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**IL-6 induces MUC4 expression through gp130/STAT3 pathway in gastric cancer cell lines.**

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## Summary

The gastric mucosal levels of the pro-inflammatory cytokine Interleukin 6 (IL-6) have been reported to be increased in *Helicobacter pylori*-infected subjects and, in gastric adenocarcinomas, the up-regulation of intestinal mucin genes (MUC2 and MUC4) has been detected. To analyse the regulatory effects of IL-6 on the activation of intestinal mucins, six gastric cancer cell lines were treated for different times with several concentrations of IL-6, and the expression of MUC2 and MUC4 was evaluated. IL-6 induced MUC4 expression, detected by quantitative RT-PCR, Western blot and immunofluorescence, and MUC2 expression was not affected. MUC4 mRNA levels decreased after blocking the gp130/STAT3 pathway at the level of the receptor, and at the level of STAT3 activation using the AG490 specific inhibitor. MUC4 presents two putative binding sites for STAT factors that may regulate MUC4 transcription after a pro-inflammatory stimulus as IL-6. We demonstrated that p-STAT3 binds to MUC4 promoter and a three-fold increase in p-STAT3 binding was observed after treating GP220 cells with IL-6.

In conclusion, IL-6 treatment induced MUC4 expression through the gp130/STAT3 pathway, indicating the direct role of IL-6 on the activation of the intestinal mucin gene MUC4 in gastric cancer cells.

## 1. Introduction

Gastric cancer is the fourth most common cancer worldwide and, approximately, 90% of gastric cancers are adenocarcinomas [1]. Chronic gastritis represents the first step of the carcinogenetic process and it is usually associated to the colonisation of the gastric mucosa by *Helicobacter pylori* (*H. pylori*) [2]. The inflammatory response to *H. pylori* includes the release of pro-inflammatory cytokines such as interleukin 6 (IL-6), which levels have been described to be increased in the gastric mucosa of *H. pylori*-infected subjects [3,4]. IL-6 belongs to a family of cytokines including interleukin-11 (IL-11), leukaemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNFT), cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC) and interleukin-27 (IL-27), that can activate target genes involved in differentiation, survival, apoptosis and proliferation [5]. These cytokines act as ligands for the signalling receptor subunit gp130. IL-6 and IL-11 signal via gp130 homodimers, and signal transduction is mediated by the JAK/STAT pathway, in particular STAT3 and to a minor extent STAT1 [5]. Tyrosine phosphorylation is essential for STAT3 dimerization and its nuclear translocation, where the STAT3 DNA-binding domain interacts with STAT-binding sites in target gene promoters [6].

Multiple sequential changes take place in the gastric mucosa previously to the development of a gastric adenocarcinoma, such as the loss of its characteristic mucin expression pattern (MUC5AC in the superficial epithelium and MUC6 in the glands [7,8]) and the activation of intestinal mucin genes, MUC2 and MUC4, which are detected in gastric tumors and in early events of the carcinogenesis process, as intestinal metaplasia [9,10].

MUC4 is a membrane-bound mucin firstly cloned from a tracheobronchial library [11]. It is composed by two subunits: the mucin subunit MUC4 $\alpha$ , and the transmembrane subunit MUC4 $\beta$ , that has been reported to be ligand for ErbB2 via EGF-like domains, providing a role for MUC4 in tumor progression [12,13]. MUC4, which is mainly expressed in normal colon tissue, is up-regulated in some pre-malignant lesions as well as in different types of tumors. In pancreatic cancer, MUC4 is overexpressed [14] and, in a recent report using a pancreatic adenocarcinoma cell line and an orthotopic mouse model, MUC4 has been described to potentiate pancreatic tumor cell proliferation, survival and invasion as well as changes in tumor cell-extra cellular matrix interactions and altered expression of growth- and metastasis-associated genes [15]. The activation of MUC4 expression has been also shown in the dysplastic cervical process [16] and, recently, we have described increased levels of MUC4 expression in endometrial hyperplasia and endometrial carcinoma [17]. In most of gastric tumors MUC4 is also up-regulated, and can be detected at early stages of the gastric carcinogenetic process [10]. However, no information about the regulatory mechanisms involved in its activation has been reported in gastric cancer cells.

In the present study we have analysed the induction of MUC4 expression by IL-6 in gastric cancer cell lines and we demonstrate that MUC4 activation occurs through gp130/STAT3 pathway.

