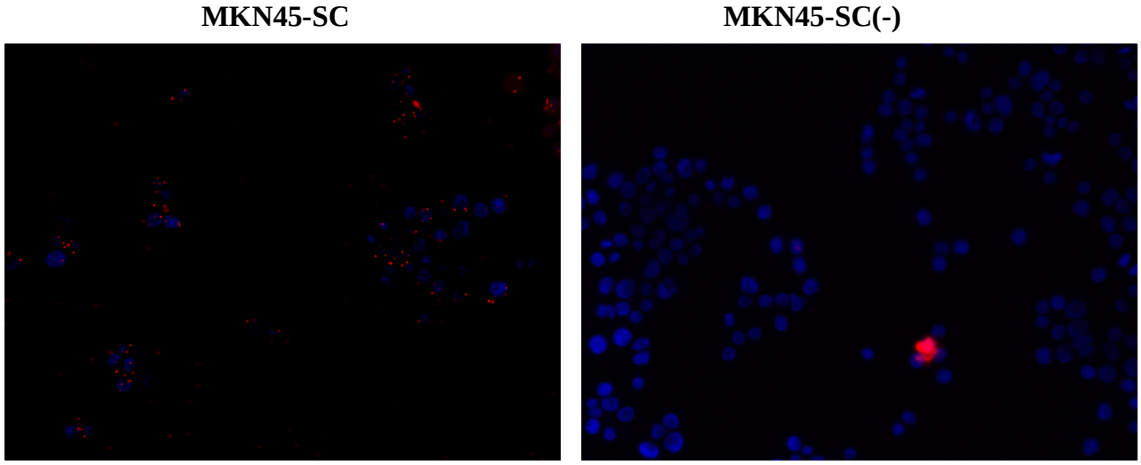


Figure 20 > *In situ* PLA assay. PLA assay was performed using MKN45-SC cells and the antibodies MUC1-CT2 and Grb2 (signal from interaction of both molecules is in red). MKN45-SC(-) – negative control.

An average of the number of interactions found between MUC1-CD and the studied MAPK signaling molecules was calculated with the Blobfinder Software (OLINK Biosciences) **(Figure 21)**.

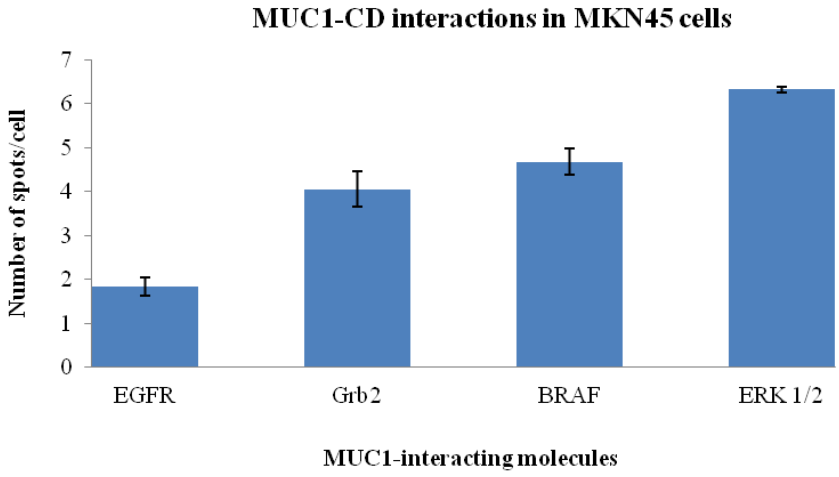


Figure 21 > Average of number of interactions between MUC1-CD and MAPK signaling effectors in MKN45-SC GC cells, as found by PLA assays.

3.3. Interaction between MUC1-CD and other possible MUC1 oncogenic signaling partners in MKN45 GC cell line - PLA assays

In an attempt to find new MUC1-CD interactions with oncogenic signaling partners in GC cells, we have studied the possible interaction between this molecule and cell cycle-related molecules such as B23 and CDK1/2, known to be involved in tumorigenic events. The interaction was found to exist for both molecules **(Figures 22 and 23)**.

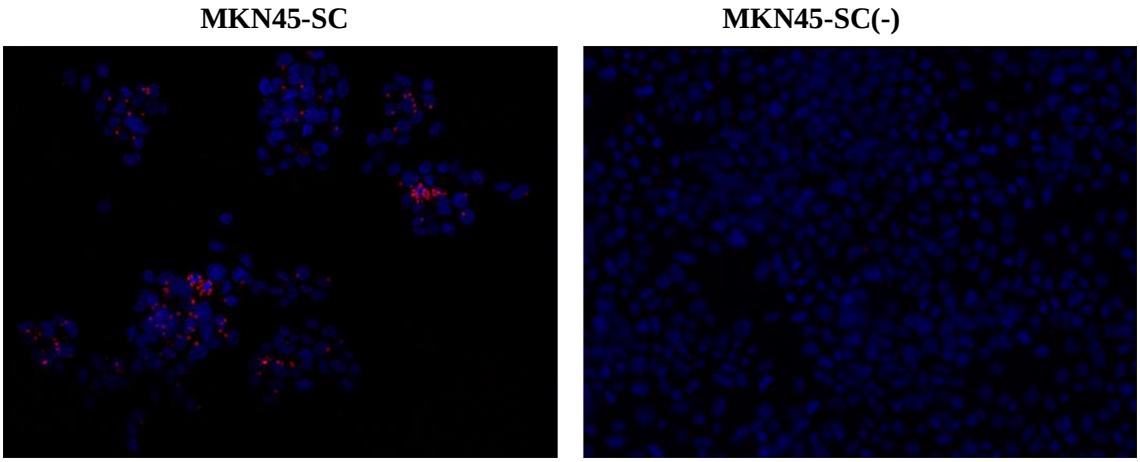


Figure 22 > *In situ* PLA assay. PLA assay was performed using MKN45-SC cells and the antibodies MUC1-CT2 and B23 (signal from interaction of both molecules is in red). MKN45-SC(-) – negative control.

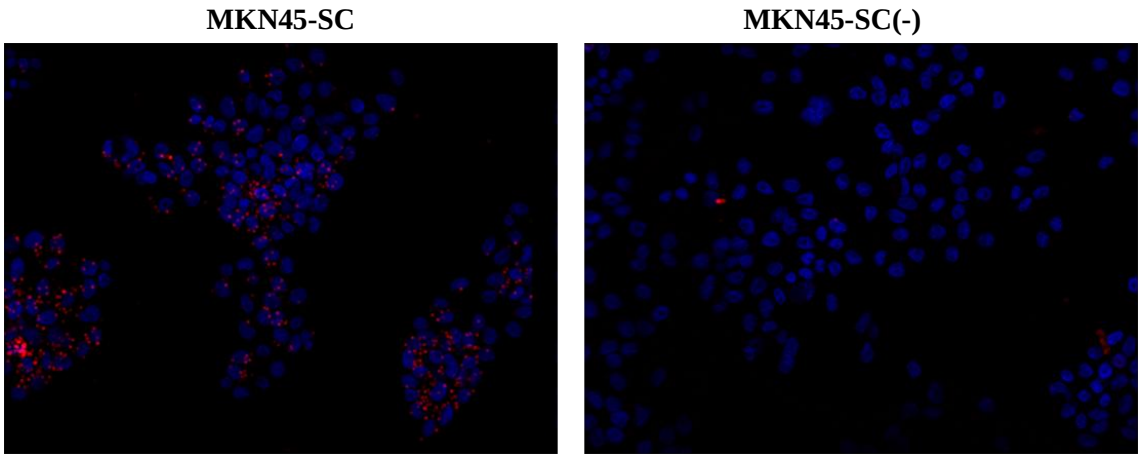


Figure 23 > *In situ* PLA assay. PLA assay was performed using MKN45-SC cells and the antibodies MUC1-CT2 and CDK1/2 (signal from interaction of both molecules is in red). MKN45-SC(-) – negative control.

We further evaluated the possible interaction of MUC1-CD with E-cadherin and β-catenin, molecules known to be deregulated in most cases of cancer. The result was positive for both molecules **(Figures 24 and 25)**.

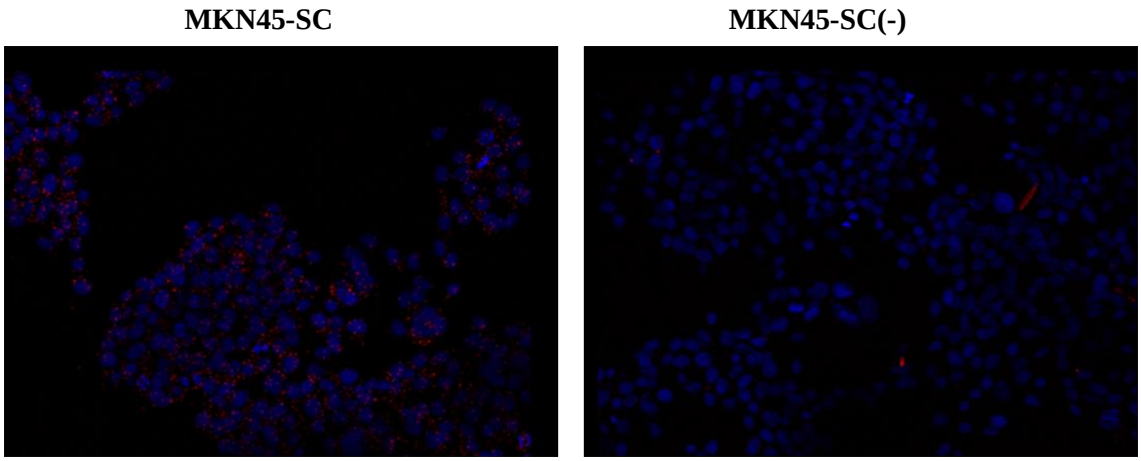


Figure 24 > *In situ* PLA assay. PLA assay was performed using MKN45-SC cells and the antibodies MUC1-CT2 and E-cadherin (signal from interaction of both molecules is in red). MKN45-SC(-) – negative control.

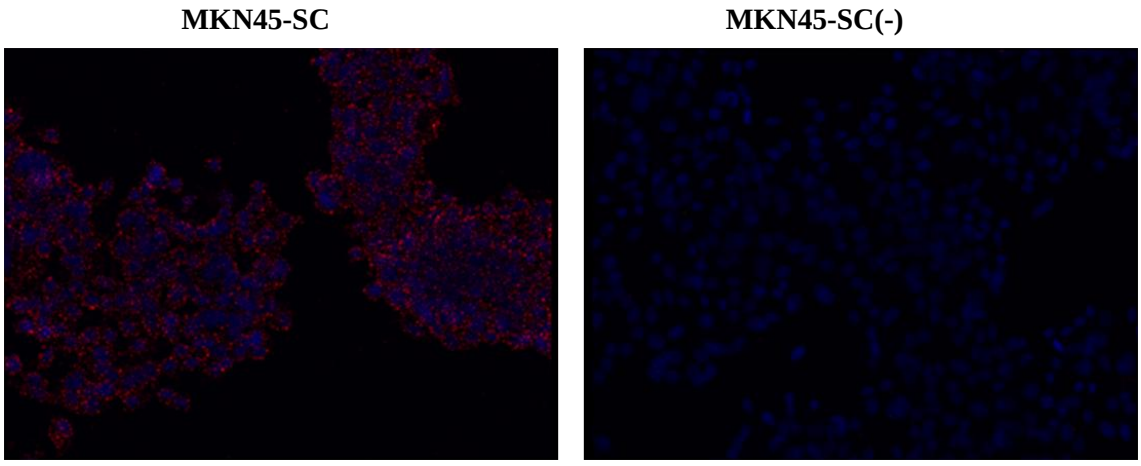


Figure 25 > *In situ* PLA assay. PLA assay was performed using MKN45-SC cells and the antibodies MUC1-CT2 and β-catenin (signal from interaction of both molecules is in red). MKN45-SC(-) – negative control.

An average of the number of interactions with MUC1-CD for all the MAPK not-related studied molecules was calculated with the Blobfinder Software (OLINK Biosciences) **(Figure 26)**.

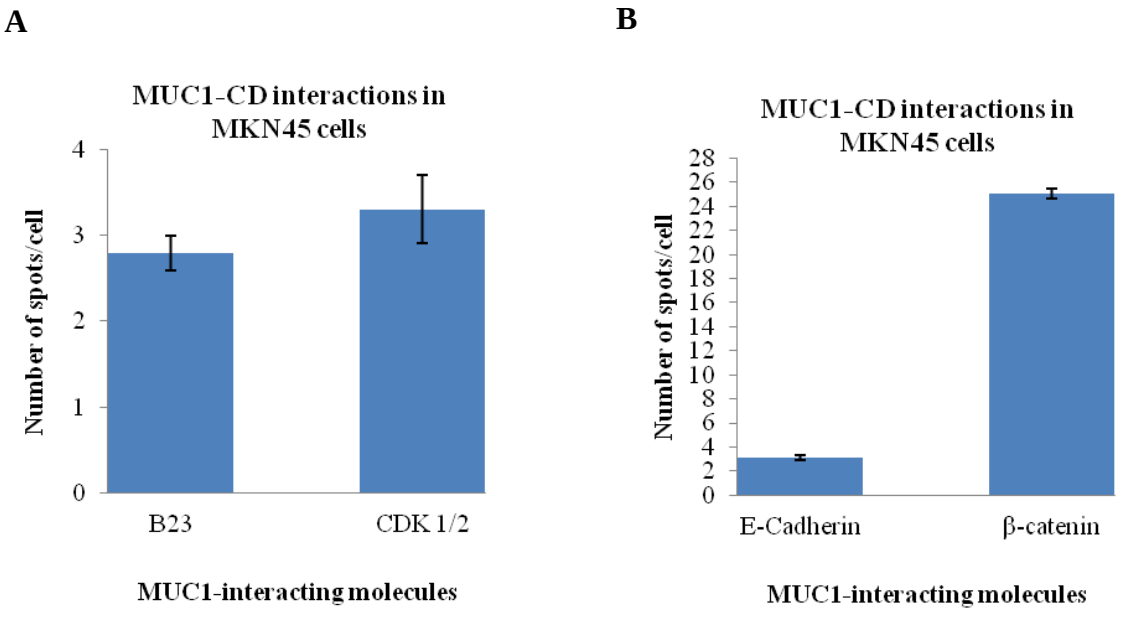


Figure 26 > (A) Average of number of interactions of MUC1-CD with B23 and CDK1/2 and **(B)** E-cadherin and β-catenin molecules in MKN45-SC GC cells, as found by PLA assays.

3.4. Interaction between MUC1-CD and MAPK signaling effectors and other oncogenic signaling partners in normal and GC tissues – PLA assays

In order to see if the interactions previously found with MUC1-CD and cell oncogenic molecules in MKN45 GC cells were cancer-specific or if they already exist in gastric normal cells, we have performed PLA assays in normal gastric tissue slides. No interactions were found between MUC1-CD and the signaling partners previously described for MKN45 GC cells (results not shown). GC tissue slides were also tested for interactions between the previously described signaling partners for MUC1-CD and no interactions were found (results not shown).

4. Evaluation of MUC1 impact in the expression and phosphorylation of gastric oncogenesis-related proteins in MKN45 GC cell line.

The expression levels of the MAPK related signaling molecules ERK1/2, EGFR, B-RAF and Grb2 were quantified by Western blot **(Figure 27)**.

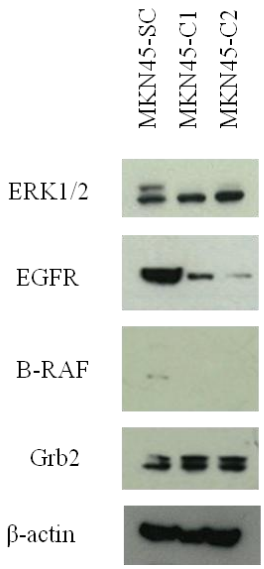


Figure 27 > MKN45-SC, C1 and C2 whole lysates were blotted with anti-ERK1/2, anti-EGFR, anti-B-RAF,anti-Grb2 and anti-β-actin.

The relative levels of ERK1/2, EGFR and B-RAF vary between the silenced clones and the scrambled control, suggesting that MUC1 may regulate not only their phosphorylation but also their transcription and/or stability.

ERK2, EGFR and B-RAF mRNA levels were studied by Real-Time PCR **(Figure 28)** in MKN45 GC cells. We observed that ERK2 upregulation and BRAF downregulation at a protein level are likely to be regulated by MUC1 at a transcriptional level, whereas EGFR protein levels are most likely regulated by MUC1 through post-transcriptional regulation mechanisms.

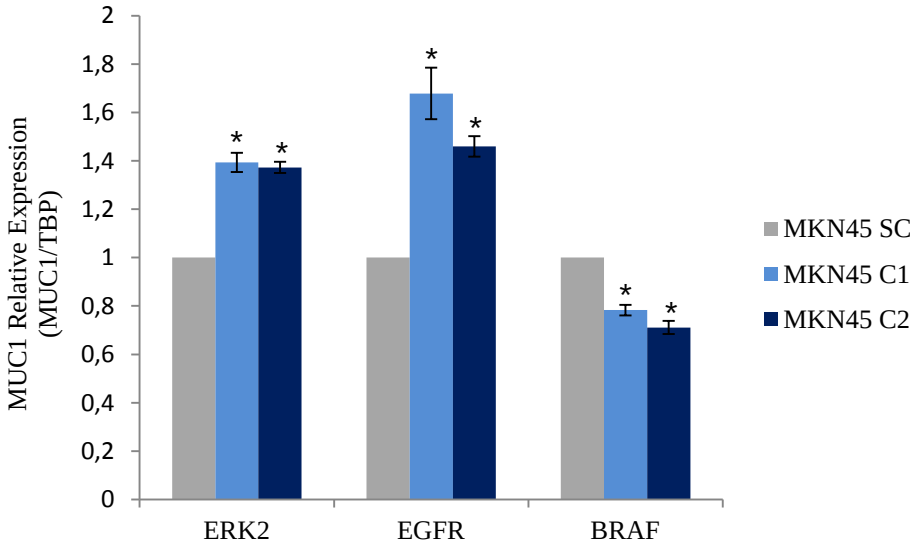


Figure 28 > Relative quantification of ERK2, EGFR and B-RAF by Real-Time PCR in MKN45-SC, C1 and C2 clones. Values were adjusted to the expression of TBP gene. * $P < 0.05$ (Mann-Whitney test), when compared to the MKN45-SC control.

Phosphorylation profiling of MKN45-SC, MKN45-C1 and MKN45-C2 by Kinexus antibody screening have shown that phosphorylation of MKN45-C1 and MKN45-C2 clones is significantly altered when compared to the MKN45-SC control in several phosphorylation sites of cell cycle related proteins **(Table 3).**

Table 3. Percentage of the increase/decrease in phosphorylation levels of some important phosphorylation sites of cell cycle related proteins between each MUC1 downregulated clone (MKN45-C1 and MKN45-C2) and the control cells.

Clones / Proteins' phospho-site	MK45-SC	MK45-C1	MKN45-C2
3-PDK1 [S244]	---	---	---
B23 [S4]	+	+ 116%	+69%
B23 [T199]	+	+ 373%	+255%
BRCA1 [S1497]	+	-27%	-19%
CDK1/2 [T14+Y15]	---/+	---/+87%	---/+264%
CDK1/2 [T161+T160]	+/+	+106%/+198	-41%/+195
CDK1/2 [Y15]	+/+	+80%/150%	-25%/93%
ERK1 [T202+Y204]	+	-73%	-59%
ERK2 [T185+Y187]	+	+59%	+33%
GSK3α [S21]	+	+5%	+25%
GSK3α [Y279]	+/---	-53%/---	-39%/---
GSK3β [S9]	---	---	---
GSK3 β [Y216]	+/---	-39%/---	-27%/---
FRAP [S2448]	---	---	---
MKK1 [S297]	+	-40%	+51%
MKK1 [S291]	+	+10%	+132%
MKK1 [T385]	+	+22%	+128%
MKK1/2 [S217/S221]	+	+1%	+183%
MKK2 [T394]	---	---	---
p27 1B [T187]	+	-68%	-35%
p70 S6-α [T229]	+	+167%	+28%
p70 S6-α [T421+S424]]	+	+365%	+270%
p85 S6 Kinase 2 [T444+S447]	---	---	---
PTEN [S380+T382+S385]	---	---	---
Akt1 [S473]	+	-26%	+9%
Akt1 [T308]	---	---	---
RAF1 [S259]	---	---	---
Retinoblastoma protein [S612]	---	---	---
Retinoblastoma protein [S780]	+	-21%	-20%
Retinoblastoma protein [S807]	+	+71%	+8%
Retinoblastoma protein [S807+S811]	+	-4%	+4%
Retinoblastoma protein [T356]	+	-32%	-47%
Retinoblastoma protein [T821]	---	---	---
Retinoblastoma protein [T826]	+	+208%	+81%

Ribossomal S6 Kinase1/2[S221/S227]	+/---/---/---	-41%/---/---/---	+42%/---/---/---
Ribossomal S6 Kinase1/2[S363/S369]	+/---/---/---	+64%/---/---/---	-8%/---/---/---
Ribossomal S6 Kinase1/2[S380/S386]	+/+/---/---	+209%/-38%/---/---	+112%/-72%/---/---
Src [Y418]	---/---	---/---	---/---
Src [Y529]	+/---	-30%/---	-34%/---
P53 [S392]	+	+11%	-7%

Different results that are shown for the same protein were obtained with different antibodies

+ phosphorylation signal detected

--- no phosphorylation was detected

Among all these proteins, we have selected the proteins nucleophosmin (B23), CDK1/2 and ERK1/2 to highlight the differences found **(Figure 29)**.

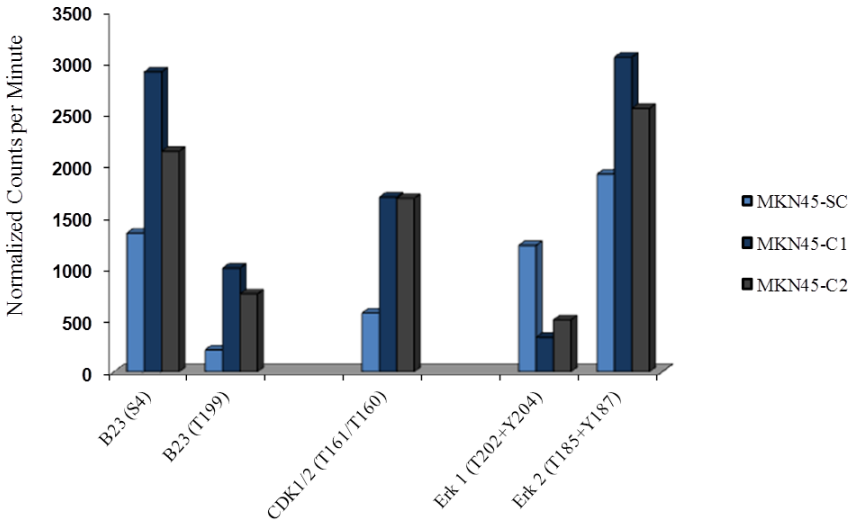


Figure 29 > Relative phosphorylation levels of important phosphorylation sites of proteins B23, CDK1/2 and ERK1/2 in MKN45-SC, MKN45-C1 and MKN45-C2 cells.

These three molecules were previously shown to interact physically with MUC1-CD, in the last step of this work.

5. *Evaluation of the impact of H. pylori binding in MUC1-mediated oncogenic signaling pathways in MKN45 GC cell line.*

In the previous part of this work we observed that MUC1-CD interacts with several cell signaling molecules that are important for the modulation of diverse cascades with oncogenic potential. ERK1/2 molecules and the MAPK pathway are the main responders to external environmental stresses. ERK1/2 activation by phosphorylation has been previously reported in many cancers and we have shown that MUC1-CD may be a crucial regulator of these molecules expression and phosphorylation. Our next step was to check if the binding of *H. pylori to* MKN45-GC cells could induce ERK1/2 activation, by phosphorylation, result already shown for other GC cell lines, and if this activation is modulated by MUC1 (since this molecule is known to be a major target for bacterial binding). Co-cultures of the *H. pylori* pathogenic strain HP26695 with MKN45-SC and MKN45-C1 and MKN45-C2 clones were performed for 1 and 6h, and the levels of phosphorylated ERK1/2 were evaluated by Western blot **(Figure 30)**.