## **2. Materials and methods**

### *2.1. Reagents and Antibodies*

IL-6 was purchased from PreproTech EC (London, UK) and Dubelcco's modified Eagle's medium (DMEM) from Invitrogen (Carlsbad, CA). The specific inhibitor of STAT3 activation AG490 was obtained from Calbiochem (Darmstadt, Germany). Anti-human gp130 receptor (CD130) antibody was purchased from Biosource (Camarillo, CA), and anti-STAT3 and anti-p-STAT3 (Tyr705) antibodies were obtained from Cell Signaling (Danvers, MA). For MUC4 detection rabbit polyclonal anti-MUC4 [10] was used. Anti-mouse or anti-rabbit HRP conjugated antibodies were purchased from Dako Cytomation (Glostrup, DK), and for fluorescent analysis anti-mouse Alexa Fluor 488 (Molecular Probes, Leiden, The Netherlands) and anti-rabbit Cy2 (Jackson ImmunoResearch, Cambridgeshire, UK) were used.

### *2.2. Cell culture and treatments*

MKN45, Kat0III and NUGC-4 human gastric cancer cell lines were obtained from ATCC. St2957, St3051, St23132 were characterised in Dr. Peter Vollmers' laboratory [18], and GP220 cells were established in Dr Sobrinho-Simões' laboratory [19]. All cell lines were maintained at 37°C in CO<sub>2</sub> atmosphere in 10% FCS supplemented DMEM. For IL-6 treatments semi confluent cells were rinsed in phosphate-buffered saline (PBS) and incubated for 2, 5, 10 or 20 hours with 20 ng/ml or 40 ng/ml IL-6 diluted in DMEM.

For gp130 blocking experiments, cells were incubated with the mouse anti-human gp130 antibody at 5 µg/ml concentration in DMEM for 3 hours and then treated with IL-6 at 40 ng/ml for 20 hours or at 20 ng/ml for 2 hours.

STAT3 phosphorylation was prevented by incubating GP220 cells with 60 µM AG490 in DMEM and cells were incubated with 40 ng/ml IL-6 for 2 or 5 hours.

All treatments were performed in duplicate and three independent experiments were done.

### *2.3. Quantitative Reverse-Transcription polymerase chain reaction analysis*

Total RNA extraction was carried out from control and IL-6 stimulated cells using GenElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich, St. Louis, MO). After rDNase I (Ambion, Austin, TX) treatment, quantitative determination of MUC4 mRNA levels was performed in quadruplicate by using QuantiTect SYBR green reverse transcription-PCR (Qiagen GmbH, Germany). MUC4 was amplified by primers 5'-CTT ACT CTG GCC AAC TCT GTA GTG-3' (sense) and 5'-GAG AAG TTG GGC TTG ACT GTC-3'. (antisense) [20]. Hypoxanthine-guanine phosphoribosyl transferase (HPRT) mRNA (GeneCards database, NCBI36:X) was analysed as an internal control by using oligonucleotides 5'-GGCCAGACTTTGTTGGATTTG-3' (sense) and 5'-TGCGCTCATCTTAGGCTTTGT-3' (antisense). RT-PCR and data collection were performed on the ABI Prism 7900HT system. All quantifications were normalized to the endogenous control (HPRT).

#### *2.4 Semi-quantitative Reverse-Transcription polymerase chain reaction*

MUC2 was amplified by primers 5'-CTT CGA CGG ACT CTA CTA CAG C-3' (sense) and 5'-CTT TGG TGT TGT TGC CAA AC-3' (antisense) [20]. As a control for mRNA levels  $\beta$ -actin cDNA was also amplified [21].

Amplification conditions MUC2 were: 94°C 1', 58°C 30" and 72°C 30" for 35 cycles. The size of the products was 387 bp for MUC2 and 349 bp for  $\beta$ -actin.

#### *2.5. Immunofluorescence*

Cells were grown on glass coverslips. Semi confluent untreated or 40 ng/ml IL-6 treated cells were fixed in 4% PFA. After washing, cells were incubated with 50 mM ammonium chloride for 30 minutes. Non-specific binding sites were blocked using 1/20 horse serum in PBS containing 1% bovine serum albumin (BSA). Cells were incubated with anti-MUC4 antibody[10] for 90 minutes. After several washes, Cy2 anti-rabbit antibody was incubated for 40 minutes. Nuclei were stained with propidium iodide. After rinsing, coverslips were mounted in an aqueous mounting medium and observed in an Olympus BX61 microscope.