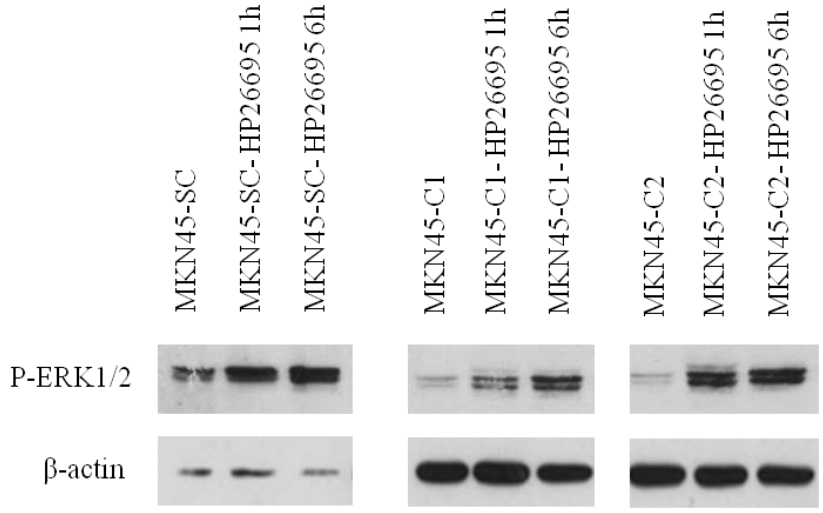


Figure 30 > Expression of phospho- ERK1/2 in MKN45-SC, MKN45-C1 and MKN45-C2 cells upon infection with HP26695 pathogenic bacteria, for 1 or 6h.

Levels of phospho-ERK1/2 expression were adjusted to the levels of β-actin expression, with the software Quantity One software (BioRad) **(Figure 31)**.

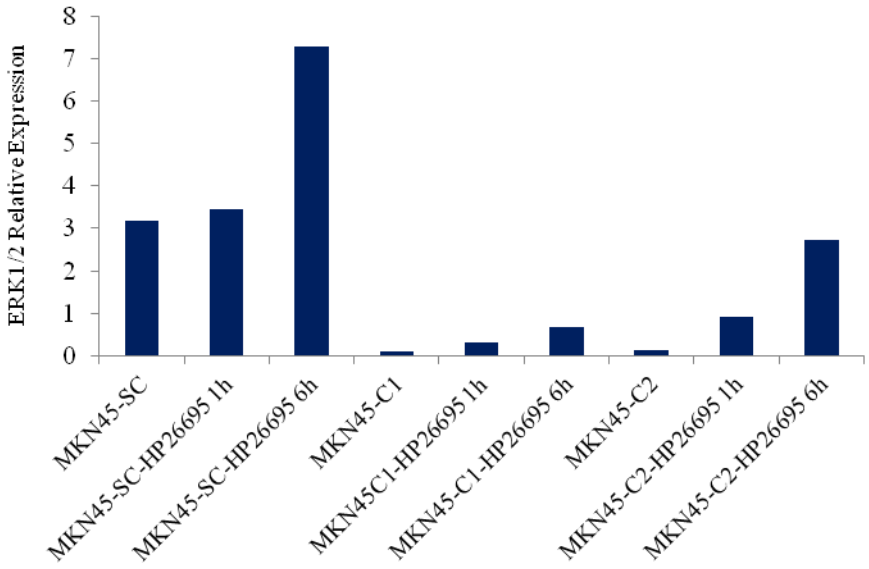


Figure 31 > Relative quantification of ERK1/2 phosphorylation in MKN45-SC, C1 and C2 cells upon infection with HP26695 pathogenic bacteria, for 1 or 6h.

Incubation of cells with bacteria was therefore shown to induce ERK1/2 phosphorylation, in a time-dependent manner, even in MUC1 downregulated clones MKN45-C1 and MKN45-C2. In MUC1 downregulated clones, the overall levels of phospho-ERK1/2 were significantly reduced when compared with the MKN45-SC control.

Discussion

Evaluation of MUC1 impact on H.pylori adhesion to GC cells.

Epidemiological studies and animal models have shown that *H. pylori* chronic infection is associated with several gastric pathologies, including gastric adenocarcinoma [6]. The different consequences of infection suggest that several factors from the host and the bacteria are involved in bacteria-host interactions, making the pathogenic potential dependent on the molecular context of the colonization of the gastric mucosa. To date, several factors involved in the *H. pylori* infection have been identified (e.g. bacterial adhesins, host mucins and pro-inflammatory cytokines). However, the complete mechanism of this infection remains to be clarified [183,184,185].

Adhesion of *H. pylori* to gastric mucosa is a fundamental step for the epithelium colonization. Different adhesion mechanisms, commonly targeting carbohydrate structures present on gastric cells surface, have been identified [186], with *H. pylori* ligands including, among others, blood group antigens Le^b and sialyl-Le^x on mucins and glycolipids [66,67,187].

The best characterized *H. pylori* adhesin is BabA, which mediates a strong adhesion between the bacteria and Le^b blood group antigen expressed on the surface of epithelial cells [66,188]. This work showed that adhesion is a relevant feature of *H. pylori* pathogenicity potential, with significantly higher adhesion levels observed for the HP26695 (pathogenic strain) when compared to the HPTx30a (non-pathogenic strain) for both cell lines studied (GP202 and MKN45). Considering that both strains don't express the adhesin BabA [189] , the observed differences cannot be explained through the BabA binding model, which suggests that other bacterial molecules are involved in the adhesion process.

Another important observation is that there is a higher adhesion of HP26695 and HPTx30a strains to GP202 cell line when compared with MKN45 cell line, a fact that reflects different expression levels and availability of ligands at the cells surface. Previous characterization of mucins and carbohydrate expression on GP202 and MKN45 cell lines showed that Le^b has a significantly higher expression in GP202 cell line [190,191], still this difference might not be relevant since BabA is not present in both bacterial strains. GP202 has a higher expression of other carbohydrate antigens

(Le^a and Le^y) [190,191] when compared to MKN45, that might be involved in *H. pylori* binding interactions. Moreover, additional ligands/interactions that are not yet explored may also exist that can explain this difference in adhesion levels between cell lines.

In order to study the influence of MUC1 VNTR variability in *H. pylori* binding, we used GP202, the cell line that showed higher bacterial adhesion, and we analysed bacterial binding to GP202 transfected clones expressing recombinant MUC1 with different number of MUC1 TR units. These clones overexpress similar levels of recombinant MUC1 [85]. We observed that MUC1 VNTR polymorphism influences the extent of *H. pylori* binding to gastric cells, with higher adhesion levels observed for clones with larger VNTR regions. This may be due to the fact that MUC1 with larger TR regions contains more potential glycan receptors, thus potentially providing more bacterial binding sites. Moreover, we have previously shown that differences in VNTR length lead to glycosylation changes in the MUC1 TR [85] , which may also contribute to the observed altered adhesion. Detailed evaluation of the results showed a small increase between the adhesion of GP202-Neo (control) and GP202-ΔTR, that may be explained by the overexpression of MUC1 in recombinant clone GP202-ΔTR [85] and by the potential presence of binding sites outside the VNTR region. No significant difference was observed between the adhesion of the bacteria to GP202-9TR and to GP202-42TR clones. We previously observed the overexpression of MUC1 underglycosylated forms in GP202-42TR [85] ,which might explain why the adhesion levels are not proportional to VNTR size.

Although we have found *in vitro* that TR alleles with more TR are able to carry more bacteria, it is known that individuals homozygous for short TR alleles have an increased risk for the development of pre-cancerous gastric lesion [13]. We can speculate that this fact can be due to MUC1 shedding from the cell surface, previously observed for other gastric cell line, and inhibition of adhesion by steric hindrance [61]. MUC1 acts as a releasable decoy, after bacterial adherence **(Figure 32)**.

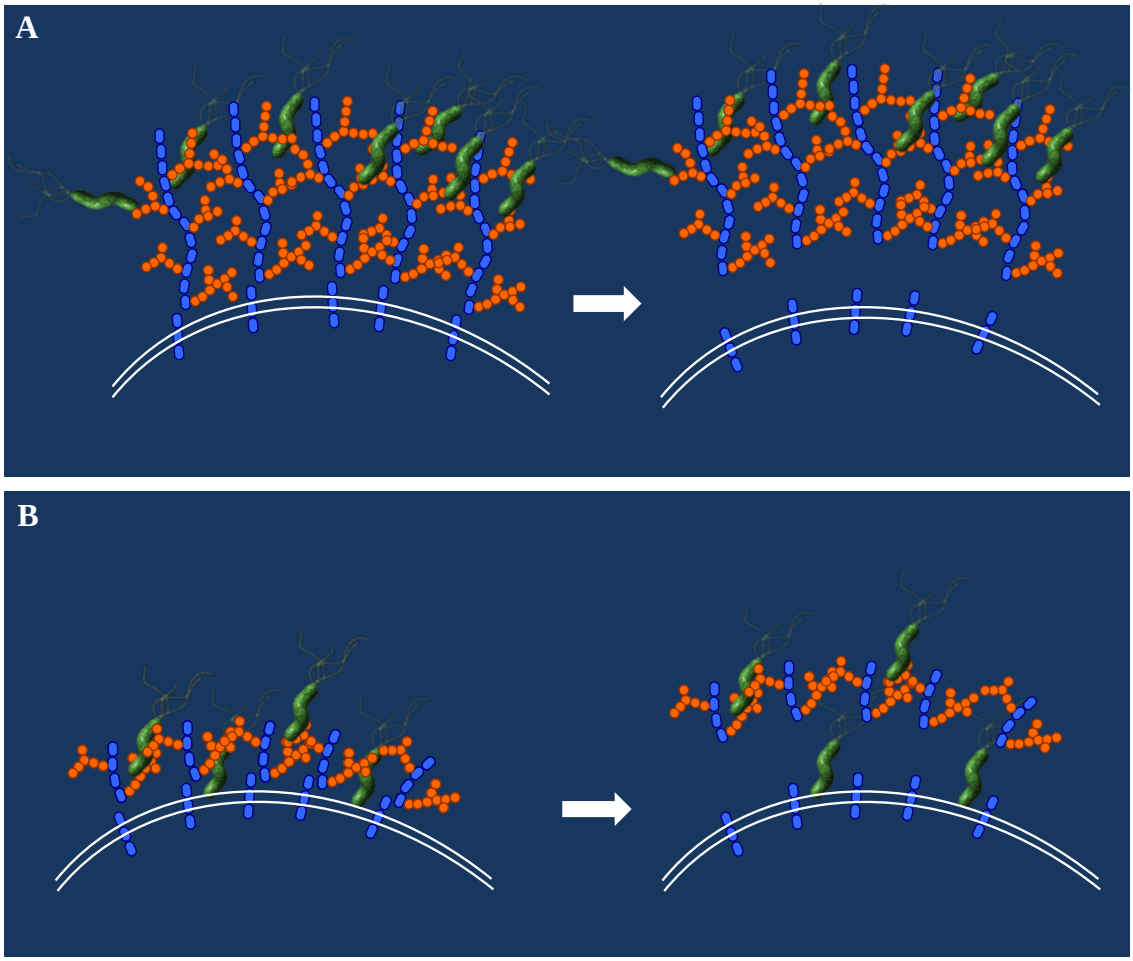


Figure 32 > Model for *H. pylori* shedding from gastric mucosa.
H.pylori adheres to the extracellular domain of MUC1. **(A)** If this region is longer, more bacteria will adhere. Upon shedding of MUC1, most of the bacteria are removed from the gastric mucus; **(B)** If this region is shorter, less bacteria will adhere but they are in closer contact with the gastric cells and therefore upon shedding of MUC1, some bacteria may remain attached to the surface of epithelial cells. Therefore, individuals with larger TR regions are less prone to have bacterial infection and further gastric diseases, as proposed before [13].

All these observations are important for understanding the bacterial and host molecular context of the colonization of gastric mucosa. Identification of a pathogenesis background, based upon host susceptibility traits like MUC1 VNTR polymorphism, will help to identify candidates more prone to bacterial colonization and patients more resilient to eradication strategies.

Evaluation of MUC1 impact on gene expression, phenotype and tumorigenicity of GC cells.

MUC1 relevance for malignant phenotype has been previously described for several tumor models [146,192,193,194,195,196], still the impact of MUC1 in gastric tumor cells biology remains to be clarified. In this work, we used sh-RNA interference to downregulate MUC1 expression in GC cells, in order to evaluate the impact of this mucin on gene expression, phenotype characteristics and tumorigenicity of MKN45 and GP202 cells. For each cell line, we established two independent clones with stable MUC1 downregulation (C1 and C2) and one scrambled control (SC). Even though MUC1 downregulation is not complete, the results obtained are consistent at protein and RNA levels. MUC1 is known to be a vital molecule for epithelial cancer cells [197] and our downregulation experiment results suggest that complete silencing of MUC1 expression may not be compatible with MKN45 and GP202 cells survival.

MKN45 MUC1 downregulated clones showed a significantly different transcriptional profile (as shown by oligonucleotide microarray analysis) when compared with MKN45-SC control. We found significant alterations in the expression levels of several genes, mainly TCN1, KLK6, ADAM29, LGALS4, TSPAN8 and SHPS-1 between MKN45-C1 and C2 cells and the MKN45-SC control. Some of these molecules have functions not yet fully clarified yet others are known to be associated with cell proliferation and migration, including KLK6, LGALS4, SHPS-1 and TCN1 [198,199,200,201,202,203], apoptosis, such as KLK6, LGALS4 and SHPS-1 [204,205,206], invasion, including KLK6 and TSPAN8 [200,207] and motility, including LGALS4 [199]. These alterations are likely due to MUC1 downregulation, since MUC1 has been shown to directly conduct signals that alter the transcriptional program of tumor cells [101,113,114,115,116,117,118,119]. In the case of GP202 cell line, the impact of MUC1 downregulation in the transcriptional profile was not significant (only three genes were found to be altered: calcium P-protein, α-protein interferon- induced and β-tubulin 2A), fact that may reflect the different cellular context of MUC1 signaling partners in this cell line (e.g. transcription factors).

MUC1 silencing by interference RNA has been previously performed for other cell lines [146,192,193,194,195,196], with different impact on cells phenotypic characteristics. MUC1 contributes to tumor progression of adenocarcinomas and therefore its downregulation was predicted to affect the malignant properties of cancer

cells, including proliferation, apoptosis, migration, invasion and cell-cell aggregation. Our next step was to evaluate the effects of MUC1 downregulation on cancer-related properties of MKN45 and GP202 GC cells.

We found that proliferation was significantly increased in MUC1 downregulated clones MKN45-C1 and MKN45-C2 when compared to the control MKN45-SC. Similar studies with breast and pancreatic carcinoma cell lines have shown respectively similar [196] and opposite results [146,195,196]. In different tumor models, such as breast, oral and human embryonic kidney gastric carcinoma cells, MUC1 was shown to regulate cell proliferation by interacting with several proteins such as ER-α, EGFR and β-catenin, respectively [100,192,208]. However, for GC cells, such interactions have not been investigated. KLK6 expression was increased in MKN45-C1 and MKN45-C2 clones, whereas LGALS-4 expression was decreased and these differences may explain the observed differences in proliferation [198,199]. The mechanisms by which KLK6 and LGALS4 expression is altered in MKN45-C1 and MKN45-C2 when compared to MKN45-SC control remains to be elucidated. Recent studies have shown that galectin-4 functions as a tumor suppressor in colorectal cancers, by interacting with the Wnt signaling pathway [205]. Its knockdown results in increased cell proliferation. KLK6 was shown to activate multiple signal transduction pathways in neurons in astrocytes, among which the activation of MAPK signaling cascade [209]. Both these results are in agreement with ours and indicate two possible candidates that may be influencing cell proliferation. In the case of GP202 cells, the results were not consistent, since one of the clones showed decreased and the other clone showed increased proliferation levels, reflecting that the clones may have different molecular contexts. Furthermore, global gene expression hasn't shown significant alterations in genes that regulate cell phenotypic characteristics, e.g., KLK6 and LGAL4.

Another important observation was that apoptosis was significantly increased in MKN45-C1 and MKN45-C2 clones when compared to the MKN45-SC control, demonstrating a MUC1 anti-apoptotic activity. MUC1 was previously shown to mediate a pro-apoptotic response in hamster ovary cells [210] and it was also attributed with anti-apoptotic functions in myeloma, breast and colorectal carcinoma cell lines [211,212,213]. However, little is known about the influence of MUC1 on cell apoptosis in GC cells. KLK6, LGALS4 and SHPS-1 are known to be involved in apoptosis events and therefore their different expression between MUC1 downregulated clones and the control may contribute to the differences observed. In the case of GP202 cells, the

differences found between apoptosis in MUC1 downregulated clones and the scrambled control were not significant. Global gene expression hasn't shown significant differences in apoptosis-related genes.

Cell-cell aggregation was decreased in MKN45-C1 and MKN45-C2 clones when compared to the MKN45-SC control in both time-points, whereas in GP202-C1 and GP202-C2 a decrease was found when compared to GP202-SC only at the 2-hours time point. Previous studies have shown that overexpression of different forms of MUC1 can lead to an increase or a decrease of cell-cell aggregation in a pancreatic carcinoma cell line [214], whereas others have shown that MUC1 downregulation induces an increase of cell-cell aggregation in an oral carcinoma cell line [192]. Modulation of cell aggregation may be explained by the fact that MUC1 contributes to both adhesive [215,216] and anti-adhesive [217,218] properties of cells. One possible explanation is that decreased expression of MUC1 extracellular domain influences the binding potential of other molecules on the surface of different cells, such as ICAM-1 [87,89,90] and galectin-3 [88,91,92].

No significant differences were found between MKN45-C1 and C2 clones and MKN45-SC control with respect to cell migration and invasion. This is in contrast to previous findings in which MUC1 was shown to influence cell migration in breast, cervical, renal and pancreatic carcinoma cell lines [90,193,194,219] and cell invasion in breast, lung, gastrointestinal, hepatic and pancreatic carcinoma cell lines [193,220,221,222]. However, in the case of GP202 cell line, invasion was found to be increased in MUC1 downregulated clones when compared to the control, whereas differences in terms of migration were not consistent between clones. Although global gene expression hasn't shown significant differences in migration or invasion-related genes, other not analysed genes may be involved in the differences observed.

The differences found between the two cell lines may be explained by their different expression of the levels of MUC1 molecule. MKN45 parental cell line expresses higher levels of MUC1 than G202 parental cell line and therefore this molecule must have a different relevance in each cell line. In terms of percentage of silencing, the levels are very similar for both cell lines, but its effects on MKN45 cell line phenotypic characteristics are clearly more pronounced that in GP202 cell line. Furthermore, each cell line has its different molecular context (e.g. MUC1 signaling partners).

MUC1 overexpression has been associated with the neoplastic progression of several tumors, including the acquisition of invasive and metastatic properties. Phenotypic studies in cell models other than GC have suggested that MUC1 influences events such as proliferation, apoptosis, migration, invasion, adhesion and cell-cell aggregation. The work presented here shows for the first time that MUC1 expression influences proliferation, apoptosis and cell-cell aggregation of MKN45 GC cells. In the case of GP202 cells, the results were not always consistent with the ones found for MKN45 cell line. These results are consistent with the view that MUC1 modulates different signaling pathways in a manner that is dependent on the expression and activity of other regulatory mechanisms and molecules, which are influenced by the cellular and biological context of the cell type that is overexpressing MUC1.

Considering all the differences found between MKN45 and GP202 cell lines and between the different MUC1 downregulated clones, we can hypothesize that the molecular context where signaling through MUC1-CD occurs influences gene expression, which in turn affects the phenotypic properties of the MKN45 and GP202 cell lines.

Considering the results obtained when studying the phenotypic effects of MUC1 downregulation in malignant properties of GC cells, we decided to perform the following studies with the MKN45 GC cell line, the one that has shown the most significant differences.

In vivo assays have shown that MKN45 cells with decreased amount of MUC1, MKN45-C2, form smaller and slowest-growing tumors than the control MKN45-SC cells. Even if MUC1 downregulated cells are more proliferative than the control, probably that fact is not enough to make them more tumorigenic. Aggregation of cells may be a fundamental step to form a cohesive mass, supportive of tumor growth, and MUC1 downregulated cells were shown to be less aggregative. Or, by another hand, the injected cells may change their behavior when in an *in vivo* context, and become less proliferative. Although some components of these mice's immune system are missing, the B cells, dendritic cells and granulocytes are all relatively intact, and there is a compensatory increase in both natural killer (NK)-cell activity and tumoricidal macrophages in these mice [223]. Other mice strain should be used as a control in future work, to evaluate the impact of mice immunological background on the growth of MUC1 downregulated tumors. Further studies would need to be performed in mice with respect to invasion and other aspects concerning the tumor aggressiveness. However,

we might speculate so far that MUC1 downregulation decreases the aggressiveness of MKN45 GC cells and this molecule may be therefore be a good candidate for GC therapy.

Identification of MUC1-mediated signaling pathways in GC cells.

We have previously observed that alterations on gene expression, cell phenotype and tumorigenicity occur upon MUC1 downregulation. These alterations may be due, among other factors, to deregulation of cell signaling pathways. MUC1 is known to interfere with some of these pathways in different cancer models [96,111,224,225,226] and its absence may have altered the normal functioning of these cells signaling pathways. Our next step was therefore to explore possible signaling pathways and molecules that could be altered in MKN45 GC cells upon MUC1 downregulation and that could justify the differences observed.

The MAPK signaling pathway has been one of the signaling pathways more often shown to be deregulated in several carcinomas, including GC [23].

The MAPK signaling pathway is composed of several cell signaling molecules. The initiator molecule is EGFR, a member of the growth factor family ErbB that works as a cell surface receptor of extracellular ligands. Ligand binding to EGFR extracellular domain leads to its activation, with subsequent homodimerization, leading to the phosphorylation of its intracellular tyrosine kinase domain. This will initiate a series of phosphorylations and intracellular signals that will ultimately activate the central MAPK signaling pathway **(Figure 33)**.

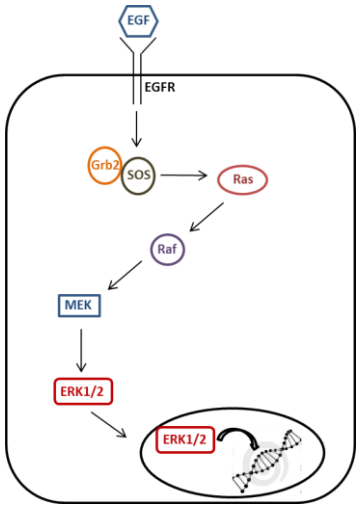


Figure 33 > MAPK signaling pathway.

EGFR molecule modulates processes of cell proliferation, migration, adhesion and proliferation and it is known to provide tumor cells with growth and survival advantages [31]. EGFR expression was found to be deregulated in several types of cancers, including GC. High EGFR levels in GC were found have been associated with the disease prognosis [32] and presence of lymph node metastasis [74]. EGFR overexpression is thought to be the main mechanism for its activation [227].

Grb2 is an important molecule that integrates the stimulatory signals of the MAPK signaling pathway [228]. This molecule has been previously shown to interact with MUC1 in breast carcinoma [105].

B-RAF is another member of the MAPK signaling pathway often overexpressed in GCs, promoting tumorigenesis and angiogenesis [229]. MUC1 was previously shown to activate the RAS-mediated signaling pathway in non-tumorigenic and tumorigenic mammary cell lines [230].

ERK1/2 molecules are the final effectors of the MAPK signaling cascade and have been shown to be activated by MUC1 in some cancer models [96,111,226], overexpressed and activated in the majority of GC cases and correlate with tumor progression and invasion [28]. These molecules were also found to be activated in *H.pylori* related cancers [29,30]. These events were found for GC cell lines, but when examining human GC biopsies, a decrease in the activation of ERK1/2 was found [231]. One possible explanation for this fact is that gastric cells start expressing molecules that attenuate ERK-mediated signaling upon its activation, or on the other hand, the cells act by activating negative feedback mechanisms. Stimulation of EGFR in cancer cells has been previously shown to activate the ERK signaling pathway [232].

Our results support an extensive involvement of MUC1-CD with MAPK signaling pathway. MUC1-CD was shown to interact with ERK1/2 in MKN45-SC control and downregulated cells, MKN45-C1 and MKN45-C2, as shown by immunoprecipitation assays. The interaction between MUC1-CD and ERK1/2 was later confirmed by proximity ligation assays. This technique also allowed us to show interactions between MUC1-CD and other MAPK signaling effectors such as EGFR, Grb2 and B-RAF.

Apart from MAPK signaling cascade, several other pathways have been shown to be deregulated in GC [23], as well as the abnormal expression of several molecules [233].

One example of a molecule found to be deregulated in GC is nucleophosmin (B23) [234]. B23 is a nucleolar protein with several cellular functions, including ribosome assembly, intracellular trafficking, DNA polymerase activity, and centrosome duplication [235]. Other well known example are Cyclin dependent kinases 1 and 2 (CDK1/2) [236,237,238]. CDK1/2 proteins are involved in several cell processes, mainly in regulating the cell cycle [239]. Both these molecules were found to interact with MUC1-CD in MKN45 GC cells. So far, there are no known interactions between these molecules and MUC1-CD in any other carcinoma cell type.

We have further decided to study the possible interaction of MUC1 with E-cadherin and β-catenin, since these are two of the most studied molecules that are involved in the development of most tumor types, including GC.

E-cadherin is expressed in all epithelial cell types and mediates cell-cell adhesion and also the maintenance of the normal epithelium architecture [240]. Loss of expression of this molecule has been found in GCs, relating with tumor dedifferentiation, invasiveness, metastasis and prognosis [241,242]. It has been shown in colorectal carcinomas that MUC1 expression was negatively correlated to E-cadherin expression [243], but to the best of our knowledge, there are no known interactions between MUC1-CD and E-cadherin in any carcinoma cell line.

β-catenin is a molecule also expressed in epithelial cells that is important in mediating the E-cadherin related cell adhesion and also by participating in Wnt signaling pathways, that has been found to be frequently deregulated in GCs [244,245,246]. Aberrant β-catenin activation has been found in several cancers [247] and by *H. pylori* in GCs [56]. Loss of this molecule has been found in metastatic GC [248]. Recent studies have shown that MUC1 overexpression can modulate the intracellular distribution of β-catenin, as well as its participation in signaling pathways [117,221,249,250]. These facts suggest that the interaction of MUC1-CD with β-catenin in GC cells has an important role in tumor progression. Interaction of β-catenin and MUC1 has been found in *H.pylori* induced GC [225], breast cancer [110], airway epithelial cells [251], among others.

Both E-cadherin and β-catenin molecules were found to interact with MUC1-CD in MKN45-SC GC cells. These are the first results that clearly show an interaction between MUC1-CD and these molecules in GC cells.

Although further studies need to be performed to better clarify the meaning and importance of these interactions, they seem to suggest that somehow MUC1-CD may

influence the role of all the referred molecules in GC cells, leading to the differences in gene expression, phenotypic characteristics and tumorigenicity that were found. Therefore, this mucin may play a crucial role in regulating mechanisms that mediate cell tumorigenesis and it is valuable to develop strategies to limit and control the development of GC.

In order to see if these interactions were restricted to MKN45 cell line, derived from a diffuse GC, we have tested the possible interaction between MUC1-CD and ERK1/2 in AGS GC cells, derived from an intestinal-type GC and the result was positive (results not shown). Therefore we may affirm that this interaction is not restricted to gastric cell lines derived from a diffuse carcinoma type.

In the next step we checked if the interactions found to exist between MUC1-CD and ERK1/2, EGFR, B-RAF, Grb2, B23, CDK1/2, E-cadherin and β-catenin were also occurring in normal gastric cells. In order to do so, we performed PLA assays in normal gastric tissue samples. However, no interaction was detected between all the mentioned molecules and MUC1-CD in the samples studied. A similar result was obtained when testing GC tissue slides.

Tissue samples and cancer cell lines have several important differences. One of these differences is that GC cell lines grow with high proliferation rates, while in normal and carcinoma tissue samples, the proliferative pool is restricted to a few set of cells. This reason can explain the fact that the results obtained by PLA assays regarding MUC1-CD interaction with cell signaling molecules are different between MKN45-SC cells and normal and GC tissue samples.

Summarizing, this is the first report that shows MUC1-CD interaction with ERK1/2, EGFR, B-RAF, Grb2, B23, CDK1/2, E-cadherin and β-catenin in MKN45 GC cell line, and with ERK1/2 in AGS GC cell line.

Evaluation of MUC1 impact in the expression and phosphorylation of GC-related proteins.

We have shown in the previous step that MUC1-CD physically interacts with several cell signaling molecules, including some that are critical effectors of the MAPK signaling pathway (such as EGFR, B-RAF, Grb2 and ERK1/2).

Our next step was to evaluate the impact of MUC1 on the expression and phosphorylation of these MAPK signaling cascade members. We have observed by

Western blot analysis that ERK2 expression is increased, whereas ERK1 expression is decreased upon MUC1 downregulation, suggesting that MUC1 may regulate the transcription and/or stability of these molecules. These two molecules were shown to have opposite effects, since ERK1 is thought to attenuate ERK2 effects [252] and therefore these results are in accordance. EGFR and B-RAF were also shown to be affected by MUC1 downregulation, whereas Grb2 levels don't seem to be altered upon MUC1 downregulation.

We can speculate that MUC1-CD is exerting a negative feedback regulation of the MAPK signaling pathway in MKN45 GC cells. Since MUC1 downregulation increases the levels of ERK2 (the crucial molecule when concerning ERK1/2 signaling), this fact may by negative feedback mechanisms decrease the levels of EGFR/B-RAF that are available.

Our next step was to perform Real Time PCR assays, to evaluate the mRNA level of ERK2, EGFR and B-RAF. The results suggested that ERK2 and B-RAF gene expression is regulated by MUC1 at a transcriptional level whereas differences observed for EGFR protein levels are probably due to MUC1-mediated post-transcriptional regulation mechanisms.

Until now, there are no references about MUC1 transcriptional regulation of expression for both ERK2 and B-RAF molecules. However, promotion of transcription of other genes by MUC1 has been previously reported [101,113,114,115,116,117,118,119,253]. It is therefore possible that MUC1-CD is interacting with the promoters of both ERK2 and B-RAF genes, regulating their transcription.

MUC1 was already shown to protect EGFR from degradation [254] and the same may be occurring in MKN45-SC cells. Previous results in oral carcinoma cells have shown that MUC1 suppression leads to a decrease of both EGFR mRNA and protein levels [192]. No binding of MUC1-CD was found for any region proximal of EGFR promoter (results not shown), result in accordance with our idea that EGFR levels are being regulated post-transcriptionally.

We know that several vital cell processes are regulated by consequent phosphorylation/dephosphorylation of proteins. This process will lead to consequent activation/deactivation of these proteins, according to the cells needs. Therefore, our next step was to see if MUC1 downregulation was interfering with the phosphorylation

of activation-related phosphorylation sites of several proteins involved in the cell cycle and therefore in possible oncogenic events.

The most significative results obtained by the antibody screening by Kinexus have shown that phosphorylation of MKN45-C1 and MKN45-C2 downregulated clones is significantly increased when compared to the MKN45-SC control in proteins: B23, CDK1/2 and ERK2, which have been all also found to physically interact with MUC1-CD. Phosphorylation of the studied specific phosphorylation sites is known to lead to an activation of the proteins B23, CDK1/2 and ERK1/2 [235,239,255,256,257,258]. These molecules, as previously referred, are involved in a multitude of signaling pathways (e.g. cell proliferation/differentiation) with oncogenic potential and therefore these results are in accordance with previously observed phenotype alterations in MUC1 downregulated clones, which show increased proliferation rates. These results are in contrast with previous found ones in mouse mammary gland, airway cells and embryonic cells have shown that MUC1 activation by phosphorylation [93,226] and also its higher expression [111] are related to an increase of ERK1/2 activity.

The differences in phosphorylation observed are rather due to physical interactions between MUC1-CD and these molecules, since MUC1 is not known to possess any kinase activity. It's also possible that MUC1-CD is interacting with kinases or phosphatases that phosphorylate/dephosphorylate B23, CDK1/2 and ERK1/2 and that this interaction is altering the phosphorylation of these proteins. MUC1 can be promoting the activity of cell kinases or, by another hand, be inhibiting the activity of cell phosphatases, a group of enzymes also found ubiquitously, which are responsible for the dephosphorylation of various proteins.

It is important to highlight that expression and activation are two different topics, and although they are usually related (higher expression is associated with higher activation), there is not any established correlation between both. We have shown that in MUC1 downregulated clones there is an increase of the expression of ERK2 and decrease of ERK1. ERK2 molecules are more phosphorylated also and therefore more activated. The opposite occurs for ERK1. The higher proliferation observed in MUC1 downregulated clones may be due to these molecules. The decreased expression observed for EGFR and B-RAF would suggest lower proliferation rates, but this fact is most probably overcomed by ERK2 increased levels and phosphorylation. Furthermore, the activation status of both EGFR and B-RAF were not studied and it is possible that, although less expressed, they can have a higher activation status.

Evaluation of the impact of H. pylori binding in MUC1-mediated oncogenic signaling pathways in GC cells.

H.pylori infection was previously shown to activate ERK1/2 molecules in GC cells [30,259,260,261,262,263]. This was observed for different multiplicities of infection, infection periods and for different pathogenicities of bacterial strains [259]. The results obtained in our co-culture assays are in accordance with these published data. Therefore, this bacterium is able to interfere with signaling pathways in which ERK1/2 molecules participate and therefore contribute to gastric carcinogenesis in MKN45 GC cells. One possible explanation is that, by binding MUC1 extracellular domains, this bacterium may promote the binding of MUC1-CD to ERK1/2 and consequent activation of these molecules (by facilitating other interactions, since it is known that MUC1-CD doesn't possess any kinase activity). The mechanism underlying this fact remains unclear, but one of two things can be occurring: the bacteria may somehow modify MUC1-CD's affinity for cytoplasmic ERK1/2 or by another hand it may originate the cleavage of MUC1-CD from the cell membrane, and this molecule will be more available to interact with both cytoplasmic and nuclear ERK1/2, facilitating its activation.

Furthermore, ERK1/2 phosphorylation seems to be MUC1 expression-dependent, since MUC1 downregulated cells express a considerably lower amount of phospho-ERK1/2 when compared to the control cells.

An interesting observation can be made at this time point of the work. ERK1/2 total phosphorylation levels are decreased in MUC1 downregulated clones when compared to the control cells. Kinexus results exhibit an increase of the phosphorylation of ERK2 and a decrease of ERK1 phosphorylation in MUC1 downregulated clones when compared to the control cells. One possible explanation for this fact is that the same cells were used at completely different confluences in the two different assays. In the co-culture assays, the cells were almost in a confluent state, whereas in kinexus assays they were at an approximate 60% confluency. This means that cells were at completely different proliferation states, with the confluent cells being at an almost quiescent state, and the cells with 60% confluency at high proliferative levels. Thus, ERK1/2 expression and activation are very likely to be regulated differently according to the proliferative status of the cells.

Summary and Conclusions

Summary and conclusions

GC is a worldwide major health problem, mostly due to the reduced knowledge of its etiological factors and pathogenesis model. This cancer is also resistant to most cancer therapies with no significant progress made during the last decades. Therefore it is critical to find new therapeutic targets to overcome resistance mechanisms. The main objective of this work was to better understand the biology of MUC1, previously described as frequently altered in GC, in the pathogenesis model.

The research work performed allowed us to:

- Establish an association between MUC1 VNTR polymorphism and *H.pylori* adhesion to GC cells.

- Demonstrate that MUC1 downregulation in MKN45 and GP202 GC cell lines conditions global gene expression and tumor phenotypic characteristics.

- Demonstrate that MUC1 downregulation in MKN45 GC cells affects their tumorigenicity *in vivo.*

- Identify molecules that directly interact with MUC1-CD in MKN45 GC cells and that may therefore be MUC1 oncogenic signaling partners, contributing for GC development. Data suggests that MUC1 acts as an integrator of signaling pathways (MAPK signaling pathway,e.g.) with oncogenic potential.

- Identify signaling molecules (ERK1/2, EGFR, B-RAF) whose expression is significantly altered by MUC1 downregulation in MKN45 GC cells.

- Identify molecules (ERK1/2, B23 and CDK1/2) whose phosphorylation is significantly altered by MUC1 downregulation in MKN45 GC cells.

- Show that *H.pylori* activates, by phosphorylation, the molecules ERK1/2 in MKN45 GC cells.

The molecular complexity of MUC1, mostly associated with the variability of its VNTR domain and with the diversity of the sugar chains that are coupled to it, has been an obstacle to understand its relevance to the tumorigenic potential of the cancer cells. MUC1 overexpression and aberrant glycosylation have been associated with the neoplastic progression of several tumors, namely to the acquisition of invasive and metastatic properties [94,264,265]. Phenotypic studies in different cell models have suggested MUC1 involvement in proliferation [192,266,267], adhesion [214,267], invasion [221,268], aggregation [192] and migration [90]. Considering the association of MUC1 overexpression with all these biological processes, it makes sense that MUC1 involvement in the signaling pathways that lead to the acquisition of these characteristics will be dependent on the expression and activity of multiple regulatory mechanisms, characteristics of a specific cellular context. We reinforced the idea that MUC1 signaling will depend on the cell context, that determines the amount of MUC1 and also the availability of its ligands.

The results found in this research work reinforce the importance of MUC1 in the tumorigenic behavior of GC cells, and therefore its importance as a new elective target for innovative therapeutic approaches.

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Papers

PAPER I

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RAPID COMMUNICATION

Relevance of MUC1 mucin variable number of tandem repeats polymorphism in *H pylori* adhesion to gastric epithelial cells

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Abstract

AIM: To evaluate the influence of MUC1 mucin variable number of tandem repeats (VNTR) variability on *H pylori* adhesion to gastric cells.

METHODS: Enzyme linked immunosorbent assay (ELISA)-based adhesion assays were performed to measure the adhesion of different *H pylori* strains (HP26695 and HPTx30a) to gastric carcinoma cell lines (GP202 and MKN45) and GP202 clones expressing recombinant MUC1 with different VNTR lengths.

RESULTS: Evaluation of adhesion results shows that *H pylori* pathogenic strain HP26695 has a significantly higher ($P < 0.05$) adhesion to all the cell lines and clones tested, when compared to the non-pathogenic strain HPTx30a. Bacteria showed a significantly higher ($P < 0.05$) adhesion to the GP202 cell line, when compared to the MKN45 cell line. Furthermore, both strains showed a significantly higher ($P < 0.05$) adhesion to GP202 clones with larger MUC1 VNTR domains.

CONCLUSION: This work shows that MUC1 mucin variability conditions *H pylori* binding to gastric cells. The extent of bacterial adhesion depends on the size of the MUC1 VNTR domain. The adhesion is further dependent on bacterial pathogenicity and the gastric cell line. MUC1 mucin variability may contribute to determine *H pylori* colonization of the gastric mucosa.

Key words: *H pylori*; MUC1; Variable number of tandem repeats; Polymorphism; Adhesion; Mucin; Gastric; Infection

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INTRODUCTION

The Gram negative bacterium *H pylori* is involved in the pathogenesis of several gastrointestinal diseases, ultimately leading to gastric carcinoma[1,2]. In the gastric mucosa, the majority of the bacteria is found within the mucus layer, but can be also attached to gastric epithelial cells[3], a crucial step for the maintenance, spreading and severity of the infection. This attachment is mediated by the interaction of bacterial molecules, such as adhesins and LPS[4], with gastric cell surface ligands such as glycolipids and glycoproteins. MUC1 is a membrane glycoprotein that protects epithelial surfaces and has been recently identified as an *H pylori* binding target[5,6]. Extracellular MUC1 variable number of tandem repeats (VNTR) domain is highly glycosylated[7], presenting carbohydrate structures (e.g. Lewis b carbohydrate antigen) involved in the binding of *H pylori* through its adhesins BabA and SabA[8,9]. Furthermore this repetitive region shows extensive allelic variation ranging from 25-125 repeat units[12]. The relevance of MUC1 VNTR variability for *H pylori* adhesion to gastric cells remains to be clarified.

In this work we tested the hypothesis that MUC1 VNTR polymorphism affects the *H pylori* adhesion to gastric cells and thus plays an important role in the colonization

of gastric mucosa. We used *H pylori* strains with different pathogenicity (strain HP26695 and strain HPTx30a) co-cultured with gastric cell lines GP202 and MKN45, and GP202 clones expressing recombinant MUC1 with different VNTR lengths. Adhesion was evaluated by an enzyme linked immunosorbent assay (ELISA)-based adhesion assay.

The results showed that MUC1 VNTR polymorphism influences the binding of *H pylori* to gastric cells. Furthermore, higher adhesion was observed in co-cultures with the pathogenic strain (HP26695) when compared to the non-pathogenic strain (HPTx30a) and GP202 cell line when compared to the MKN45 cell line. This work contributes to the understanding of the interplay between host and bacterial factors in *H pylori* infection pathogenesis.

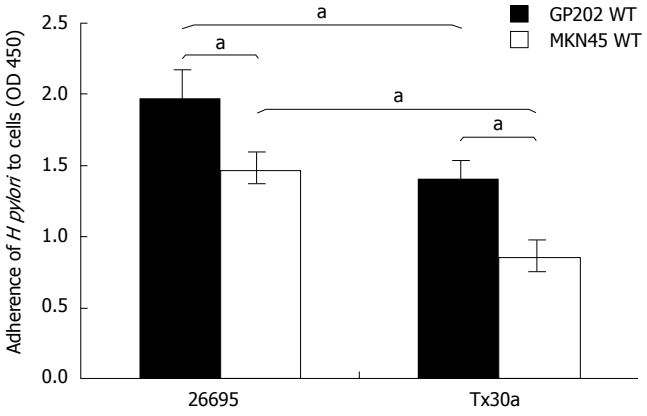

Figure 1 Adhesion of HP26695 and HPTx30a *H pylori* strains to GP202 and MKN45 gastric cell lines. $^{a}P < 0.05$.

MATERIALS AND METHODS

Cell lines

We used two gastric carcinoma cell lines: GP202, previously established in our laboratory[13] from a signet ring cell gastric carcinoma that constitutively expresses MUC1 and MKN45 (Japan Health Sciences Foundation).

GP202 clones expressing recombinant MUC1 with different VNTR lengths[14] were previously established by stable transfection with an eukaryotic expression vector pHb-APr1-neo containing subcloned epitope-tagged MUC1 (FLAG-MUC1) cDNAs with different number of TR units (0, 3, 9 and 42 repeats, respectively GP202-dTR, GP202-3TR, GP202-9TR and GP202-42TR)[15]. GP202-Neo was obtained by transfection with empty vector.

The parental cell lines and transfectants were cultured in 150 cm^2 flasks at 37℃ in a humidified 5% CO_2 incubator and maintained in RPMI 1640 medium (with Glutamax and 25 mmol/L Hepes) supplemented with 10% fetal bovine serum and 50 μg/mL gentamicin. Media was changed every 3 d to 4 d, and the cells were passaged when they reached 80% to 90% confluence using 0.05% trypsin-0.53 mmol/L ethylenediamine tetra-acetic acid in Hank's balanced salt solution. Cell culture reagents were obtained from Invitrogen (Carslbad, CA, USA).

H pylori strains

Two *H pylori* strains were used in this study: the pathogenic strain HP26695 (*vacA* s1/m1, *cag* PAI+, ATCC 700392) and the non-pathogenic strain HPTx30a (*vacA* s2/m2, *cag* PAI-, ATCC 51932). Bacteria were grown on Trypticase soy agar with 5% sheep blood (BioMérieux) at 37℃ in microaerobic conditions.

ELISA assay

Quantitative evaluation of *H pylori* adhesion to gastric cells was performed by ELISA, as previously described[16], with some modifications. Briefly, cells were cultured in 96 well plates and allowed to form confluent monolayers. Cells were washed and *H pylori* suspension was added in a 200:1 bacteria to cell ratio (MOI) and incubated for 60 min. Cells were washed and fixed at 4℃ with 8% paraformaldehyde for 60 min. Endogenous peroxidase was inactivated by addition of 1% H_2O_2 in methanol. After washing with PBS, anti-*H pylori* monoclonal antibody MAB922 (Chemicon, USA) was added overnight, 4℃, followed by addition of peroxidase-conjugated goat anti-mouse immunoglobulins (Santa Cruz Biotechnology) 30 min, RT. Tetramethylbenzidine (TMB) (Sigma, USA) was added and reaction stopped with 1 mol/L HCl. Plates were read in a 680 ELISA microplate reader (Bio-Rad, USA) at 450 nm. OD values were used as the index of the number of *H pylori* adhering to cells[16]. Two sets of triplicates were made for each assay.

Statistical analysis

Statistical analysis was performed using the Mann-Whitney test, StatView Software version 5.0 (SAS Institute). A *P* value of less than 0.05 was accepted as statistically significant.

RESULTS

Evaluation of *H pylori* adhesion shows that pathogenic strain HP26695 has significantly ($P < 0.05$) higher adhesion values for both GP202 and MKN45 cell lines (1.97 ± 0.10 and 1.47 ± 0.06) when compared with the non-pathogenic strain HPTx30a (1.40 ± 0.15 and 0.85 ± 0.15; Figure 1). This statistically significant association between pathogenicity and higher adhesion (strain HP26695 *vs* HPTx30a) is also observed for the GP202 MUC1 recombinant clones (GP202-Neo 1.1 ± 0.10 *vs* 0.72 ± 0.06; GP202-dTR 1.32 ± 0.09 *vs* 1.0 ± 0.10; GP202-3TR 1.45 ± 0.08 *vs* 1.18 ± 0.05; GP202-9TR 2.2 ± 0.12 *vs* 1.96 ± 0.12; and GP202-42TR 2.3 ± 0.07 *vs* 1.89 ± 0.11; Figure 2). Furthermore, GP202 cell line shows higher adhesion levels than MKN45 cell line for both bacteria strains (HP26695 strain 1.97 ± 0.10 *vs* 1.47 ± 0.06; HPTx30a strain 1.40 ± 0.15 and 0.85 ± 0.15; Figure 1).

Adhesion of both *H pylori* strains (HP26695 and HPTx30a) is significantly higher in all the GP202-MUC1 transfectants over-expressing MUC1 (GP202-dTR 1.32 ± 0.09 and 1.0 ± 0.10; GP202-3TR 1.45 ± 0.08 and 1.18 ± 0.05; GP202-9TR 2.2 ± 0.12 and 1.96 ± 0.12; GP202-42TR 2.3 ± 0.07 and 1.89 ± 0.11) when compared with the control, GP202 Neo (1.1 ± 0.10 and 0.72 ± 0.06, Figure 2). There is also an association between the increased number of Tandem Repeats (GP202-9TR and GP202-42TR) and the increased adhesion, for both strains (Figure 2).