#### *2.6. Flow Cytometry*

GP220 and MKN45 cultured cells were treated with 40 ng/ml IL-6 for 20 hours.  $5 \times 10^5$  viable cells were incubated for 30 minutes at 4°C with 1  $\mu$ g/ $\mu$ l anti-gp130 antibody diluted in PBS-1% BSA. Cells were rinsed in PBS-1% BSA and incubated with the secondary anti-mouse Alexa Fluor 488 antibody for 30



minutes at 4°C. After washing, fluorescent analysis was performed by using a FACScan (Becton Dickinson, Franklin Lakes, USA).

### *2.7. Cell lysates and Western Blot Analysis*

Cytoplasmic cell lysates were obtained for gp130 and MUC4 detection by lysing the cells in 50 mM Tris pH8, 62.5 mM EDTA and 1% Triton X-100 lysis buffer. For STAT3 and p-STAT3 detection, total cellular pellets were solubilized in 2X SDS gel sample buffer (20 mM dithiothreitol, 6% SDS, 0.25 M Tris pH 6.8, 10% glycerol, and bromophenyl blue) and sonicated using 3-4 bursts of 5 seconds each. Extracts were boiled at 100°C for 5 minutes and immediately cooled on ice.

For MUC4 detection Western blot was performed on 2% SDS-agarose gels as previously described [22]. Anti-MUC4 antibody [10] was incubated for 2 hours and bound secondary antibody was detected using the ECL Western Blotting Substrate (Pierce, Rockford, IL).

For gp130, STAT3 and p-STAT3 detection, lysates were electrophoresed on 8% SDS-polyacrylamide gels. Separated proteins were blotted onto nitrocellulose membranes (Protran, Dassel, Germany), blocked for 1 hour at RT, and incubated overnight with the specific primary antibodies following manufacturer's instructions. ECL Western Blotting Substrate, for gp130 and STAT3, or Supersignal West Femto, for p-STAT3, were used.

### *2.8. Electrophoretic Mobility Shift Assay (EMSA)*

Nuclear extracts were obtained from IL-6 treated (20 ng/ml for 2 hours) and untreated GP220 cells. Pellets from cultured cells were resuspended in

nuclear extraction buffer 1 (10 mM Hepes pH 7.6, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM DTT, 0.5% NP-40 and phosphatases and proteases inhibitors) and centrifuged. Pelleted nuclei were resuspended in nuclear extraction buffer 2 (20 mM Hepes 7.6, 1.5 mM MgCl<sub>2</sub>, 840 mM KCl, 0.5 mM DTT, 0.2 mM EDTA, 25% glycerol and phosphatases and proteases inhibitors). Supernatants obtained after centrifugation were dialyzed (20 mM Hepes 7.6, 100 mM KCl, 0.5 mM DTT, 0.2 mM EDTA, and 20% glycerol) for 5 hours and the protein content was measured by Bio-Rad Protein Assay.

30 µg of protein were incubated with the radiolabeled probes in incubation buffer (10 mM Hepes 7.9, 1 mM EDTA, 5 mM MgCl<sub>2</sub>, 10% glycerol, 0.5 mM DTT, 1 mg/ml BSA and 1 µg PolydI-dC (Amersham Biosciences)) for 1 hour on ice. The radiolabeled probes used were: **T51**: 5'-TCTTCCCCATTCTTCCCA**GCAG**CCCAA-3', and **T63**: 5'-CCTGGCCCC**TTCGGAGAA**ACGCA-3', corresponding to the MUC4 promoter regions (NCBI sequence, accession number AF241535 [23]) 3287–3313 and 3582–3604, respectively. Unlabeled cold oligonucleotides, mutated T51 (5'-CTTCCCCATTCA**AGAGTTCC**CAGCC-3') or mutated T63 (5'-CTGGCCCC**GCAGGATGG**ACGCA-3') probes were used for competition experiments (100x and 300x the concentration of the labeled probe). For supershift assays, extracts were incubated with anti-p-STAT3 antibody or with irrelevant immunoglobulins for 15 additional minutes. Samples were run on a 6% acrylamide gel. Gels were dried and subjected to autoradiography.