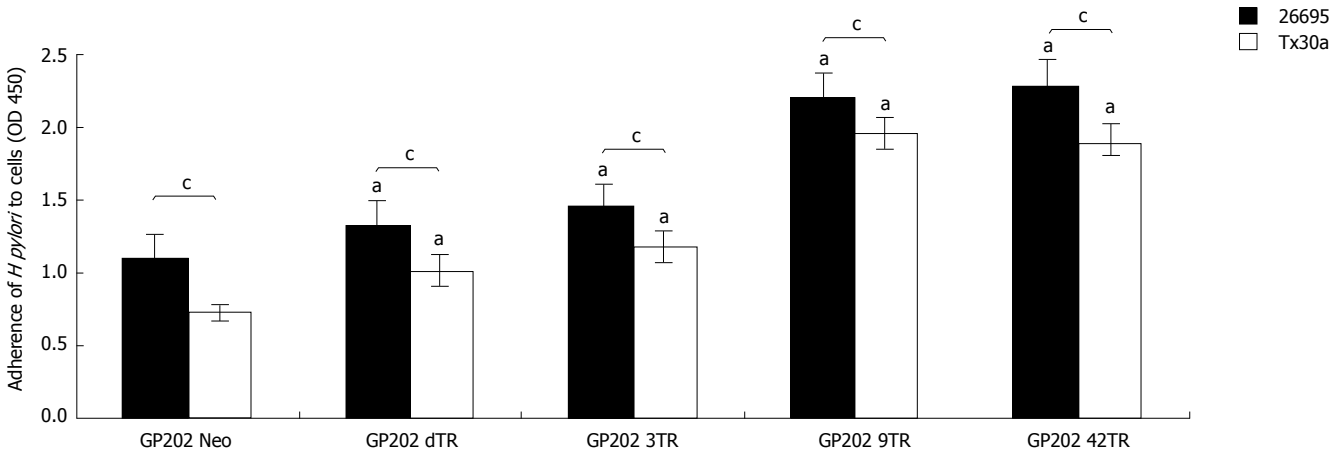

Figure 2 Adhesion of HP26695 and HPTx30a *H pylori* strains to GP202 transfectants GP202-Neo, GP202-dTR, GP202-3TR, GP202-9TR and GP202-42TR. $^{a}P < 0.05$, compared to the control (GP202 Neo) and $^{c}P < 0.05$.

DISCUSSION

Epidemiological studies and animal models have shown that *H pylori* chronic infection is associated with several gastric pathologies, ranging from asymptomatic gastritis to gastric adenocarcinoma and MALT lymphoma[1,2]. The different consequences of the infection suggest that several factors from the host and the bacteria are involved in the bacteria-host interactions, being the pathogenic potential dependent upon the molecular context of the colonization of gastric mucosa. To date several factors involved in the *H pylori* infection have already been identified (e.g. bacterial adhesins, host mucins and pro-inflammatory cytokines) however the complete mechanism remains to be clarified[17-19].

Adhesion of *H pylori* to gastric mucosa is a fundamental step for epithelium colonization. Different adhesion mechanisms, commonly targeting carbohydrate structures present on gastric cells surface, have been identified[4] with *H pylori* ligands including, among others, blood group antigens on mucins and glycolipids[8-11,20-26].

The best-characterized *H pylori* adhesin is BabA, that mediates a strong adhesion between the bacteria and Le^b blood group antigen expressed on the surface of epithelial cells[8,27]. This work showed that adhesion is a relevant feature of *H pylori* pathogenicity potential, with significantly higher adhesion levels observed for the HP26695 (pathogenic strain) when compared to the HPTx30a (non-pathogenic strain) in both cell lines. Considering that both strains don't express BabA adhesin[28], the observed differences can not be explained through the BabA binding model, what suggests that other bacterial molecules are involved in the adhesion process.

Another important observation is that there is a higher adhesion of HP26695 and HPTx30a strains to GP202 cell line when compared with MKN45 cell line. This reflects different expression levels and availability of ligands at the cells surface. Previous characterization of mucins and carbohydrate expression on GP202 and MKN45 cell lines showed that Le^b has a significantly higher expression in GP202 cell line[29]. Still, this difference might not be relevant since BabA is not present in both bacterial strains[28]. In addition, the MUC1 expression is identical for both cell lines[29] and therefore can not be held responsible for the observed differences. GP202 has a higher expression of other carbohydrate antigens (Le^a and Le^y)[29,30] compared to MKN45, that might be involved in *H pylori* binding interactions. Moreover, additional ligands/interactions that are not yet explored may also exist that can explain this difference in adhesion levels between cell lines.

In order to study the influence of MUC1 VNTR variability in *H pylori* binding, we used GP202, the cell line that showed higher bacteria adhesion and we analyzed GP202 transfected clones expressing recombinant MUC1 with a different number of repeats. These clones overexpress similar levels of recombinant MUC1[14]. We observed that MUC1 VNTR polymorphism has influence in the extent of *H pylori* binding to gastric cells, with the higher adhesion levels observed in clones with larger VNTR regions. This may be due to the fact that MUC1 with larger Tandem Repeat regions contains more potential glycan receptors, thus potentially providing more bacterial binding sites. Moreover, we have previously shown that differences in VNTR length lead to glycosylation changes in the MUC1 Tandem Repeat[14], which may also contribute to the altered adhesion observed. Detailed evaluation of the results showed a small increase between the adhesion of GP202-NEO (control) and GP202-dTR that may be explained by the overexpression of MUC1 in recombinant clone GP202-dTR[14] and by the potential presence of *O*-glycosylated binding sites outside the VNTR region. No significant difference was observed between the adhesion of the bacteria to GP202-9TR and to GP202-42TR clones. We have previously observed the overexpression of MUC1 underglycosylated forms in GP202-42TR[14], which might explain why the adhesion levels are not proportional to VNTR size.

All these observations are important for understanding the bacterial and host molecular context of the colonization of gastric mucosa. Identification of a pathogenesis background, based upon host susceptibility traits like MUC1 VNTR polymorphism, will help to identify candidates more prone to bacterial colonization and patients more resilient to eradication strategies.

COMMENTS

Background

More than half of the world population is persistently infected by *H pylori*. Adhesion of the bacteria to the gastric mucosa is essential for attachment and infection. Therefore it is important to know host and bacterial factors that condition the adhesion.

Innovations and breakthroughs

The study of host factors that influence the binding of *H pylori* to gastric cells may help to identify candidates more prone to bacterial colonization and patients more resilient to eradication strategies.

Applications

These findings may help to develop screening methods to identify candidates more prone to bacterial colonization and to develop more efficient eradication strategies, as well as to develop strategies to prevent or minimize *H pylori* binding to the gastric mucosa.

Peer review

This is a good study designed to elucidate that MUC1 VNTR polymorphism affects *H pylori* adhesion to gastric cells. The results are informative and potentially helpful for prevention of *H pylori* binding to the gastric mucosa.

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PAPER II

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PLoS one

Impact of MUC1 Mucin Downregulation in the Phenotypic Characteristics of MKN45 Gastric Carcinoma Cell Line

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Abstract

Background: Gastric carcinoma is the second leading cause of cancer-associated death worldwide. The high mortality associated with this disease is in part due to limited knowledge about gastric carcinogenesis and a lack of available therapeutic and prevention strategies. MUC1 is a high molecular weight transmembrane mucin protein expressed at the apical surface of most glandular epithelial cells and a major component of the mucus layer above gastric mucosa. Overexpression of MUC1 is found in approximately 95% of human adenocarcinomas, where it is associated with oncogenic activity. The role of MUC1 in gastric cancer progression remains to be clarified.

Methodology: We downregulated MUC1 expression in a gastric carcinoma cell line by RNA interference and studied the effects on cellular proliferation (MTT assay), apoptosis (TUNEL assay), migration (migration assay), invasion (invasion assay) and aggregation (aggregation assay). Global gene expression was evaluated by microarray analysis to identify alterations that are regulated by MUC1 expression. *In vivo* assays were also performed in mice, in order to study the tumorigenicity of cells with and without MUC1 downregulation in MKN45 gastric carcinoma cell line.

Results: Downregulation of MUC1 expression increased proliferation and apoptosis as compared to controls, whereas cell-cell aggregation was decreased. No significant differences were found in terms of migration and invasion between the downregulated clones and the controls. Expression of TCN1, KLK6, ADAM29, LGAL4, TSPAN8 and SHPS-1 was found to be significantly different between MUC1 downregulated clones and the control cells. *In vivo* assays have shown that mice injected with MUC1 downregulated cells develop smaller tumours when compared to mice injected with the control cells.

Conclusions: These results indicate that MUC1 downregulation alters the phenotype and tumorigenicity of MKN45 gastric carcinoma cells and also the expression of several molecules that can be involved in tumorigenic events. Therefore, MUC1 should be further studied to better clarify its potential as a novel therapeutic target for gastric cancer.

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Introduction

Gastric cancer is one of the most common and life-threatening cancers worldwide (for review see [1]).The poor prognosis of this disease reflects our poor understanding of its etiological factors and pathogenesis and the lack of effective treatments.

MUC1 is a high molecular weight transmembrane protein that is expressed at the apical surface of most glandular epithelial cells [2]. MUC1 is overexpressed in almost 95% of cancer cells [3], a molecular pathological feature that is associated with carcinogenesis and poor prognosis [4,5,6,7,8]. Moreover, aberrant glycosylation and loss of apical expression of MUC1 have been reported for gastric carcinomas [9,10,11].

MUC1 protein consists of a highly variable extracellular domain composed of a variable number of tandem repeats (VNTR), and a highly conserved cytoplasmic domain (CD), which are both essential for MUC1-driven oncogenic activities [12,13]. The MUC1 extracellular domain can be extensively glycosylated [14] and was shown to interact with several extracellular ligands, including ICAM-1[15] and galectin-3[16]. These interactions influence cell adhesion [16], motility and migration [17,18], metastasis [19] and cell-cell aggregation [20], which contribute to the maintenance of a normal cell phenotype, and upon disregulation contribute to tumor progression. The MUC1 cytoplasmic domain (MUC1-CD) engages in signal transduction through several residues that can be phosphorylated by receptor tyrosine kinases (and other kinases), which in turn regulate MUC1-CD affinity to other mediators of signal transduction and transcriptional regulation [for review see [21] and [22]. MUC1-CD associates with molecules such as β-catenin, c-Src, Grb2/Sos,

p53, GSK-3β, EGFR and PKC-δ [for review see [22], Lyn [23] , Lck and Zap 70 [24] , ER-α [25], NFKβ [26], c-Abl [27], ATM [28] and CAML [29], that regulate processes of cell survival, proliferation, apoptosis, adhesion, migration and cell-cell aggregation. These functions of MUC1 are known to contribute to tumor progression and poor survival of cancer patients [for review see [6,30]. Nonetheless, the relevance of MUC1 in gastric cancer progression has not been previously investigated.

In the report presented here we used retrovirus-mediated transfection of short-hairpin RNAs (shRNA) to induce a stable downregulation of MUC1 in the gastric carcinoma- derived cell line MKN45. The effects of MUC1 downregulation were studied *in vitro* with respect to cell proliferation, apoptosis, migration, invasion and cell-cell aggregation. MUC1 downregulated cells were more proliferative and apoptotic than the controls and exhibited lower degrees of cell-cell aggregation. No significant differences were found in terms of cell migration and invasion. Global gene expression analysis, evaluated by oligonucleotide microarrays, identified several genes influenced by MUC1 downregulation that may contribute to the observed phenotypic alterations. *In vivo* studies have shown that MUC1 downregulation impacts tumor development.

Materials and Methods

Cell culture

A human cell line derived from diffuse-type gastric carcinoma – MKN45 (poorly differentiated adenocarcinoma, Japan Health Sciences Foundation [31]) was grown in RPMI 1640 containing Glutamax^{TM}I and 25mM HEPES, supplemented with 10% fetal bovine serum (FBS) and 50 μg/ml gentamicin (Invitrogen). The packaging cell line PhoenixGP [32] was maintained in Dulbecco's Modified Eagle Medium containing Glutamax^{TM}I, 4,500 mg/l D-Glucose and Sodium Pyruvate, supplemented with 10% FBS and 1% (v/v) penicillin/streptomycin. Stable MUC1 downregulated clones derived from MKN45 cells were grown in standard growth medium supplemented with 5 μg/ml puromycin (Sigma). After evaluation of MUC1 levels at different time points in culture, all the assays were performed considering the time of cell culture in which the downregulation was higher, at 96 hours of cell culture. Cells were grown at 37°C with 5% CO_2 in humidified atmosphere.

MUC1 downregulation strategy

MUC1 downregulated cells were produced using a retroviral expression system with short hairpin RNAs. Briefly, a 21-nucleotide sequence of the MUC1 gene, with no homology to other DNA sequences detected in a BLAST search, was chosen according to standard RNAi rules [33]. The scramble control was designed and tested for homology in a BLAST search as well. Sense and antisense oligos (Proligo) were ligated and inserted in the pSUPER.retro.puro vector (Oligoengine). The oligos used were the following: MUC1 Exon 2 (*sense*: GATCCCCACCTCCAGTTTAATTCCTCTTCAAGAGAGAGGAATT AAACTGGAGGTTTTTTA; *antisense*: AGCTTAAAAAACCTCCAGTTTAATTCCTCTC TCTTGAAGAGGAATTAAACTGGAGGTGGG; the MUC1-cDNA target region is underlined) and scramble control (*sense*: GATCCCCATCACCTTCGTACTCCTTA TTCAAGAGATAAGGAGTACGAAGGTGATTTTTTA, *antisense*: AGCTTAAAAAATC ACCTTCGTACTCCTTATCTCTTGAATAAGGAGTACGAAGGTGATGGG; the "unpaired"-cDNA target region is underlined). The MUC1 specific target or the scrambled control constructs were transfected into PhoenixGP packaging cell line by calcium-phosphate mediated transfection and transfected cells were selected using puromycin. Stable transfectants were seeded in a 6-well plate ($1x10^6$cells/well) and incubated for 24 hours at 32°C. The media containing the virus was collected, filtered through a 0.45 μm filter to remove remnant cells, and used to infect MKN45 cells, during 24 hours at 37°C. The viral supernatant was then replaced by the standard growth medium and cells were incubated 48 hours at 37°C. Efficiently transduced cells were selected and grown in standard media supplemented with puromycin. Two independent MUC1 downregulated clones (C1 and C2) were isolated and expanded for three times using cloning rings.

Immunofluorescence

MKN45 cells at 96h in culture were harvested, seeded in 12-well slides (Cell Line) and air-dried overnight at room temperature. Cells were then fixed in ice cold acetone for 5 minutes, washed twice with PBS and blocked with normal rabbit serum (DAKO) diluted 1:5 in 10% bovine serum albumin (BSA) for 30 minutes. Serum was then replaced by the MUC1 monoclonal antibody HMFG1 (NovoCastra) diluted 1:50 in 5% BSA, and incubated overnight at 4°C. After three washes with PBS, cells were incubated with a rabbit anti-mouse FITC labeled antibody (DAKO) diluted 1:70 in 5% BSA for 30 minutes in the dark at room-temperature. Cells were washed 3 times with PBS and mounted in vectashield (Vectorlabs). Images were acquired in a Leica DMIRE2 fluorescent microscope. Results are representative of three independent experiments.

Protein extraction and Western blot

MKN45 cells were cultured in 60-mm dishes to 80–90%-confluence at 96h in culture. After washing twice with PBS, lysis buffer (10mM Tris pH 7.4, 150mM NaCl, 0.1% (p/v) SDS, 1mM PMSF, 1% (v/v) Triton X-100) was added and cells were scraped. Lysates were incubated on ice for 1 hour and centrifuged for 2 minutes at 4°C to collect the supernatants. Protein content was assessed by the bicinchoninic acid method (Pierce), as described in the manufacturer's instruction manual. Protein extracts were analyzed by a 4–10% SDS-PAGE (Invitrogen), transferred to a nitrocellulose membrane (Amersham Biosciences), and blotted overnight at 4°C with anti MUC1-Ab5 monoclonal antibody (1:300, ThermoScientific), anti-beta-actin polyclonal antibody (1:8,000, Sigma), anti ERK1/2(1:1,000, Cell Signaling Technology) and anti β-catenin (1:1,000, BD Transduction Laboratories) in 5% non-fat milk in TBS-0.1%Tween20 (Sigma). Membranes were washed 3 times with TBS-0.1%Tween20 and the primary antibodies were revealed using goat anti-mouse peroxidase-conjugated antibody (1:1,000, DAKO) in 5% non-fat milk in TBS-0.1%Tween20, followed by ECL detection kit (BioRad). Results are representative of three independent experiments.

RNA extraction and Real-Time PCR

Total RNA was isolated from MKN45 cells at 96h in culture using TriReagent^{TM} (Sigma), according to the manufacturer's instructions. 5 μg of RNA were primed with random hexamers (Invitrogen) and reverse transcribed with Superscript II (Invitrogen) in a final volume of 20 μl. 2 μl of a 1:10 dilution of cDNA were amplified with 300nM of each primer and SYBRGreen (Applyed BioSystems) in a final volume of 20 μl, using the fluorescence reader ABI Prism 7000. Each sample was run in duplicate. The primers used were the following: MUC1 (*sense*: CTCCTTTCTTCCTGCTGCTG, *antisense*: CTGGAGAGTACGCTGCTGGT) and 18S (*sense*: CGCCGCTAGAGGTGAAATTC, *antisense*: CATTCTTGGCAAATGCTTTCG), and their specificity was confirmed using the software BLASTn on-

line and by melt curve analysis. For each sample, the level of 18S RNA was measured and used for normalization of target genes abundance. Relative mRNA levels were then calculated using the comparative C_t method [34]. Data is expressed as a ratio of the results obtained with each clone and the scramble control, from three independent experiments. Statistical analysis was performed using the Mann-Whitney test.

MTT proliferation assay

Cells were plated in triplicate in 96-well plates at 5,000 cells per well and incubated at normal conditions for 96h for MKN45 cells. At each time point, the medium was removed and cells incubated with 20 µl of MTT solution (5mg/ml, Sigma) for 3 hours at normal conditions. MTT was removed and 200 µl of DMSO were added to each well to dissolve formazan. Finally, formazan optical density was measured using a microplate reader at a wavelength of 540nm. The relative growth was defined as the following formula: Relative Growth = (A_{540nm} at Tn / A_{540nm} at $T0_{24h}$). Data is expressed as a ratio of the results obtained with each clone and the scramble control, from three independent experiments. Statistical analysis was performed using the Mann-Whitney test.

Terminal Transferase dUTP Nick End Labeling (TUNEL) assay

Post-confluent cells at 96h in culture were harvested and fixed with 4% paraformaldehyde in PBS for 15 minutes. Fixed cells were seeded in 12-well slides (Cell Line) and air-dried overnight at room temperature. Following washing with PBS, cells were permeabilized with ice-cold freshly-made PBSTrCit solution (PBS + 0.1%TritonX + 0.1% Sodium Citrate) for 2 minutes on ice. Cells were washed again twice, and incubated with TUNEL reaction mix (enzyme solution, label solution and dilution buffer,

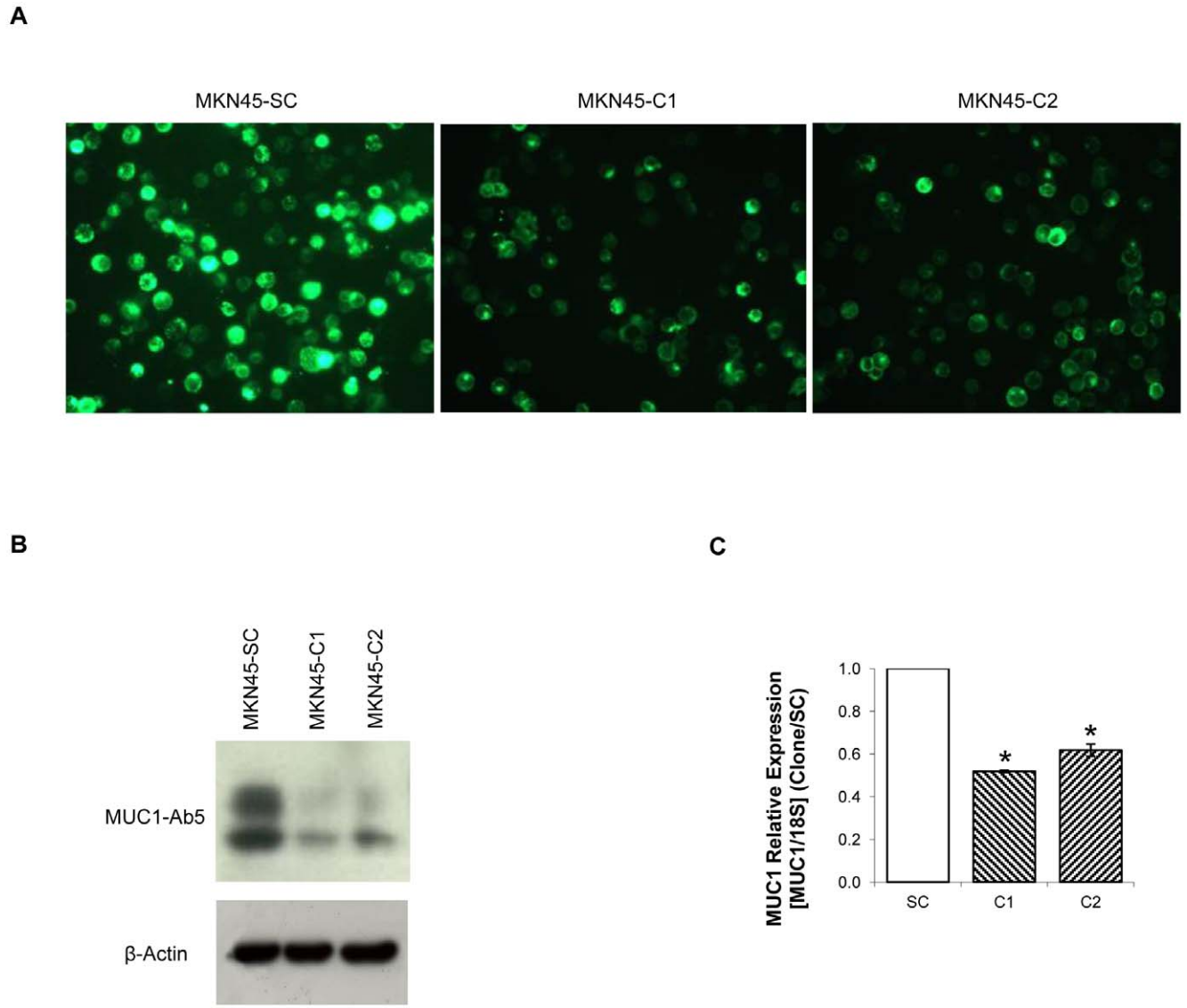

Figure 1. MUC1 downregulation by shRNA. (**A**) MUC1 detection by immunofluorescence with HMFG-1 antibody in MKN45-C1 and MKN45-C2 and MKN45-SC control; (**B**) MUC1 protein detection by western-blot with MUC1-Ab5 antibody of total protein extracts from MKN45-C1 and MKN45-C2 and MKN45-SC control; (**C**) Quantification of MUC1 RNA in MKN45-C1 and MKN45-C2 and MKN45-SC control by real-time PCR. MUC1 expression was corrected to the house-keeping gene 18S and normalized to the data obtained with the scrambled control. *$P<0.01$.
doi:10.1371/journal.pone.0026970.g001

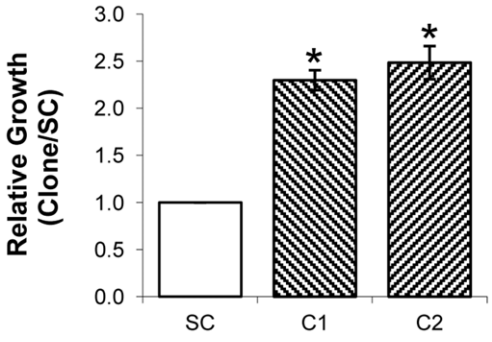

Figure 2. Quantification of cell proliferation by MTT assay. Quantification of metabolically active cells by MTT assay in MKN45-C1 and MKN45-C2 clones and MKN45-SC control at 96h in culture. Data from 24 hours was used to set time zero and results were normalized to the data obtained with the scrambled control.*$P<0.01$.
doi:10.1371/journal.pone.0026970.g002

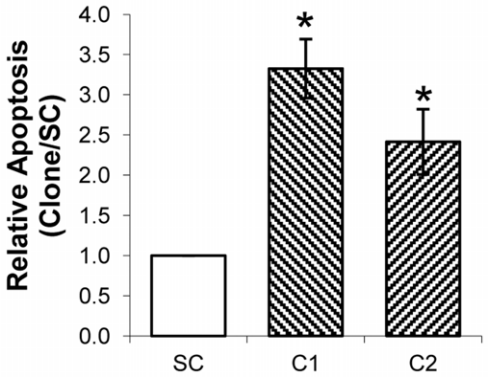

Figure 3. Quantification of apoptotic cells by TUNEL assay. Apoptosis of MKN45-C1 and MKN45-C2 and scramble control (SC) were evaluated at 96h in culture by the TUNEL assay. Results were normalized to the data obtained with the scrambled control. *$P<0.01$.
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1:9:10, In Situ Death Detection Kit, Fluorescein, Roche) for 1 hour at 37°C. Two additional washing steps were performed and slides were mounted in Vectashield with DAPI (Vectorlabs). Results were analyzed under a Leica DMIRE2 fluorescent microscope and data is expressed as a ratio of the results obtained with each clone and the scramble control, from three independent experiments. Statistical analysis was performed using the Mann-Whitney test.

Migration assay

MKN45 cells were cultured in 60-mm dishes for a full-confluence at 96h in culture. The epithelial cells monolayer was then washed with PBS and wounded with a 10 µl micropipette tip. Non-adherent cells were removed by washing twice with PBS. Images of cells at the edge of the wound were acquired automatically at 20x magnification in a Leica DMIRE2 fluorescence microscope with a Leica DFC Twain camera for 144 frames at 10-minute intervals (corresponding to 24 hours) controlled by Leica FW4000 software. Frames from 0, 6, 12, 18 and 24 hours were used to quantify the percentage of migration: a grid of 50x30 squares was used to fulfill the wound space and the percentage of migration was calculated by the number of squares occupied by cells at each time point. Data is expressed as a ratio of the results obtained with each clone and the scramble control, from three independent experiments. Statistical analysis was performed using the Mann-Whitney test.

Matrigel invasion assay

Cell invasion was studied by using BD Biocoat™ Matrigel™ invasion chambers with 8-µm size pores (BD Biosciences), according to the manufacturer's instructions. MKN45 cells at 96h in culture were harvested and seeded in duplicate at 250,000 cells per insert (sized for 24-well plates) in 1% FBS containing medium, and 20% FBS containing medium was added to the bottom of the growth well, as an attractant. Cells were allowed to invade for 22 hours (37°C, 5% CO_2 atmosphere). The non-invading cells were then swabbed from the top of the inserts and the invading cells on the lower surface were fixed with 100% methanol and stained with DAPI for 15 minutes in the dark. The membranes were removed and cells were counted under a Leica DMIRE2 fluorescence microscope. Data are expressed as a ratio of the results obtained with each clone and the scramble control, from three independent experiments. Statistical analysis was performed using the Mann-Whitney test.

Cell-cell Aggregation assay

MKN45 cells at 96h in culture were harvested and seeded in duplicate at 250,000 cells per well in 24-well plates. Plates were placed at 37°C with constant stirring (150rpm) for 1 and 2 hours. Cells were fixed with 100 µl of 25% glutaraldehyde at time zero and at the end of the incubation. Aggregates were photographed under a light microscope and isolated cells were counted (cells in duplicates were counted as isolated cells). The aggregation index was defined as the following formula: Aggregation index = 1-(number of isolated cells at Tn / number of isolated cells at T0). Data are expressed as a ratio of the results obtained with each clone and the scramble control, from three independent experiments. Statistical analysis was performed using the Mann-Whitney test.

Gene expression analysis

The expression of 12,135 genes in MUC1 downregulated clones and the respective scramble control were evaluated following the same protocol as before [35]. Briefly, following RNA extraction (as described previously), cDNA was obtained by reverse transcription, during which labeled nucleotides were incorporated: MKN45-C1 and MKN45-C2 cDNAs were labeled with Cy3 (green emission) and MKN45-SC control with Cy5 (red emission). After hybridization, the mixture was hybridized with the array overnight and then the array was digitalized with the ScanArray4000 (Perkin-Elmer) system and fluorescence analyzed by the QuantArray software package (Perkin-Elmer).

Normalization and background subtraction were performed and ratios for downregulated clones /Scrambled Control and Scrambled Control/downregulated clones were calculated using Microsoft Excel software. Gene expression with a ratio higher than 2 was considered statistically significant.

All data is MIAME compliant and that the raw from the microarray experiments were uploaded onto the Gene Expression Omnibus Database http://www.ncbi.nlm.nih.gov/geo (Geo accession numbers: GSM717858 and GSM717859).

In Vivo tumor growth Assays

Six-week-old female N:NIH(s)II:nu/nu nude mice were obtained previously from the Medical School, University of Cape Town in 1991 and maintained and housed at IPATIMUP Animal House at the Medical Faculty of the University of Porto, in a pathogen-free environment under controlled conditions of light

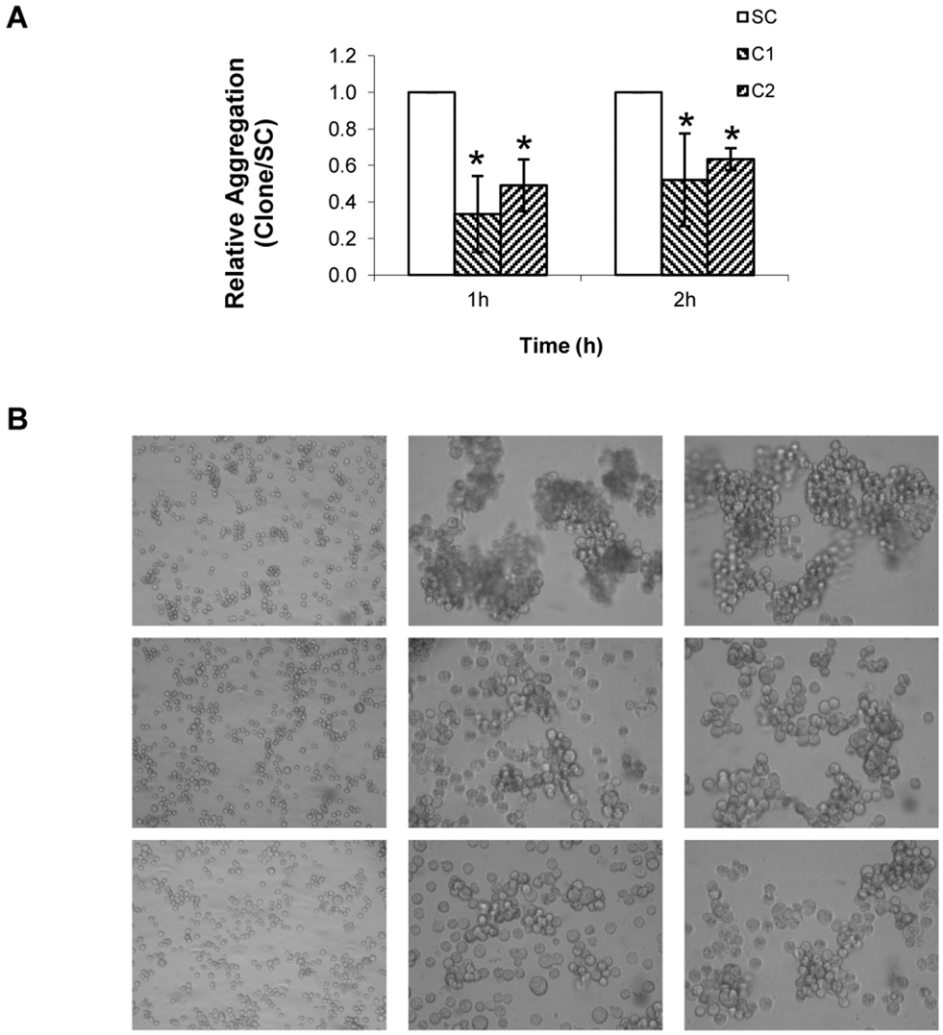

Figure 4. Quantification of cell-cell aggregation. (**A**)Quantification of the cell-cell aggregation index in MKN45-C1 and MKN45-C2 and MKN45-SC control. The cell-cell aggregation index was assessed by the observed decrease in the number of isolated cells over time, and normalized to the data obtained with the scrambled control. *$P<0.01$; (**B**) Images of the aggregates formed after 1 and 2 hours of constant stirring. First column shows isolated cells at time 0h (20x magnification) and second and third columns show aggregates formed after 1h and 2h of incubation (40x magnification), in MKN45-C1 and MKN45-C2 and MKN45-SC control.
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and humidity. Males and females, aged 6–8 weeks, were used for *in vivo* experiments. Animal experiments were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals, directive 86/609/EEC. Mice were subcutaneously injected in the dorsal flanks using a 25-gauge needle with $1x10^5$ of MKN45-SC (2 male and 2 female mice) or MKN45-C2 cells (3 male and 3 female mice). Mice were weighed, and tumor width and length were measured with calipers every week. Mice were euthanized 21 days after cell injection, at the time when the first tumor reached maximum allowable volume. For statistical analysis, the Mann Whitney test-StatView Software version 5.0 (SAS Institute, Cary, NC) was used. A P value of less than 0.05 was considered as statistically significant.

Results

MUC1 downregulation by shRNA

We established two independent MUC1 downregulated clones, MKN45-C1 and MKN45-C2 and one scramble control, MKN45-SC. MUC1 downregulation was verified by immunofluorescence (Figure 1A), Western Blot (Figure 1B) and Real-Time PCR (Figure 1C).

There was a significant downregulation of MUC1 expression in MKN45-C1 and MKN45-C2 clones as compared to the MKN45-SC control. The expression of MUC1 at the protein level was detected with two different antibodies, one that binds the VNTR extracellular domain (HMFG-1, Figure 1A) and other that recognizes a 14–28 KDa sequence in MUC1 cytoplasmic domain (MUC1-Ab5, Figure 1B). Both showed a significant reduction in the amount of MUC1 protein in MKN45-C1 and MKN45-C2 clones when compared to the scramble control. Real-Time PCR results indicate that the MUC1 downregulation was 48% (MKN45-C1) and 38% (MKN45-C2) (Figure 1C). MUC1 RNA levels were evaluated at 48, 72 and 96h of cell culture and the highest downregulation occurred at 96h (results not shown).

Effects of MUC1 downregulation on MKN45 cells

Cell proliferation. MKN45-C1 and MKN45-C2 cells showed significantly increased proliferation rates ($P<0.01$) when

Table 1. Oligonucleotide microarray results by comparison between MKN45-C1/MKN45-C2 and MKN45-SC control cells, by order of magnitude.

Genes upregulated >2 fold in MUC1 downregulated clones
Transcobalamin 1 (TCN1)
Kallikrein-related peptidase 6 (KLK6)
Desintegrin and metalloproteinase 29 (ADAM29)
Keratoepithelin (TGFBI)
MRP family of ATP transport member 2 (ABCC2)
Amyloid beta precursor-like protein 2 (APLP2)
Mitochondrial ATP synthase (ATP5I)
Sulfide dehydrogenase like protein (SQRDL)
Sarcoglycan, epsilon (SGCE)
Hypothetical protein (FLJ20323)
Galectin 1 (LGALS1)
Proline-histidine rich protein (PHLDA1)
Trypsin 2 (PRSS2)
Mesotrypsin (PRSS3)
SP2 transcription factor (SP2)
Ubiquitin-conjugating enzyme (UBE2L6)
Vitellogenic-like carboxypeptidase (CPVL)
Genes downregulated >2 fold in MUC1 downregulated clones
Galectin 4 (LGALS4)
Tetraspanin 8 (TSPAN8)
Tyrosine phosphatase SHP substrate (SHPS-1)
Polymerase (DNA-directed), delta 4 (POLD4)
H2B histone family, member J (HIST1H2BH)
H2B histone family, member T (HIST1H2Bk)
Carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM5)
Annexin IV (ANXA4)
Intercellular adhesion molecule 4 (ICAM4)
DEAD (Asp-Glu-Ala-Asp) box polypeptide 39 (DDX39)
Apolypoprotein B (APOBEC2)
Clusterin (CLU)
GDP-mannose 4,6-dehydratase (GMDS)
Serine/threonine kinase 38 like (STK38L)
CD55 (CD55)
Apolipoprotein B-catalytic polypeptide-like 3C (APOBEC3C)
Cell adhesion related-molecule (CDON)
Villin-1 (VIL1)

MKN45-C1 and MKN45-C2 and the MKN45-SC control were analysed by oligonucleotide microarrays. Listed are genes with expression increased or decreased more than 2 fold in both MUC1 downregulated clones when compared to the control.
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compared to the MKN45-SC control (2.29 and 2.48 *vs* 1), when evaluated by MTT assay (Figure 2).

Cell apoptosis. MKN45-C1 and MKN45-C2 cells showed significantly increased levels of apoptosis ($P<0.01$) when compared to the MKN45-SC control (3.32 and 2.41 *vs* 1), when evaluated by a TUNEL assay (Figure 3).

Cell-cell aggregation. MKN45-C1 and MKN45-C2 cells showed significantly decreased cell-cell aggregation levels ($P<0.01$), when compared to the MKN45-SC control (0.34 and 0.49 *vs* 1 at 1h; 0.52 and 0.64 *vs* 1 at 2h), when evaluated by a cell aggregation assay (Figures 4A and 4B).

Cell migration and invasion. There were no significant differences in cell migration and invasion of MKN45-C1 and MKN45-C2 cells when compared to the MKN45-SC control, when evaluated by motility and invasion assays (results not shown).

Effects of MUC1 downregulation in MKN45 cells gene expression

The observed phenotypic modifications associated were likely due in part to alterations in signal transduction pathways mediated by MUC1-CD, since overexpression of MUC1 has been shown to modulate gene expression through reprograming transcription of multiple genes [36,37]. We evaluated the net effects of downregulating MUC1 in the MKN45 gastric carcinoma cell line by performing a global analysis of gene expression by oligonucleotide microarrays (Table 1). The results revealed that a number of genes that influence proliferation, migration, invasion and motility were differentially expressed in MKN45-C1 and MKN45-C2 and the MKN45-SC control. The most significant differences were found for TCN1, KLK6 and ADAM29 (>10 fold upregulated between the MKN45-C1 and MKN45-C2 clones and the MKN45-SC control) and LGALS4, TSPAN8 and SHPS-1 (>3.5 fold downregulated between the MKN45-C1 and MKN45-C2 clones and the MKN45-SC control).

In vivo tumor growth assays

In vivo tumorigenicity assays showed that mice injected with MUC1-downregulated cells (MKN45-C2) developed smaller and slower-growing tumors, when compared to mice injected with the MKN45-SC control cells (Figure 5).

Discussion

In the work presented here, we evaluated the effects of MUC1 downregulation on cancer-related properties of MKN45 gastric carcinoma cells. Stable downregulation of MUC1 expression was achieved in MKN45 gastric carcinoma cell line by RNA interference. MUC1 contributes to tumor progression of adenocarcinomas and therefore its downregulation was predicted to affect the malignant properties of cancer cells, including proliferation, apoptosis, migration, invasion and cell-cell aggregation.

We found that proliferation was significantly increased in MUC1 downregulated clones MKN45-C1 and MKN45-C2 when compared to the control MKN45-SC. Similar studies with breast and pancreatic carcinoma cell lines have shown similar [38] and opposite [35,38,39] results. In different tumor models, MUC1 was shown to regulate cell proliferation by interacting with several proteins such as ER-α, β-catenin and EGFR [25,40,41]. However, for gastric carcinoma cells, such interactions have not been investigated. Results obtained by an oligonucleotide microarray analysis showed that expression of molecules affecting cell proliferation such as KLK6 and LGAL-4 [42,43,44] were significantly altered in MKN45-C1 and MKN45-C2 clones when compared to the MKN45-SC control. KLK6 expression was increased in MKN45-C1 and MKN45-C2 clones, whereas LGAL4 expression was decreased and these differences may explain the observed differences in proliferation. The mechanisms by which KLK6 and LGAL4 expression is altered in MKN45-C1 and MKN45-C2 when compared to MKN45-SC control remains to be elucidated.

Another important observation was that apoptosis was significantly increased in MKN45-C1 and MKN45-C2 clones when compared to the MKN45-SC control. MUC1 was previously shown to mediate a pro-apoptotic response in hamster ovary cells [45] and it was also attributed with anti-apoptotic functions in

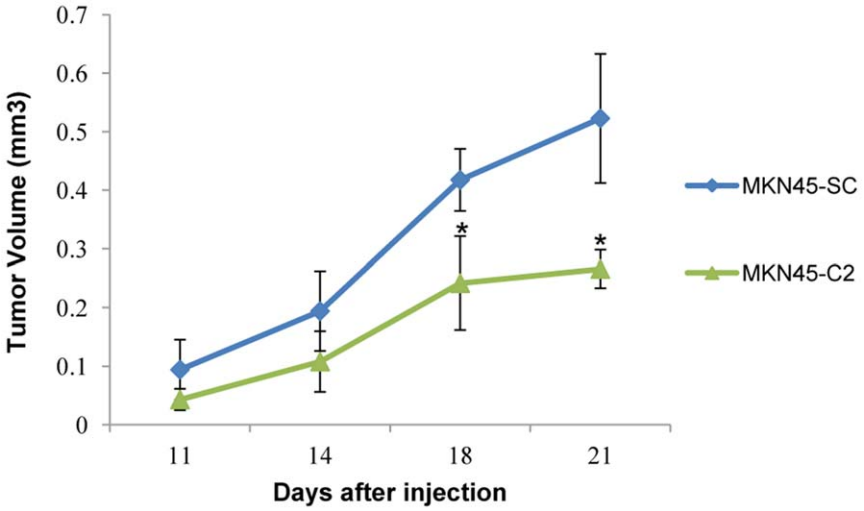

Figure 5. Study of the tumorigenicity of MKN45 gastric carcinoma cells *in vivo*. Tumor growth curves. 1×10^5 cells were subcutaneously injected in mice at day 0. The curves show tumor growth until day 21, the day on which all mice were sacrificed.* $P<0.05$, when compared to the MKN45-SC control cell line.
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myeloma, breast and colorectal carcinoma cell lines [46,47,48]. However, little is known about the influence of MUC1 on cell apoptosis in gastric carcinoma cells.

No significant differences were found between MUC1-C1 and C2 clones and MKN45-SC control with respect to cell migration and invasion. This is in contrast to previous findings in which MUC1 was shown to influence cell migration in breast, cervical, renal and pancreatic carcinoma cell lines [18,49,50,51] and cell invasion in breast, lung, gastrointestinal, hepatic and pancreatic carcinoma cell lines [3,52,53].

Cell-cell aggregation was decreased in MKN45-C1 and MKN45-C2 clones when compared to the MKN45-SC control. Previous studies have shown that overexpression of different forms of MUC1 can lead to an increase or a decrease in cell-cell aggregation in a pancreatic carcinoma cell line [54], whereas others have shown that MUC1 downregulation induces an increase of cell-cell aggregation in an oral carcinoma cell line [41]. MUC1 interactions with other adhesion molecules have been shown to contribute to both adhesive [55,56] and anti-adhesive [57,58,59] properties of cells. Our results showed that MUC1 plays a relevant role in MKN45 cell-cell aggregation, contributing to gastric cells adhesive properties.

Another possibility is that signaling through the MUC1-CD influences gene expression, which in turn affects the phenotypic properties of the MKN45 cell line. By oligonucleotide microarray analysis we found alterations in the transcriptional profile of cells following MUC1 downregulation when compared to control cells. These alterations are likely due to MUC1 downregulation, since MUC1 has been shown to directly conduct signals that alter the transcriptional program of tumor cells [36,37,60]. MUC1 cytoplasmic domain can be phosphorylated in several sites, modulating its interaction with cell signalling partners and transcription factors [21]. The phosphorylation of MUC1-CD will be dependent on the amount and availability of its signaling partners and therefore on the cell type in question. We found significant alterations in the expression levels of several genes, mainly TCN1, KLK6, ADAM29, LGALS4, TSPAN8 and SHPS-1. Some of these molecules have functions not yet fully clarified yet others are known to be associated with cell proliferation and migration, including KLK6, LGAL4 and SHPS-1 [42,43,59,61,62], invasion, including KLK6 [59] and motility, including LGAL4 [43]. MUC1 may be facilitating the transcription of these genes and therefore be contributing to the observed phenotypic alterations observed.

In vivo assays confirmed that cells with decreased levels of MUC1 form smaller and slower-growing tumors than the control cells. This result emphasizes that MUC1 contributes to gastric tumor progression in the context of the multicellular environment of tumor growth in vivo.

MUC1 overexpression has been associated with the neoplastic progression of several tumors, including the acquisition of invasive and metastatic properties. Phenotypic studies in cell models other than gastric cancer have suggested that MUC1 influences events such as proliferation, apoptosis, migration, invasion, adhesion and cell-cell aggregation. Previous studies of MUC1 in breast carcinogenesis models show mixed results for different breast cancer cell lines [38], which reinforces the relevance of the molecular context on the MUC1-mediated cancer progression. The effects of MUC1 in gastric carcinogenesis will thus be dependent on MUC1 and the molecules interacting with MUC1, which will significantly differ between cell lines. Evaluation of different gastric cell lines will complement the data regarding the impact of MUC1 gastric carcinogenesis.

The work presented here shows for the first time that MUC1 expression influences proliferation, apoptosis and cell-cell aggregation of MKN45 gastric carcinoma cells. The results are consistent with the view that MUC1 modulates different signaling pathways in a manner that is dependent on the expression and activity of other regulatory mechanisms and molecules, which are influenced by the cellular and biological context of the cell type that is overexpressing MUC1.

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Author Contributions

Conceived and designed the experiments: NRC PP TC MAH FSS. Performed the experiments: NRC PP. Analyzed the data: NRC PP FSS. Contributed reagents/materials/analysis tools: NRC PP TC MAH FSS. Wrote the paper: NRC MAH FSS.

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PAPER III

MUC1 mucin conditions MAPK signaling pathway in MKN45 gastric carcinoma cells

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Abstract

Background: Gastric cancer (GC) is one of the most common and life-threatening cancers worldwide. The poor prognosis of this disease reflects the poor understanding of its etiological factors and pathogenesis and the lack of effective treatments.

MUC1 is a high molecular weight glycoprotein that protects epithelial surfaces. MUC1 highly conserved cytoplasmic domain (MUC1-CD) is involved in cell signaling cascades in different tumor models, being phosphorylated by several kinases and interacting with several cell signaling oncogenic molecules. Overexpression, aberrant glycosylation and loss of apical expression of MUC1 have been reported for gastric carcinomas. However, there is no information so far about MUC1-CD participation in cell signaling pathways in GC cells. The aim of this work was therefore to identify MUC1 oncogenic signaling partners in MKN45 gastric carcinoma cell line and to evaluate the impact of MUC1 expression in its signaling partners expression and phosphorylation. **Methodology:** Identification of MUC1-CD signaling partners was performed by

immunoprecipitation and proximity ligation assays. Evaluation of MUC1 downregulation impact in MUC1-CD signaling partners expression and phosphorylation was performed using immunoblots, Real Time PCR and phospho-antibody screening (Kinexus). **Results:** Immunoprecipitation and proximity ligation assays have shown that MUC1-CD interacts with EGFR, Grb2, B-RAF, and ERK1/2 proteins, all important effectors of the MAPK signaling pathway in MKN45 GC cells and with ERK1/2 in AGS GC cells.

MUC1 downregulation conditions ERK1/2, EGFR and B-RAF protein levels, suggesting that MUC1 is also involved in the transcription and/or stability of these molecules. The evaluation of mRNA expression by Real-Time PCR has shown that ERK2 and B-RAF expression is likely regulated by MUC1 at a transcriptional level, whereas EGFR expression is most likely regulated by post-transcriptional mechanisms. Furthermore, MUC1 downregulated clones have shown a general significant increase in the phosphorylation of ERK2 molecule and a decrease in the levels of ERK1, when compared to the control cells.

Conclusions: MUC1-CD physically interacts and affects the expression and phosphorylation of MAPK-related oncogenic signaling proteins in gastric carcinoma cells.

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Introduction

Gastric cancer (GC) is one of the most common and life-threatening cancers worldwide [1] and nowadays the third cause of cancer-related deaths all over the world and the fourth in Portugal ([2] GLOBOCAN 2008, IARC) . The poor prognosis of this disease with a 5-year survival rate of 20% [3] reflects the reduced understanding of its etiological factors and pathogenesis, the absence of specific symptoms and consequent late diagnosis and lack of effective treatments. Mucins that cover the gastric epithelium and that are the first contact barrier between cells and the external environment are thus elective targets to study gastric oncogenesis.

MUC1 mucin is a high molecular weight transmembrane protein that is expressed at the apical surface of most glandular epithelial cells [4] and one of the major mucins expressed in the stomach. Immediately after translation, MUC1 protein undergoes proteolytic cleavage in the extracellular part of the protein, 71 to 53 amino acids upstream of the transmembrane domain. The two resulting proteins remain associated in a tight heterodimer complex that is expressed at the cell membrane [5, 6].

The extracellular domain of this protein contains a region of variable number of tandem repeats (VNTR) rich in serine and threonine residues that constitute targets of O-glycosylation. In epithelial adenocarcinomas of several tissues, including lung, breast, ovary, prostate, pancreas, stomach and colon, immunohistochemical analysis of MUC1 expression with antibodies against epitopes of the VNTR region of the mucin shows overexpression of this molecule on the entire cell membrane and also its aberrant glycosylation [7]. Although the biological relevance of the VNTR domain of the mucin is still

unknown, it was shown that the variability of the VNTR length is associated with the risk for cancer development [8, 9]. This association is related with the expression of tumor-associated carbohydrate antigens that are coupled to the VNTR region of MUC1 and other mucins [10, 11].

Contrasting to the variation observed in the extracellular domain, the cytoplasmic domain of MUC1 mucin (MUC1-CD) is highly conserved among species, suggesting its involvement in basic cellular processes [12]. In fact, most of the oncogenic features of MUC1-overexpressing tumor cells are thought to be driven by MUC1-CD interaction with proteins involved in signal transduction. Tyrosine residues present in MUC1-CD can be phosphorylated by several tyrosine kinases, including the epithermal growth factor receptor (EGFR) and members of the Src family, leading to the activation of the MAPK pathway through the RAS-MEK-ERK2 pathway in breast, COS-7 and mouse mammary gland [13-15]. Phosphorylation of the MUC1-CD by these kinases also protects β-catenin from the degradation mediated by GSK3-β, ultimately leading to the nuclear translocation of the complex MUC1-CD/ β-catenin, and to the activation of transcription events in breast, colon and pancreatic carcinoma cell lines [16-18]. However, in a recent report on MUC1-CD interaction with β -catenin it was shown that expression of this molecule in human embryonic kidney-derived cells decreases β-catenin translocation into the nucleus, reducing the activation of the LEF-1 transcription factor and blocking the expression of cyclin-D1, thus, inhibiting cell proliferation [19].

MUC1-CD is also involved in the inhibition of oxidative stress-induced apoptosis. In the presence of high levels of reactive oxygen species, MUC1-CD expression is up-regulated, leading to an increase in the expression of anti-

oxidant enzymes and to the dephosphorylation-mediated activation of FOXO3a, a member of the forkhead family of transcription factors that induces oxidant scavenging and DNA repair in breast, colon and cervical carcinoma cell lines [20, 21]. The role of MUC1 in cell survival is also suggested by MUC1-CD interaction with p53 in response to genotoxic agents. MUC1-CD binds directly to p53 regulatory domain, suppressing p53-dependent apoptotic response to DNA damage [22]. Despite these reports, it was also shown, in a completely different model, that MUC1-overexpressing tumor cells are more sensitive to FasL-induced apoptosis than their negative counterparts, through an increase in the expression of Fas receptor [23].

The apparently contradictory data suggests that the complexity of the MUC1 mucin as a molecule involved in different signaling pathways may be dependent on the cell type and on the molecular context (e.g. amount and availability of signaling partners) where it is expressed.

Data about MUC1-CD signaling partners or MUC1-CD participation in oncogenic cell signaling cascades in GC cells is very limited. There is one study that shows that interaction between MUC1-CD and β-catenin leads to upregulation of cyclin-D1 in *H. pylori* derived gastric cancer [24].

In the present study we aimed to identify MUC1-CD signaling partners by immunoprecipitation and proximity ligation assays (PLAs) and to evaluate the impact of MUC1 expression levels in possible MUC1-CD-signaling partners expression (by immunoblots and real Time PCR) and phosphorylation (by Kinexus phospho-antibody screening, using MKN45 clones with stable downregulation of MUC1 previously established [25].

We have found that MUC1-CD binds EGFR, Grb2, B-RAF and ERK1/2 proteins. Furthermore, ERK1/2, EGFR and B-RAF mRNA and protein levels are altered upon MUC1 downregulation, suggesting that this mucin is involved in the transcription and/or stability/recycling of these signaling partners. Moreover, MUC1 downregulated clones have shown a significantly increased phosphorylation of ERK2 and decreased phosphorylation of ERK1 proteins.

Our work allowed us to identify MUC1-CD signaling partners in GC cells and to show the impact of MUC1 downregulation in the expression and phosphorylation of some of these MUC1-CD signaling partners.

Material and methods

Cell culture

Human cell line derived from diffuse-type gastric carcinoma – MKN45 (poorly differentiated adenocarcinoma, Japan Health Sciences Foundation [26]) was grown in RPMI 1640 containing Glutamax^{TM}I and 25mM HEPES, supplemented with 10% fetal bovine serum (FBS) and 50µg/ml gentamicin (Invitrogen). MUC1 downregulated stable clones derived from MKN45 cells were grown in standard growth medium supplemented with 5µg/ml puromycin (Sigma). Cells were grown at 37ºC with 5% CO_2 in humidified atmosphere.

Clinical Samples

Normal gastric tissues were obtained from the University of Nebraska Medical Center's Tissue Bank through the Rapid Autopsy Pancreatic program in compliance with IRB 091-01. To ensure minimal degradation of tissue, organs

are harvested within three hours post mortem and the specimens flash frozen in liquid nitrogen or placed in formalin for immediate fixation. After processing the sections were cut from paraffin blocks of formalin fixed tissue into 4µm thick sections and mounted on charged slides. For gastric carcinoma samples, we have used paraffin embebbed slides from the antrum part of the stomach treated in a similar way as the normal samples and obtained from the Medical Faculty of Porto University from gastric biopsies.

Confocal Microscopy

MKN45-SC and MKN45-C2 cells were grown until confluency in round slides, washed with PBS and fixed with 4% paraformaldehyde/120mM sucrose for 15 min. 0.1M glycine was added after paraformaldehyde removal for 15 min. Cells were washed with PBS 1% BSA twice and permeabilized with 0.15% Triton-X100/1% BSA in PBS for 15 min. After washing with PBS 1% BSA, the primary antibodies were added in PBS 1% BSA: MUC1-Ab5 monoclonal antibody (1:300, ThermoScientific), and calreticulin 1:50 (Cell Signaling) and incubated overnight at 4ºC. After washing with PBS, the secondary antibodies were added (Alexa Dylight-488 anti-hamster 1:500 and donkey anti-rabbit-649 1:500) for 1h at RT. After PBS washing, nuclei were stained with DAPI (1:10,000 in PBS for 2min) cells were washed again with PBS and mounted in Vectashield (Vectorlabs).

Phospho-antibody screening (Kinexus)

Cells were lysed by the addition of lysis buffer (20 mM Mops, pH 7.0, 2 mM EGTA, 5mM EDTA, 30 mM sodium fluoride, 60 mM β-

glycerophosphate, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM PMSF, 3 mM benzamidine, 5 µM pepstatin,10 µM leupeptin, and 0.5% Triton X-100; final pH 7.0). Cells sonication was performed twice for 15 s and the homogenate was subjected to ultracentrifugation for 30 min at 50,000 rpm.

Protein concentration was measured by the Bio-Rad assay and phospho-kinase screening was performed by Kinexus (Vancouver, BC; KPSS-10.1).

Immunoprecipitations and immunoblots

Subconfluent cells were lysed in appropriate ice-cold lysis buffer. Lysates were cleared by centrifugation at 4,000 rpm for 30 min. Lysates were incubated for 3 h at 4ºC with anti MUC1-Ab5 monoclonal antibody (1:300, ThermoScientific), and normal armenian hamster IgG (EBioscience) previously precipitated with Protein G Sepharose (Sigma) for 1 h at 4ºC. After washing with non-denaturing lysis buffer, the immunoprecipitates and lysates were separated in 12% Tris-Glycine Gels (Invitrogen). Proteins were transferred to PVDF membranes and were blocked with 5% nonfat dry milk in phosphate-buffered saline (PBS) containing 0.01% Tween 20. Membranes were then incubated with anti-ERK1/2 (1:1,000, Cell Signaling), anti-EGFR (1:200, Santacruz Biotec.), anti B-RAF (1:200, Santacruz Biotec.), anti-Grb2 (1:200, Santacruz Biotec.) and anti-β-actin (1:8,000, Sigma) in 5% non-fat milk in PBS-0.1%Tween20 (Sigma). Membranes were washed 3 times with PBS-0.1%Tween20 and the primary antibodies were revealed using goat anti-mouse/rabbit peroxidase-conjugated antibody (1:1,000, DAKO) in 5% non-fat

milk in PBS-0.1%Tween20, followed by ECL detection kit (BioRad). Results are representative of three independent experiments.

RNA extraction and Real Time PCR

Total RNA was isolated from MKN45 cells using TriReagent™ (Sigma) according to the manufacturer's instructions. 5µg of RNA were primed with random hexamers (Invitrogen) and reverse transcribed with Superscript II (Invitrogen) in a final volume of 20µl. 2µl of a 1:20 dilution of cDNA were amplified with 300nM of each primer and SYBRGreen (Applyed BioSystems) in a final volume of 20µl, using the fluorescence reader ABI Prism 7000. Each sample was run in triplicate. The primers used were the following: MUC1 (*sense*: CTCCTTTCTTCCTGCTGCTG, *antisense*: CTGGAGAGTACGCTGCTGGT); TBP (*sense*: GCACAGGAGCCAAGAGTGAA, *antisense*: TCACAGCTCCCCACCATATT) , EGFR (*sense*: GAGCGACTGCCTGGTCTGCC, *antisense*: CACGCAGGTGGCACCAAAGC) , ERK2 (*sense*: GACACAACACCTCAGCAATGACCA, *antisense*: GGCTTGAGGTCACGGTGCAGA) and B-RAF (*sense*: TTAGTGAGCCAGGTAATGAGGCA; *antisense*: ATCAATTTGGGCAACGAGACCGA) and their specificity was confirmed using the software BLASTn on-line and by melt curve analysis. For each sample, the level of TBP RNA was measured and used for normalization of target genes abundance. Relative mRNA levels were then calculated using the comparative C_t method [27]. Data is expressed as a ratio of the results obtained with each clone and the scrambled control, from three independent experiments.

Statistical analysis was performed using the Mann Whitney test -StatView Software version 5.0 (SAS Institute, Cary, NC).

Conjugation of MUC1-CT2 antibody with DuolinkII Probemarker

MUC1-Ab5 monoclonal antibody (1:300, ThermoScientific), is produced in hamster. Since there are no probes against hamster antibodies commercialized by O-link Bioscience, we have first labelled it with a kit, the Duolink II Probemarker Plus (OLink Bioscience), according to the manufacturer's instructions.

Relative Quantification of interaction between MUC1-CD and cell signaling molecules by Proximity Ligation Assays (PLAs)

MKN45-SC clones were grown until subconfluency in round slides, fixed with paraformaldehyde 4% 120mM sucrose and permeabilized with Triton-X at RT and washed with PBS. We have followed the instructions of the kit DII anti-rabbit minus (OLink Bioscience) or the DII anti-mouse minus (OLink Bioscience) (depending on the origin of the secondary antibodies used), followed by the detection kit DII Det. Reag.Orange (OLink Bioscience). Assays were made in duplicate and for each one, MUC1-Ab5 antibody was incubated with ERK1/2 (1:100, Cell Signaling), EGFR (1:50, SantaCruz Biotec.), B-RAF (1:50, SantaCruz Biotec.) and Grb2 (1:50, SantaCruz Biotec.). Fluorescence spots were observed under a fluorescence microscope. The negative controls are cells incubated with no MUC1-CD antibody.

Results

Interactions between MUC1-CD and oncogenic signaling effectors in gastric carcinoma cells

Immunoprecipitation and PLA assays have shown that MUC1-CD interacts with ERK1/2 molecules in the diffuse type MKN45 GC cells **(Figures 1 and 2)**. We have also studied the possible interaction between MUC1-CD and ERK1/2 in AGS intestinal gastric carcinoma cells and it was clearly positive (results not shown). PLA assays have also shown an interaction between MUC1-CD and EGFR, B-RAF and Grb2 **(Figure 3, 4 and 5)**, other members of the MAPK signaling pathway. We have performed the same PLA assays in gastric carcinoma and also normal gastric tissues and the results were negative.

Impact of MUC1 downregulation in MAPK oncogenic signaling partners expression and phosphorylation

MUC1 expression in MKN45 gastric carcinoma cells has been previously obtained, with the isolation of one scrambled control (MKN45-SC) and two MUC1 downregulated clones (MKN45-C1 and MKN45-C2) [25]. MUC1 silencing is shown in MKN45-SC and MKN45-C2 cells by confocal microscopy **(Figure 6)**.

The relative levels of ERK1/2, EGFR and B-RAF vary between the silenced clones and the scrambled control, suggesting that MUC1 may regulate their transcription and/or stability **(Figure 7)**.

ERK2, EGFR and B-RAF mRNA levels were studied by Real-Time PCR **(Figure 8)** in MKN45 GC cells. mRNA levels of ERK2 and EGFR increased, whereas mRNA levels of B-RAF decrease between MUC1 dowregulated clones and the control.

Phosphorylation profiling of MKN45-SC, MKN45-C1 and MKN45-C2 by Kinexus antibody screening have shown that phosphorylation of MKN45-C1 and MKN45-C2 clones is significantly altered when compared to the MKN45-SC control in ERK1/2 proteins. There is an increase in the phosphorylation levels of active sites of ERK2 while ERK1 shows decreased phosphorylation levels **(Figure 9).**

Discussion

Interactions between MUC1-CD and oncogenic signaling effectors in gastric carcinoma cells

The MAPK signaling pathway has been one of the signaling pathways more often shown to be deregulated in several carcinomas, including GC [28]. Our aim was to study the impact of MUC1-CD in this signaling cascade in MKN45 gastric carcinoma cells.

The MAPK signaling pathway is composed of several cell signaling molecules. The initiator molecule is the epidermal growth factor receptor (EGFR), a member of the growth factor family HER that works as a cell surface receptor of extracellular ligands. Ligand binding to EGFR extracellular domain leads to its activation, with subsequent homodimerization, leading to the phosphorylation of its intracellular tyrosine kinase domain. This will initiate a

series of phosphorylations and intracellular signals that will ultimately activate the central Ras/RAF/MEK/ERK mitogen activated protein kinase (MAPK) signaling pathway.

EGFR molecule modulates processes of cell proliferation, migration, adhesion and proliferation and it is known to provide tumor cells with growth and survival advantages [29]. EGFR expression was found to be deregulated in several types of cancers, including GC. High EGFR levels in GC were found have been associated with the diseases' prognosis [30] and presence of lymph node metastasis [31].

Grb2 is an important molecule that integrates the stimulatory signals of the MAPK signaling pathway [32]. This molecule has been previously shown to interact with MUC1 in breast carcinoma [33].

B-RAF is another member of the MAPK signaling pathway often overexpressed in GCs, promoting tumorigenesis and angiogenesis [34]. MUC1 was previously shown to activate the RAS-mediated signaling pathway in non-tumorigenic and tumorigenic mammary cell lines [35].

ERK1/2 molecules are the final effectors of the MAPK signaling cascade and have been shown to be activated by MUC1 mucin in some cancer models [15, 36, 37]. These molecules were also shown to be overexpressed and activated in the majority of GC cases and correlate with tumor progression and invasion [38]. These molecules were also found to be activated in *H.pylori* related cancers [39, 40]. These events were found for gastric carcinoma cell lines, but when examining human gastric carcinoma specimens, a decrease in the activation of ERK1/2 was found [41]. One possible explanation for this fact is that gastric cells start expressing molecules that attenuate ERK-mediated

signaling upon its activation, or on the other hand, the cells act by activating negative feedback mechanisms.

MUC1 was shown to interact with ERK1/2 in MKN45 control and downregulated cells, as shown by immunoprecipitation assays. The interaction between MUC1-CD and ERK1/2 was later confirmed by a proximity ligation assay. This technique also allowed us to show interactions between MUC1-CD and other MAPK signaling effectors such as EGFR, Grb2 and B-RAF.

Although further studies need to be performed to better clarify the relevance of these interactions, this finding suggest that MUC1-CD is directly associated with an oncogenic signalling pathway and that might be valuable to develop new strategies to limit and control the development of GC.

In order to see if these interactions were restricted to MKN45 cell line, derived from a diffuse gastric carcinoma, we have tested the possible interaction between MUC1-CD and ERK1/2 in AGS gastric carcinoma cells, derived from an intestinal-type gastric carcinoma and the result was also positive (results not shown). Therefore, this interaction is not restricted to cells from diffuse gastric carcinomas.

We checked if the interactions found to exist between MUC1-CD and ERK1/2, EGFR, B-RAF and Grb2 were also occurring in gastric normal cells. In order to do so, we used normal gastric tissue samples in PLA assays. However, no interactions seem to exist between all the mentioned molecules and MUC1-CD in the samples studied. A similar result was obtained when testing the same interactions in gastric carcinoma tissue samples.

Tissue samples and cancer cell lines have several important differences. One of these differences is that gastric carcinoma cell lines grow with high

proliferation rates, while in normal and carcinoma tissue samples, the proliferative pool is restricted to a few set of cells. This reason can explain the fact that the results obtained by PLA assays regarding MUC1-CD interaction with cell signaling molecules are different between MKN45-SC cells and normal and gastric carcinoma tissue samples.

This is the first report that shows MUC1-CD interaction with ERK1/2, EGFR, B-RAF and Grb2 in MKN45 gastric carcinoma cell line, and with ERK1/2 in AGS gastric carcinoma cell line (results not shown).

Impact of MUC1 downregulation in MAPK oncogenic signaling partners expression and phosphorylation

The relative levels of ERK1/2, EGFR and B-RAF vary between the silenced clones and the scrambled control, suggesting that MUC1 may regulate not only their phosphorylation but also their transcription and/or stability.

We have observed that ERK2 upregulation and B-RAF downregulation at a protein level, considering Real Time PCR results, are likely due to MUC1-mediated transcriptional mechanisms, whereas EGFR protein levels difference is most likely due to post-transcriptional regulation mechanisms.

Kinexus antibody screening has shown that phosphorylation profiling of MKN45-C1 and MKN45-C2 clones is significantly altered when compared to the MKN45-SC control in ERK1/2 proteins important activation sites (T202/Y204; T185/Y187). Phosphorylation of the specific phosphorylation sites analyzed by Kinexus is known to lead to an activation of the proteins [42, 43].

The differences in phosphorylation observed are rather due to physical interactions between MUC1-CD and ERK1/2, since MUC1 is not known to

possess any kinase activity. It's also possible that MUC1-CD is interacting with kinases that phosphorylate ERK1/2 and that this interaction is altering the phosphorylation of these proteins.

Conclusions

Gastric cancer is a worldwide major health problem, mostly due to the reduced knowledge of its etiological factors and pathogenesis model. This cancer is also resistant to most cancer therapies with no significant progress made during the last decades. Therefore it is critical to find new therapeutic targets to overcome resistance mechanisms. The main objective of this work as to better understand the biology of MUC1 mucin, previously described as frequently altered in gastric cancer, in this disease pathogenesis model.

This research work allowed us to identify molecules that directly interact with MUC1-CD in MKN45 GC cells and that may therefore be MUC1 oncogenic signaling partners, contributing for GC development (EGFR, B-RAF, Grb2 and ERK1/2). We were also able to identify signaling molecules (ERK1/2, EGFR, B-RAF) whose expression is significantly altered by MUC1 downregulation in MKN45 GC cells. Furthermore, we have shown that ERK1/2 phosphorylation and consequent activation is significantly altered by MUC1 downregulation in MKN45 GC cells.

The results found in this research work reinforce the participation of MUC1 in the MAPK signaling pathway of gastric carcinoma cells and therefore its importance as a new elective target for innovative therapeutic approaches.

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Figures

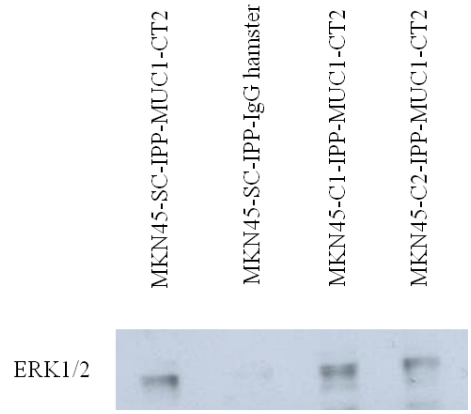

Figure 1: Immunoprecipitation assays. MKN45-SC, C1 and C2 lysates were immunoprecipitated with anti-MUC1-CT2 and with normal hamster IgGs as a control and blotted with anti-ERK1/2 antibody.

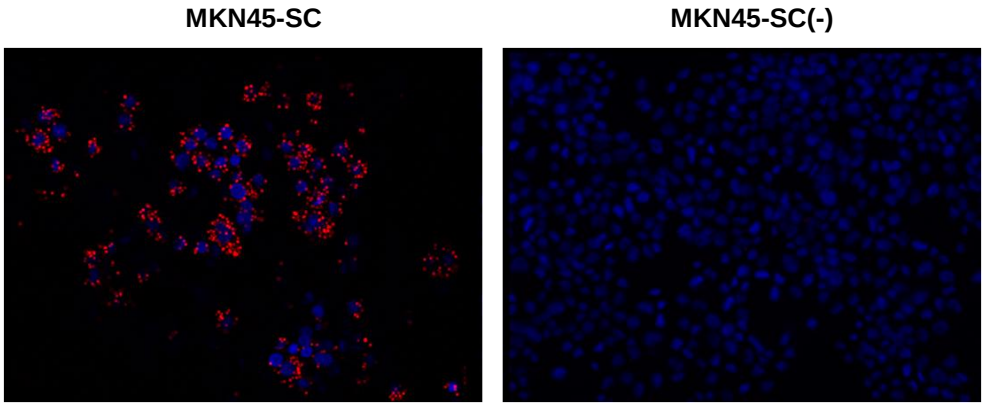

Figure 2: *In situ* PLA assay. PLA assay was performed using MKN45-SC cells and the antibodies MUC1-CT2 and ERK1/2. MKN45-SC(-) – negative control.

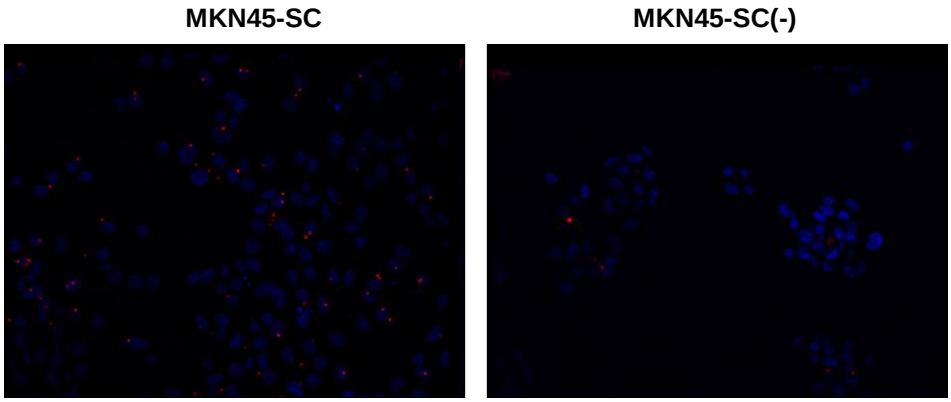

Figure 3: *In situ* PLA assay. PLA assay was performed using MKN45-SC cells and the antibodies MUC1-CT2 and EGFR. MKN45-SC(-) – negative control.

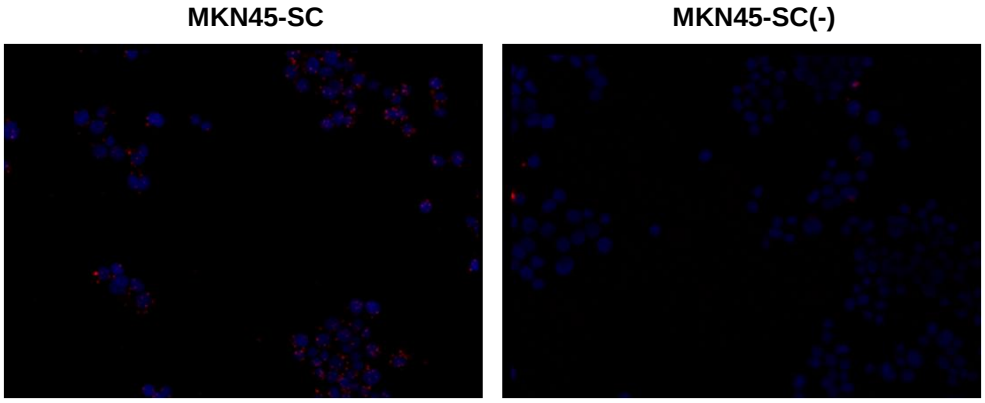

Figure 4: *In situ* PLA assay. PLA assay was performed using MKN45-SC cells and the antibodies MUC1-CT2 and B-RAF. MKN45-SC(-) – negative control.

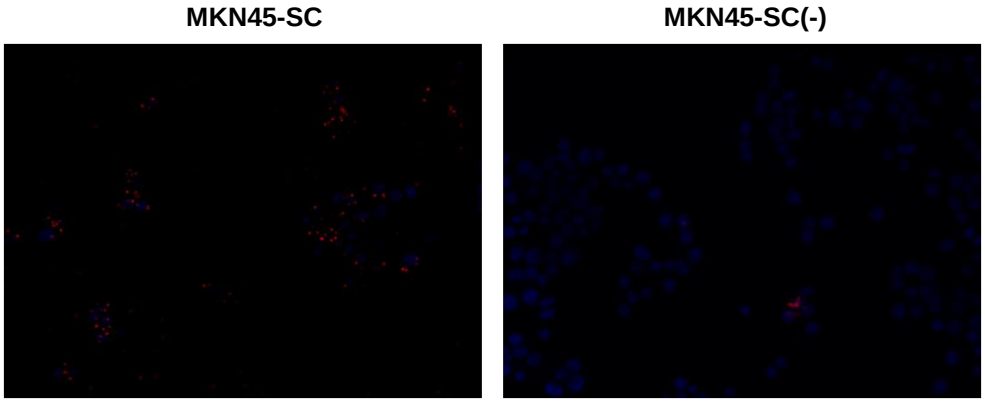

Figure 5: *In situ* PLA assay. PLA assay was performed using MKN45-SC cells and the antibodies MUC1-CT2 and Grb2. MKN45-SC(-) – negative control.

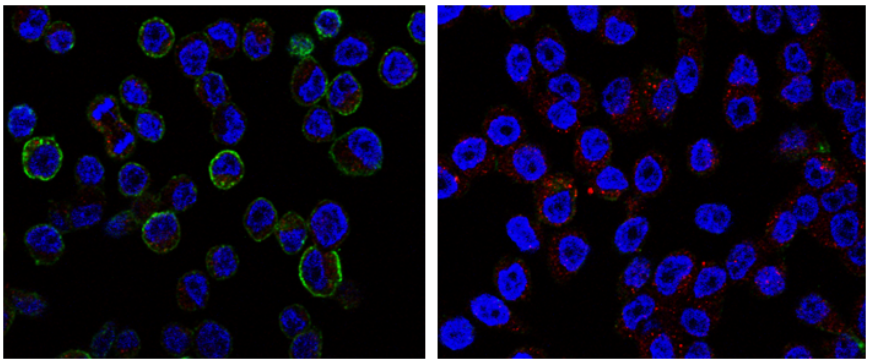

Figure 6: MUC1 downregulation by siRNA. Effect of siRNA on MUC1-CD expression in MKN45 gastric carcinoma cell line assessed by confocal microscopy. A) MKN45-SC control cells and B) MKN45-C2 downregulated clone. Nuclei are stained with DAPI, MUC1-CD is shown in green and the endoplasmic reticulum in red.

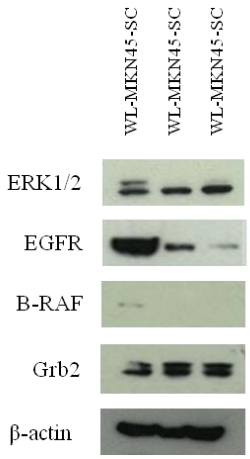

Figure 7: Immunoblots assays. MKN45-SC, C1 and C2 lysates were blotted with anti-ERK1/2, anti-EGFR, anti-B-RAF, anti-Grb2 and anti-β-actin. WL-Whole Lysates.

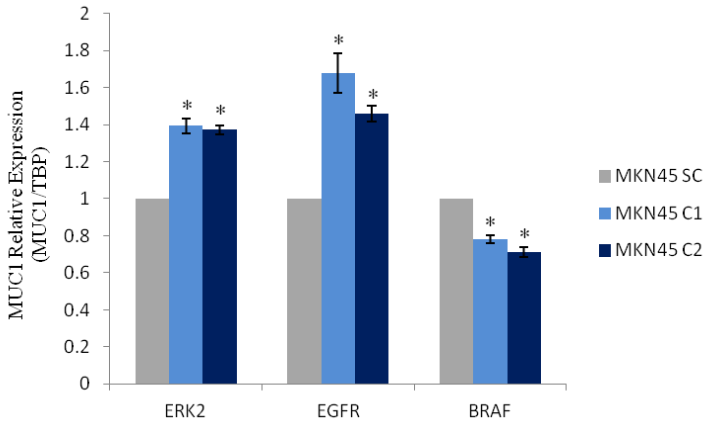

Figure 8: Relative quantification of ERK2, EGFR and B-RAF by Real-Time PCR in MKN45-SC, C1 and C2 clones. Values were adjusted to the expression of TBP gene. * $P < 0.05$, when compared to the MKN45-SC control.

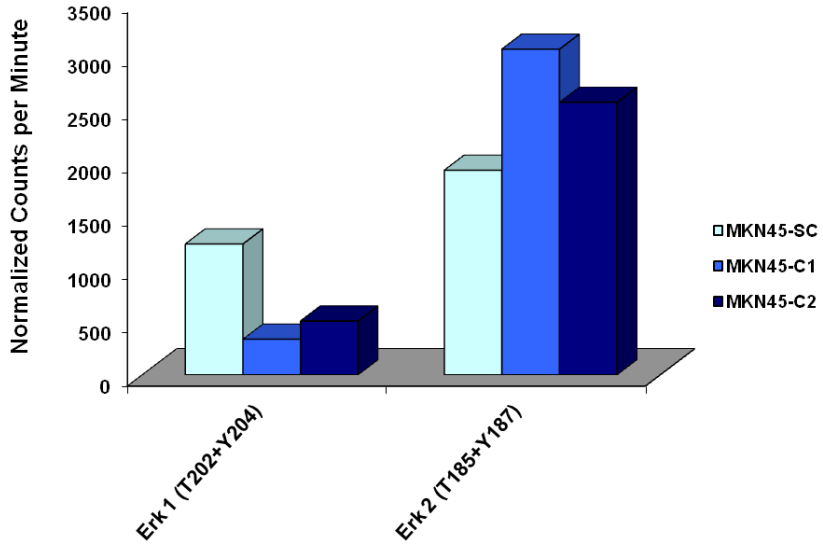

Figure 9: Phospho-site kinexus assay. Relative phosphorylation levels of important phosphorylation sites of the proteins ERK1/2 in MKN45-SC, MKN45-C1 and MKN45-C2 cells.

Invited Review

1

Oncogenic Signaling in Gastric Cancer

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Portugal

1. Introduction

Gastric cancer is one of the most common and life-threatening cancers worldwide (for review see Power et al., 2010). The late diagnosis and high mortality of this disease reflects the reduced understanding of its etiological factors and pathogenesis model and the lack of effective treatments. This disease results from the complex interplay between genetic and environmental factors at the gastric mucosa level that deregulates cell potentially oncogenic signaling pathways, leading to gastric cancer development. It is known that more than 80% of gastric cancer cases can be attributed to deregulation of signaling pathways caused by *Helicobacter pylori* infection (Houghton & Wang, 2005). Some of these signaling pathways are important during gastric embryogenesis and during the normal lifetime of a gastric cell, but can be tumorigenic if deregulated and therefore their better understanding is crucial for the development of new therapeutic drugs. In this review we will summarize the most important intracellular signaling pathways that have been found to be deregulated in gastric carcinoma and we will include new data recently obtained in our laboratory, focused on MUC1 mucin-mediated signaling pathways.This new information is a relevant contribution for the understanding of the gastric oncogenic signaling scenario and opens new perspectives for the development of innovative therapeutic strategies against the disease.

2. Signaling pathways involved in gastric carcinogenesis

2.1 EGFR and the extracellular signal-regulated MAPK pathway

The mitogen-activated protein kinases (MAPKs) pathway is activated either by extracellular ligand binding or intracellular stimuli, and regulates a series of cell activities such as proliferation, differentiation and cell death. The extracellular signal-regulated kinase (ERK) MAPK pathway has been often found to be deregulated in cancers and consists of several kinases (Ras, Raf, MEK) that are activated by phosphorylation upon ligand binding to a membrane receptor, ending up in the activation of several proteins involved in processes of cell invasion, apoptosis, transcription, survival and drug resistance (for review see Kim EK & Choi E, 2010). Members of this cascade have been found to be deregulated in gastric cancers, such as RAS family members (Adjei, 2001; Velho et al., 2010). Ras/MAPK activation was found to be associated with cell proliferation in gastric carcinomas (Regalo et al., 2010). ERK1/2, the final effectors of this pathway, were also found to be activated in gastric cancers (Liang et al., 2005) and in *Helicobacter pylori* related gastric cancers (Hatakeyama ,

2006; Chen et al., 2006). In contrast, ERK2 activity was found to be reduced by nonsteroidal anti-inflammatory drugs NSAIDS, therefore inhibiting the proliferation of gastric carcinoma cells (Husain et al., 2001). All of these events were found in cell lines, but when examining human gastric carcinoma specimens, a decrease in the activation of ERK1/2 was found (Wu et al., 2008). One possible explanation for this fact is that gastric cells start expressing molecules that attenuate ERK-mediated signaling upon its activation, or on the other hand, the cells act by activating negative feedback mechanisms.

The epidermal growth factor receptor (EGFR) is a member of the growth factor family HER and works as a cell surface receptor of extracellular ligands. Ligand binding to EGFR extracellular domain leads to its activation, with subsequent homodimerization, leading to the phosphorylation of its intracellular tyrosine kinase domain. This will initiate a series of intracellular signals, including activation of the central Ras/Raf/mitogen activated protein kinase (MAPK) signaling pathway (Figure 1). This molecule modulates processes of cell , migration, adhesion and proliferation and it is known to provide tumor cells with growth and survival advantages (for review see Nicholson et al., 2001). EGFR expression was found to be deregulated in several types of cancers, including gastric cancer. High EGFR levels in gastric carcinoma have been associated with the disease prognosis (Kim et al., 2008) and presence of lymph node metastasis (Choi et al., 2009). Therapies against EGFR have been developed and are active in gastric cancer treatments (Pinto et al., 2007; Liu et al., 2011), although not completely effective. The EGFR/MAPK pathway has also been shown to be activated in gastric carcinomas with microsatellite instability (Corso et al., 2011).

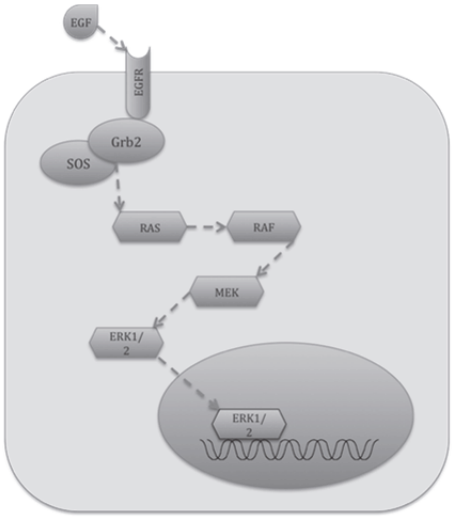

Fig. 1. EGFR/MAPK signaling pathway.

2.2 E-cadherin and Wnt/beta-catenin pathway

E-cadherin is a calcium-dependent cell-cell adhesion molecule that is essential for the maintenance of the normal epithelium architecture (for review see Van Roy & Berx , 2008). Loss of expression of this molecule has been found in gastric cancers, relating with tumor

dedifferentiation, invasiveness, metastasis and prognosis (Shino et al.,1995; Gabbert et al., 1996). Mutations of this molecule have been often found in familial gastric cancers (for review see Oliveira et al., 2006). The cytoplasmic domain of this molecule interacts with the molecule beta-catenin, forming strong cohesive nets between the actin cytoskeleton (Leckband & Sivasankar, 2000), essential for processes of cell-cell adhesion and cell shape, polarity, migration and invasion.

EGFR and E-cadherin were found to interact through the respectives extracellular domains and signaling mediated by EGFR was found to be inhibited by E-cadherin (Qian et al., 2004). EGFR was found to be hyper-activated in cells where the extracellular domain of E-cadherin is not present (Bremm et al., 2008). Therefore, it may be of therapeutic value to use EGFR inhibitors in the treatment of gastric cancers in which there is a deregulation of E-cadherin.

Beta-catenin is important in mediating the E-cadherin related cell adhesion and also by participating in Wnt signaling pathways. The Wnt signaling pathway regulates several processes during development, such as determination of cell fate, morphology, polarity, adhesion and growth. Wnt signaling can be divided into canonical and non-canonical pathways. In the canonical one, wnt signals (extracellular ligands, such as wnt-1) stabilize beta catenin, therefore activating gene transcription by interaction of beta-catenin with transcriptional factors (Figure 2).

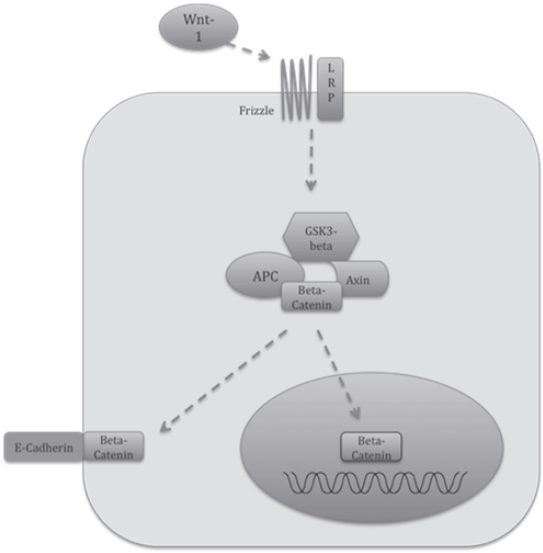

Fig. 2. Wnt signaling pathway (canonical).

This pathway was found to be deregulated in several cancers (for review see Polakis, 2007), including gastric carcinoma (Katoh et al., 2001; Clements et al., 2002; Nabais et al., 2003). The non canonical pathway is not related to beta-catenin and is involved in embryonic development and cell polarity and has also been linked to the development of gastric cancers (Kurayoshi et al., 2006; Gencer et al., 2010). This pathway seems to be repressed by Notch1 receptor in keratinocytes (Nicolas et al., 2003). Beta catenin was found to be activated by the bacterium *Helicobacter pylori* in gastric cancers (Franco et al., 2005).

In gastric cancer tissues, the expression of Wnt-1, beta-catenin and E-cadherin was found to be increased when compared to normal gastric tissue, as well as tumor size, tumor invasive depth, lymph node metastasis, pTNM stage, differentiation and five-year survival rate (Zhang & Xue , 2008). Therefore, these molecules are promising therapeutic targets for gastric carcinoma.

2.3 Hedgehog pathway

Hedgehog (Hh) signaling plays an important role during embryonic development and differentiation, proliferation and maintenance of adult tissues through the maintenance of stem cells population. Until now, three different members of the Hh family have been identified: Sonic Hedgehog (Shh), Indian Hedgehog (Ihh) and Desert Hedgehog (Dhh). All of them are secreted-type glycoproteins with a N-terminal signal peptide, a Hedgehog signaling domain and a Hint domain that signals through ligation to the hedgehog receptor Patched (Ptch), which usually acts by inhibiting the seven-span transmembrane receptor Smoothened (Smo) (Katoh and Katoh, 2005).

Of all Hh members, Shh is the most studied signaling pathway in vertebrates and plays a crucial role in stomach development. Shh protein expression is increased in parietal cells of the normal adult, gastric corpus and antrum (Saqui-Salces and Merchant, 2010). It is believed that Shh is important for regulation of gastric epithelial differentiation and its silencing causes gastric atrophy and subsequent disruption of glandular differentiation (Van den Brink, 2007). Recently, with the development of a mouse model expressing a parietal cell-specific Shh deletion, the function of this protein in adult stomach has been better clarified (Xiao et al., 2010).

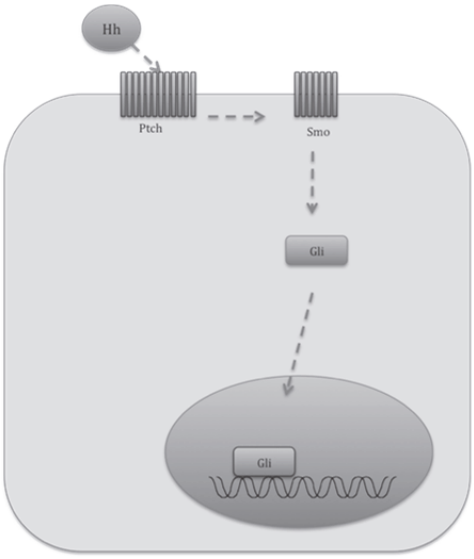

Fig. 3. Human Hedgehog signaling pathway.

Shh ligand is expressed as a 45-kDa precursor that is cleaved autocatalytically to yield a 19kDa amino terminal fragment, that contains all the signaling functions and a 26-kDa

carboxy-terminal fragment (ShhC), that acts as a cholesterol transferase (Goetz et al., 2006; Zavros et al., 2007). This activation process depends on the acid-activated protease pepsin A. Gastrin increases acid secretion in parietal cells leading to conversion of pepsinogen A to pepsin A, mediating Shh processing (Zavros et al., 2007). In the stomach, Shh binds directly to Ptch receptor but not to Smo being the activity of Smo controlled indirectly by Ptch (Figure 3). In Shh absence Ptch supresses Smo activity. The binding of Shh to Ptch results in loss of Ptc activity and consequently Smo activation. Smo activation triggers Hh signal into the cytoplasm by triggering activation of Glioblastoma family transcription factors (Gli1, Gli2 and Gli3) that induce transcription of signaling targets like Wnt and the zinc finger transcription factor, Snail (Martin et al., 2010). Gli 1 induces the transcription of Snail that inhibits E-cadherin transcription. The inhibition of E-cadherin, a protein that plays an important role in cell adhesion, is associated with an increase in nuclear beta-catenin, triggering the activation of Wnt pathway targets like CD44, c-Myc and cyclin D1 (Li et al., 2006; Medici et al., 2008; Tanaka et al., 2002).

Alterations in Hh signaling pathway activation are related to different types of cancer such as gastric cancer, breast cancer, small-cell lung cancer, skin and pancreatic cancer (Kubo et al., 2004; Thayer et al., 2003; Watkins et al., 2003; Yang et al., 2010). The expression of Hh ligands, Ptch1, Smo and the three Gli transcription factors (Gli1, Gli2 and Gli3) has been related with more than two-thirds of primary gastric cancers and correlated with poorly differentiated and more aggressive tumors (Fukaya et al., 2006; Lee et al., 2007). Hh signaling activation is triggered by expression of hedgehog ligands like Shh and Ihh or an increased Ptch receptor expression (Wu et al., 2010; Zavros, 2008). Inhibition of Shh signaling pathway using Smo or antagonists like cyclopamine or Hedgehog neutralizing antibody 5E1 causes growth inhibition and regression of xenograft tumors in vivo (Berman et al., 2003). However, in the presence of precancerous lesions such as gastric atrophy (loss of parietal cells) or intestinal metaplasia, Shh protein expression is reduced or totally lost (Shiotani et al., 2005; Suzuki et al., 2005; van den Brink et al., 2001). This observation is associated with *Helicobacter pylori* infection, which is directly linked to the development of gastric cancer (Correa et al., 1975; Uemura et al., 2001). Taken together, these evidences may indicate that the inactivation of Hh signaling mediates in part the precancerous tissue alterations induced by *Helicobacter pylori* infection while its re-activation confers survival advantages in later stages of gastric carcinogenesis.

2.4 Notch pathway

Notch signaling pathway is evolutionary conserved and plays a role in many important and fundamental processes in cell and tissues such as proliferation, differentiation, apoptosis, cell fate determination, and maintenance of stem cells (Koch and Radtke, 2007; Leong and Karsan, 2006; Radtke and Raj, 2003). Notch signaling is activated during cell-to-cell contact through four receptors (Notch1-4) that can interact with ligands of the Delta (Dll-1, Dll-3, Dll-4) and Jagged (Jagged-1 and Jagged-2) family (Bray, 2006). Notch-ligand binding induces the cleavage of Notch receptor through a cascade of proteolytic cleavages by the metalloprotease tumor necrosis factor-α-converting enzyme (TACE) and γ-secretase, releasing the intracellular domain of Notch (NICD) (Katoh, 2007a; Wang et al., 2009). The NICD is translocated into the nucleus to associate with CSL transcription factor triggering the activation of Notch target genes (Androutsellis-Theotokis et al., 2006; Miele, 2006). Until now, few Notch target genes have been identified in different cellular and developmental contexts (Borggrefe and Oswald, 2009), such as Hes-1 (Hairy enhance of split-1), Cyclin D1, Nuclear factor-κB (NF-κB) and c-myc (Miele, 2006) (Figure 4).

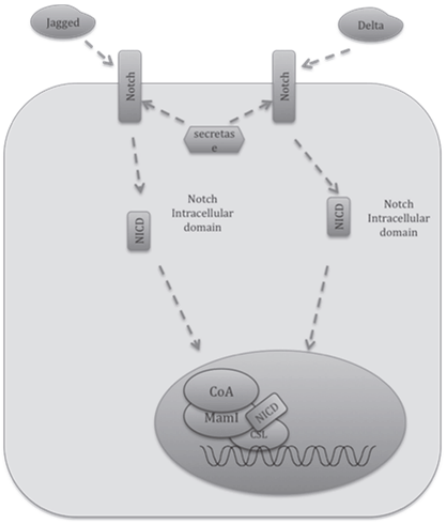

Fig. 4. Notch signaling pathway.

Recently, it has been described an association between Notch signaling and progression of gastric cancer. Three Notch receptors (Notch1-Notch3) and Notch ligand Jagged1 are expressed in human gastric cancer (Katoh, 2006; Sander and Powell, 2004; Sekine et al., 2006) and Notch signaling pathway is activated after infection with *Helicobacter pylori* in gastric cancer (Katoh, 2007b). Gastric cancer patients with Jagged1 expression in tumor tissues have more aggressive tumors and poor survival, suggesting an important role of this pathway in gastric cancer progression (Yeh et al., 2009). This aggressiveness seems to be correlated with the interaction between Notch signaling pathway and COX-2, an independent prognostic factor of gastric cancer (Shi et al., 2003). Notch signaling induces Cox-2 expression by directly binding to it through the intracellular domain of Notch1 receptor producing a stimulatory effect on cancer growth and invasion (Yeh et al., 2009). Nuclear factor-κB is also involved in Notch signaling and mediates COX-2 expression to regulate cell proliferation of human gastric cancer cells (Espinosa et al., 2003). Therefore, COX-2 inhibitors may be a new strategy to be used for treatment of gastric cancer in the future.

Twist, another transcription factor regulated by Notch signaling, was also shown to regulate cell motility and invasion in gastric cancer cell lines, probably through N-cadherin and fibronectin (Yang et al., 2007). Possibly, these EMT mediators induced by NICD could lead to an increased expression of COX-2. Whether N1ICD induces expression of COX-2 to modulate metastasis in gastric cancer through EMT mediators remains unknown.

Furthermore, activation of Notch signal pathway is involved in epithelial-mesenchymal transition (EMT), during development and tumorigenesis. Notch signaling increases Snail-1 expression and elevates EMT in cardiac development, kidney tubular cell differentiation, and hypoxia (Sahlgren et al., 2008; Timmerman et al., 2004; Zavadil et al., 2004). The Jagged1-activated Notch signaling also promotes EMT through E-cadherin repression by Slug (Leong et al., 2007). Jagged1 and Hey1, a target gene of Notch signal pathway, are also involved in mediating transforming growth factor-β–induced EMT (Zavadil et al., 2004). All these findings together indicate that Notch signaling plays a multitude of important roles in gastric carcinogenesis.

2.5 COX-2/PGE2 pathway

The regular use of nonsteroidal anti-inflammatory drugs (NSAIDs) is associated with a reduced risk of cancer development in the gastrointestinal tract (Thun et al., 1993; Farrow et al., 1998; Oshima et al., 2009). The major target of NSAIDs is cyclooxygenases (COXs), such as COX-2, which is a rate-limiting enzyme responsible for the conversion of arachidonic acid to prostaglandins (PGs) (Chan et al., 2007; Williams et al., 1999). The anticancer effect of these agents is thought to be caused by the inhibition of COX-2 and, consequently the reduction of PG synthesis (Wu et al., 2010). Regular use of NSAIDs is associated with a decreased incidence of gastric cancer (Oshima et al., 2009, as cited in Thun et al, 1993; Zaridze et al., 1999).

The over-expression of COX-2 was reported in several common human malignancies, such as in lung, colon, pancreas, bladder, head and neck cancers, being its main expression in gastrointestinal tract (Pereira et al., 2009, as cited by Fujimura et al., 2006 and van Rees & Ristimaki, 2001; Schuller et al., 2006). Several studies have shown that the treatment with NSAIDs or COX-2 selective inhibitors (COXIBs) suppressed chemically induced tumor formation and xenografted tumor growth, so these results show that the COX-2 pathway plays an essential role in cancer development (Oshima & Taketo, 2002; Oshima et al., 2009).

The inducible enzyme, COX-2 is responsible for catalyzing the biosynthesis of prostaglandin (PG) H2, which is further converted to PGE2 by microsomal PGE synthase-1 (mPGES-1), a PGE2 converting enzyme that is functionally coupled with COX-2 (Murakami et al., 2000; Seno et al, 2002). Cox-2-derived PGE2, a stable prostanoid synthesized by prostaglandin E synthase (PGES) can modulate inflammation (Huang & Chen, 2011, as cited in Harizi et al., 2000), relax vascular smooth muscles (Huang & Chen, 2011, as cited in Smyth et al., 2009) and act as a promoter of cancer progression (Huang & Chen, 2011, as cited in Iniguez, 2008). This molecule also has the ability to stimulate tumor-associated angiogenesis (formation of new blood vessels that supply oxygen and nutrients), promote cellular proliferation, inhibit apoptosis and enhance cellular invasiveness, facilitating the progression of cancers (Gross et al, 2005). Among the COX-2 downstream prostanoids, PGE2 is the one that is better studied, concerning its potential role in tumor progression (Huang & Chen, 2011) and mediates most, if not all, of the oncogenic effect of COX-2 in gastric cancer (Muller-Decker & Furstenberger, 2007). Such as for COX-2, an up-regulation of PGE2 in most of the gastrointestinal cancers also occurs (Huang & Chen, 2011). Therefore, it is crucial for gastric carcinogenesis an increased level of PGE2 through the induction of COX-2 and mPGES-1. Simultaneous induction of COX-2 and mPGES-1 is observed in gastric cancer tissues, which suggests the induction of PGE2 pathway in gastric tumors (Oshima et al., 2006). Several studies using mouse models have elucidated the roles of the PGE2 pathway in gastric tumorigenesis in the Wnt-activated and BMP-suppressed gastric mucosa (Oshima et al., 2009).

It is known that *Helicobacter pylori* infection causes chronic gastritis, as well as an over-expression of COX-2 and mPGES-1(Oshima et al., 2009). Concordantly, after eradication of *H. pylori*, COX-2 expression is suppressed (McCarthy et al., 1999), with correlation with decreased levels of mPGES-1, indicating that *H. pylori* infection induces the PGE2 pathway through induction of both COX-2 and mPGES-1. Several studies have found over-expression of COX-2 in gastric precancerous lesions and in gastric cancer (Tatsuguchi et al., 2000; Wambura et al., 2002). The molecular mechanism for COX-2 induction in tumors has not been totally elucidated, however there is a possibility that *H. pylori* can stimulate Toll-like receptors (TLRs) leading to the activation of the nuclear factor-κB (NF-κB) pathway that in turn induces the expression of COX-2. Another possibility is that the cytokine network can

be activated by infection and, as a result, an induction of COX-2 expression occurs (Chang et al., 2004; Smith et al., 2003). Transgenic mice over-expressing COX-2 and mPGES-1 simultaneously develop intestinal metaplasia and hyperplastic tumors in the glandular stomach, which is associated with macrophage infiltration, so these results suggest that increased levels of PGE2 enhance infiltration of macrophages, whose activation by *H. pylori* may enhance gastric carcinogenesis (Oshima et al., 2004; Wu et al., 2010).

2.6 NF-κB pathway

NF-κB (Nuclear Factor kappa B) is a critical regulator of genes involved in cell survival and proliferation, cellular stress response, innate immunity and inflammation (Baeuerle & Baltimore, 1996; Barnes & Karin, 1997). The NF-κB family is composed by five closely related DNA binding proteins: RelA (p65), RelB, c-Rel, NF-κB1/p50 and NF-κB2/p52, which function as various homodimers and heterodimers. All share a highly conserved domain called the Rel homology domain (RHD), responsible for their dimerization, nuclear translocation, DNA binding and also interaction with the inhibitors of NF-κB (IκBs) (Gilmore, 2006). This family can be subdivided according to differences in synthesis and C-terminal sequences. While members of the "Rel subfamily" (RelA, RelB and c-Rel) have a transactivation domain (TAD) at their C-termini and are synthesized directly as mature forms, p50 and p52 from "NF-κB subfamily" are generated from large precursor proteins, p105 and p100, respectively, by limited proteolysis or arrested translation. Although lacking a TAD, the precursors of the second subfamily contain a C-terminal with multiple copies of ankyrin repeats - ankyrin repeat domain (ARD) - the typical domain of IκBs. Characteristic NF-κB dimmers usually involve one member from each subfamily, although all NF-κB members may form various homo- or heterodimers. p50 or p52 homodimers inhibit NF-κB target gene expression due to lack of a TAD, therefore, members of the NF-κB subfamily are generally not activators of transcription but function as IκB-like inhibitors of NF-κB, except when they form heterodimers with members of the Rel subfamily, participating in target gene transactivation (Li & Verma, 2002).

NF-κB is essencial in cellular response regulation being an example of transcription factors that are present in cells in an inactive state and do not require new protein synthesis to be activated. The activation of NF-κB requires phosphorylation of IκBs, resulting in their ubiquitin-dependent degradation. Therefore, NF-κB can enter the nucleus and activate the genes in response to certain stimuli, including reactive oxygen species (ROS), tumor necrosis factor alpha (TNFα), interleukin 1-beta (IL-1β) and bacterial lipopolysaccharides (LPS) - a component of the outer membrane of Gram-negative bacteria including *H. pylori* (Thanos & Maniatis, 1995). In unstimulated cells, the NF-κB dimers are sequestered in the cytoplasm by IκBs. There are two pathways leading to NF-κB activation: the canonical/classical and non-canonical/alternative. The canonical pathway can be activated by several stimuli including inflammation cytokines and antigens that induce the phosphorylation and activation of an IκB kinase (IKK) complex, consisting of catalytic kinase subunits (IKKα and/or IKKβ) and a scaffold, sensing protein termed NF-κB essential modulator (NEMO). The activated IKK promotes phosphorylation of IκBα and its ubiquitin-dependent degradation by the proteasome. The released NF-κB is able to enter the nucleus and regulate the expression of a wide range of genes including activation of its own repressor, IκBα (Nelson et al., 2004) (Figure 5).

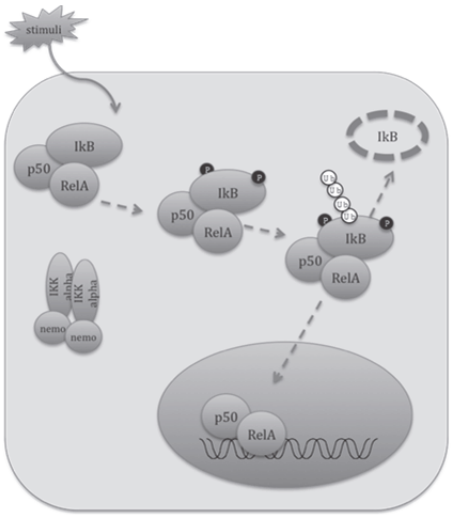

Fig. 5. NF-kB signaling pathway (canonical activation).

The non-canonical pathway is induced by certain receptor signals like B-cell activating factor (BAFF), Lymphotoxin β (LTβ), CD40 ligand, TNF-like weak inducer of apoptosis (TWEAK) and receptor activator of NF-κB ligand (RANKL) (Xiao et al., 2006). It is a slow process that depends on NF-κB-inducing kinase (NIK) protein synthesis. Despite the fact that its mRNA expression is abundant, protein levels are usually low due to its constitutive degradation by a TRAF3-dependent mechanism (Qing et al., 2005). When non-canonical NF-κB stimuli occur, the key components of this mechanism are degraded by the proteasome and NIK is activated and then able to activate an IKKα complex (an homodimer lacking NEMO), that consequentially phosphorylates p100, leading to its partial proteolysis (in the proteasome) and formation of p52. The p52/RelB complex then translocates into the nucleus to modulate gene expression (Zarnegar et al., 2008) (Figure 6).

There is evidence that NF-κB is constitutively activated in gastric cancer tissues, with higher levels in gastric carcinoma cells in comparison to normal adjacent epithelial cells (Sasaki et al., 2001) although it is RelA and not NF-κB that is used as a prognostic indicator of gastric carcinoma. It has also been reported that patients with highly activated NF-κB levels in cancer cells would have a lower survival potential when compared to those with low NF-κB activation (Yamanaka et al., 2004). In gastric cancer, abnormal NF-κB activation has been shown to lead to enhanced proliferation, evasion of apoptosis, genomic instability, increased rate of glycolysis and drug resistance (Cho et al., 2008; Kang et al., 2008; X. Liu et al., 2010).

Regarding drug resistance, a study has been performed in order to evaluate the effect of 5-Fluorouracil (5-FU) and irinotecan (CPT-11) in NF-κB activation. It led to the conclusion that these components are inducing two different pathways: apoptosis through direct effect on nucleic acids, and inhibition of apoptosis through activation of NF-κB. Moreover, the same authors used an inhibitor of NF-κB and predicted that its combination with 5-FU and CPT-11 may be a more effective treatment option instead of chemotherapy alone for gastric cancer (Camp et al., 2004).

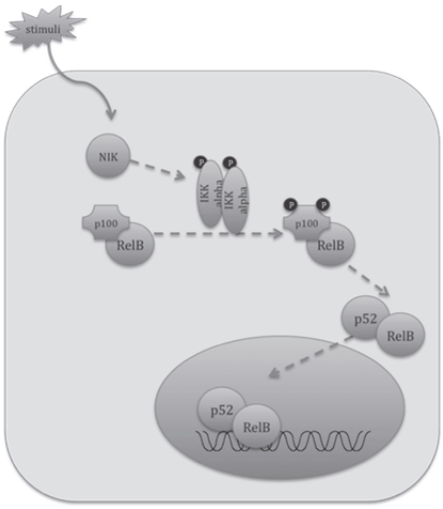

Fig. 6. NF-kB signaling pathway (non-canonical activation).

Since the discovery that blocking NF-κB can cause tumor cells to stop proliferating, to die, or enhance their sensitivity to the action of anti-tumor agents NF-κB has been widely used as a target for anti-cancer therapy (Escarcega et al., 2007). HIF-1α, one of the components of hypoxia-inducible factor 1 (HIF-1), has been directly implicated in tumorigenesis including angiogenesis, tumor cell proliferation, metastasis, as well as chemo- and radiotherapy response, particularly in gastric cancer (Liu et al., 2008; Weidemann & Johnson, 2008). Interestingly, a recent study showed that NF-κB inhibition in gastric cancer suppressed hypoxia-induced HIF-1α protein expression (but not at the mRNA level), suggesting that this protein is a downstream molecule of NF-κB in the angiogenesis pathway in gastric cancer. Also, NF-κB seems to be activated by hypoxia leading to HIF-1α protein accumulation at the translational level, but not at the transcriptional or post-translational levels. Thus, NF-κB/HIF-1α pathway may be a fitter candidate target for inhibition in gastric carcinoma therapy (Nam et al., 2011).
Using a different approach, other investigators described a new mechanism in gastric tumor cells associated with NF-κB inhibition responsible for impairment of cell proliferation and induction of apoptosis of cancer cells. By blocking NF-κB (with a RelA inhibitor, SN50) they achieved an increase in p53 expression, which led to the induction of pro-apoptotic and autophagic proteins. Thus, p53 contributes to NF-κB inhibitor-induced apoptosis of cancer cells by activation of autophagic mechanisms (Zhu et al., 2011).

2.7 Transforming growth factor- β, bone morphogenic protein pathway

Transforming growth factor (TGF)-β is a multifunctional cytokine that controls differentiation, apoptosis, cell growth and immune reactions (Roberts, 2002; Shi & Massagué, 2003). TGF-β1, -β2, and -β3 are three isoforms of TGF-β that are present in mammals. In most types of cells, TGF-β is a potent growth inhibitor, so alterations on TGF-β signaling lead to tumor progression by the induction of angiogenesis, extracellular matrix accumulation and immunosuppression (Blobe et al., 2000; Derynck et al., 2001; Wakefield & Roberts, 2002).

This pathway is considered to be a tumor suppressor pathway that negatively regulates cell growth and promotes apoptosis of epithelial cells (Siegel & Massagué, 2003). In early stages of cancer, TGF- β signaling acts as a tumor-suppressor and in later stages promotes invasion and metastasis (Wu et al., 2010). TGF-β signaling pathway is composed of two distinct receptors with intrinsic serine/threonine kinase activity, TGF-β type I and type II receptors (TbRI and TbRII) and Smad proteins. The binding of TGF-β to TbRII leads to recruitment and transphosphorylation of TbRI (heteromeric complex). Cytoplasmatic Smad2 and Smad3 are then phosphorylated by activated TbRI kinase, allowing them to form a heteromeric complex with Smad4, that is translocated into the nucleus acting as transcription factors. (Massagué, 1998; Miyazono et al., 2000; Miyazono et al., 2003) (Figure 7).

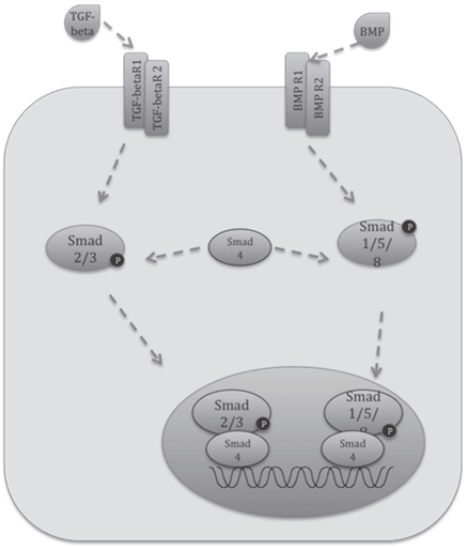

Fig. 7. Wnt signaling pathway (canonical).

Several studies demonstrated that the over-expression of TGF-β, in gastric cancer, is correlated with lymph node metastasis and poor prognosis (Maehara et al., 1999; Saito et al., 2000), as well as promotion of invasion and metastasis (via Smad3-, ERK- and JNK-dependent signal pathways) (Fu et al., 2009; Wang et al., 2006; Yoo et al., 2008). TGF-β induces RUNX3, a transcription factor that is involved in the formation of a variety of cancers (Ito, 2004). RUNX3 is expressed in glandular stomach epithelial cells, however, the loss of expression of this gene is associated with the progression, differentiation, metastasis and poor prognosis of gastric cancer (Li et al., 2002; Sugiura et al., 2008; Wei et al., 2005). Vogiatzi and colleagues (2006) demonstrated that RUNX3 interacts with FoxO3a /FKHRL1 to activate Bim and induce apoptosis in gastric cancer cells. *H. pylori* causes methylation of RUNX3 gene and its loss of expression in gastric epithelial cells (Katayama et al., 2009). Moreover, RUNX3, Smad4 inactivation has been documented in gastric cancer (Wu et al., 2010 as cited in Powell et al., 1997). Another study carried out by Shinto and colleagues (2011) demonstrated that the expression of p-Smad2 is associated with malignant phenotype and poor prognosis in patients with advanced gastric carcinoma.

Bone morphogenetic proteins (BMPs) are members of the TGF-β superfamily (von Bubnoff & Cho, 2001). They were originally identified as osteoinductive cytokines that regulate bone and cartilage formation (Balemans & Van Hul, 2002; Chen et al., 2004; Hogan, 1996). The BMPs mediate their effects by binding to type I and II serine–threonine kinase receptors (BMPR), leading to the phosphorylation of Smad1, Smad5 and Smad8. These phosphorylated Smads heterodimerize with Smad4 and this complex is translocated to the nucleus to activate the transcription of downstreams targets (Derynck et al., 1998; Kretzschmar et al., 1997; Heldin et al., 1997). Several studies have demonstrated that BMPs play important roles in the regulation of cell motility, proliferation, apoptosis, differentiation, self-renewal of embryonic stem cells and remodeling of the extracellular matrix (Hardwick et al., 2004; Hogan, 1996; Li et al., 1996; Massagué, 1996; Nissinen et al., 1997; Von Bubnoff, 2001). BMP proteins are expressed in adult stomach (Peek & Blaser, 2002; van den Brink et al., 2001).

BMP signaling in the stomach is down-regulated in cancer and upregulated during inflammation (Wu et al., 2010). *H. pylori* infection leads to an increase in BMP expression, mainly caused by an influx of BMP2-producing cells. The influx correlates with an increase in the activity of the BMP pathway (Bleumeing et al., 2006). A study carried out by Wen and colleagues (2004) demonstrated that BMP-2, a BMPR ligand, caused cell cycle arrest in the G1-phase in MKN74 and OUMS37 cells, and that this growth inhibitory action may be mediated by $p21^{Waf1/Cip1}$ (BMP-2 suppresses gastric cancer cells proliferation). Moreover, this BMP is suppressed by tumor methylation in gastric cancer cells (Wen et al., 2006). Taken together, these results suggest that the inhibition of BMP signaling contributes to gastric tumorigenesis through the suppression of differentiation. (Oshima et al., 2009). However, recent studies have discovered that BMP-2 can accelerate the migration and invasiveness of gastric cancer cells and may correlate with disease progression (Kang et al., 2010; Park et al., 2010).

3. MUC1 mucin-mediated signaling pathways in gastric cancer

The stomach is continuously subjected to a harsh acidic environment and several external aggressions. The mucus layer produced by the gastric epithelium has a crucial protective role against these adverse conditions. Three major heavily glycosylated proteins (mucins) line the stomach epithelium under normal conditions: one membrane associated mucin, MUC1, and two secreted mucins, MUC5AC and MUC6. They all contribute to the formation and maintenance of a cohesive "mucin net" that covers the entire epithelium, working as an efficient barrier. Abnormal expression and glycosylation have been described for these highly polymorphic mucins in gastric carcinoma and pre-neoplastic lesions (Reis et al., 1999; Teixeira et al., 2002). MUC1 polymorphism defines different susceptibility backgrounds associated with the development of conditions that precede gastric carcinoma: chronic atrophic gastritis and intestinal metaplasia (Silva et al., 2001).

MUC1 and MUC4 have been recently identified as participating in intracellular signaling pathways, by their cytoplasmic domains (Carraway et al., 2003; Hollingsworth and Swanson, 2004). The phosphorylation of MUC1 cytoplasmic domain (MUC1-CD) has been found to modulate its interaction with several molecules, such as EGFR, β-catenin, p53, ER-α, ICAM-1, among other molecules (for review see Singh & Hollingsworth , 2006). These interactions have been mainly found for breast, pancreatic and lung cancer cells and so far the data about MUC1-mediated signaling pathways or MUC1 signaling partners in gastric

carcinoma cells is limited. MUC1-CD is known to interact with beta-catenin and upregulate the Wnt signaling pathway in CagA *Helicobacter pylori*-infected gastric carcinoma (Udhayakumar et al., 2007).

Our group has been studying the MUC1-dependent signaling pathways in gastric cancer cells. We have stably down-regulated MUC1 expression in MKN45 gastric carcinoma cell line by shRNA and we evaluated MUC1 down-regulation impact in potential MUC1-mediated signaling pathways. We observed that MUC1-downregulation leads to abnormal expression levels of ERK1/2 proteins and an increased phosphorylation of these kinases. We further characterized the association between MUC1 and ERK1/2 and we showed by proximity ligation assays that MUC1-CD directly interacts with ERK1/2 kinases in these cells. The impact of MUC1 in the transcription and stability of these kinases and the interaction with other signaling partners (e.g. EGFR) are being currently evaluated, nonethless this clearly suggests that MUC1-CD is involved at different levels in the regulation of the MAPK signaling pathway in gastric carcinoma cells (Figure 8).

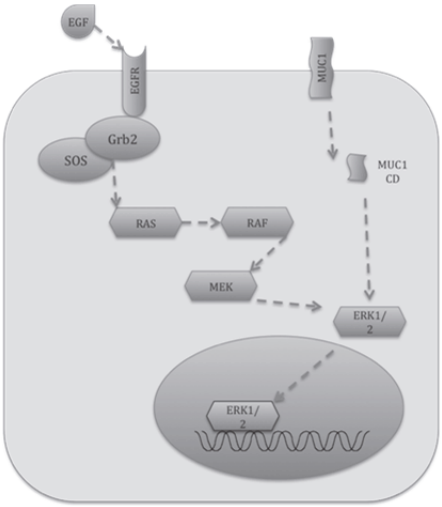

Fig. 8. MUC1 and EGFR/MAPK signaling pathway.

The kinases of EGFR/MAPK signaling pathway are crucial effectors responsible for cell proliferation and oncogenic transformation. Recent data from other tumor models further reinforce the relevance of MUC1 as a key player in cell-cell (microenvironment) signaling contexts (Behrens et al., 2010). Therefore, it assumes critical relevance an extensive characterization of MUC1-mediated signaling events in this pathway and their impact in gastric carcinoma phenotype. These results suggest MUC1 as a new and promising candidate to be targeted by therapies against gastric cancer.

4. Conclusions

Gastric cancer is a leading cause of cancer-related death worldwide. Given the limited options currently available for gastric cancer therapy and prevention, it becomes urgent to better understand the oncogenic signaling pathways beyond the emergence of the disease.

There is an increased knowledge about the alterations occurring in multiple signaling pathways and the acquisition of gastric cancer phenotype. The complex interplay between environmental factors and oncogenic signaling pathways involving cell proliferation, differentiation, apoptosis and invasion, remains however elusive. Emerging evidence in other models has brought new evidences on the complex interaction among different oncogenic signaling pathways. Whether such phenomena occur in gastric cancer, remains unclear.

The elucidation of individual interactions is thus required to develop a more consistent understanding of the gastric oncogenic signaling networks and will help to identify novel targets for anticancer drug development. The reviewed signaling pathways are relevant contributors for gastric carcinogenesis and encompass a multitude of potential therapeutic targets. In addition to these signaling-related targets we included new data on MUC1 mucin, previously described as being involved in gastric cancer susceptibility phenotype. The characterization of the complete spectrum of MUC1-dependent oncogenic signaling interactions in gastric cancer cells, will offer the molecular basis for the development of innovative therapies using MUC1 as an elective target. Furthermore an integrative perspective, of these MUC1-mediated signaling pathways, will be critical to design therapeutic strategies that inhibit multiple signaling pathways enhancing the efficacy of gastric cancer therapies and probably prevent the development of drug resistance phenotype.

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