## 2.9. Chromatin Immunoprecipitation (ChIP) Assay

Chromatin immunoprecipitation assay was performed as described.[25] Briefly, IL-6 treated (20 ng/ml IL-6 for 2 hours) and untreated GP220 cells were cross-linked with 1% formaldehyde, lysed and sonicated. After pre-clearing the chromatin with protein A-agarose and irrelevant IgGs, p-STAT3 was immunoprecipitated by incubating with the specific antibody overnight at 4°C. Blocked agarose was added to the samples and incubated for 2 hours at 4°C. Immunoprecipitates were washed, eluted with elution buffer (0.1 M Na<sub>2</sub>CO<sub>3</sub> and 1% SDS) and digested with K proteinase for 30 minutes at RT and 30 minutes at 55°C. 5 M NaCl was added to the samples and they were incubated overnight at 65°C. DNA purification was performed using GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK).

Quantitative PCR was performed using two different sets of primers: **Primer Set 1:** 5'-TCA TAC AGC CCC AAG GTC GC-3' (sense), 5'-TAG CCG GGT TCC TGG GTC C-3' (antisense), corresponding to the MUC4 promoter region 3251-3373 (NCBI sequence, accession number AF241535 [23]) and **Primer Set 2:** 5'-GAA AAG GGT GAT TAG CGT GG-3' (sense) and 5'-TCC CCT CAG GCG GCT GGC C-3' (antisense), corresponding to the 3528-3632 region of MUC4 promoter. As internal control primers for the exon 1 of the RNA polymerase 2 were used. The sequences were: 5'-ACT CCA GGC TAG AGG GTC AC-3' (sense) and 5'-CCG CAA GCT CAC AGG TGC TTT GCA GTT CC-3' (antisense).

Quantifications were also normalized to input and calculated as a percentage of the input.

### 2.10. Site-directed Mutagenesis

Quick Change site-directed mutagenesis kit (Stratagene) was used to generate site-specific mutations in the STAT binding site (T63) present in the MUC4 promoter construct 2150 [23]. Mutagenesis was performed as described [24]. The oligonucleotide containing the desired mutations was designed according to manufacturer's instructions (mutated nucleotides are underlined and italicized): T63 (3008): CCCTGGCCCCTT*AGCGT*AAACGCACTTGG

### 2.11. Luciferase reporter assays

Reporter assays were carried out in GP220 cells by using 400ng of the human wild type MUC4 promoter construct 2150 (-1187/-1) [23], 400ng of the mutated 2150 construct 3008 or 400ng of pGL3 basic vector (Promega). Cells were cotransfected with 1ng of simian virus 40-Renilla luciferase plasmid as the control for transfection efficiency. 24 hours post-transfection cells were incubated with IL-6 (40 ng/ml) for 20 hours. The expression of Firefly and Renilla luciferases was analysed 48 hours after transfection, according to manufacturer's instructions.

### 3. Results

#### 3.1. Induction of MUC4 expression by IL-6

GP220, MKN45, St23132, St2957 and St3051 gastric cancer cells, that express the gastric mucin MUC5AC but do not express MUC6 [26], and NUGC-4 gastric cancer cells expressing both, MUC5AC and MUC6, were subjected to different treatments with IL-6 (20 ng/ml and 40 ng/ml) to test the induction of the intestinal mucins MUC4 and MUC2.

The selected gastric cancer cell lines do not express MUC4 (Figure 1A), and after 20 hours IL-6 treatment (20 ng/ml and 40 ng/ml) its expression was induced.

GP220 and MKN45 cells, which expressed MUC4 mRNA at different levels after 20 hours IL-6 stimulation, were selected to further characterise MUC4 expression induced by IL-6. For this reason, these cells were treated with IL-6 at different times and doses. In GP220 cells MUC4 mRNA levels increased in a dose- and time-dependent manner. After 10 and 30 minutes of IL-6 treatment there is not induction of MUC4 expression (data not shown). MUC4 was detected after 2 hours and 20 ng/ml IL-6 treatment, and the maximum levels of expression were achieved when cells were incubated with 40 ng/ml IL-6 for 20 hours. In MKN45 cells, the expression of MUC4 was induced at lower levels than in GP220 cells. MUC4 was detected at 2 hours and 20 ng/ml IL-6 stimulation, and the maximum levels were obtained after 5 hours IL-6 treatment (Figure 1B). MUC4 protein expression was analysed by Western blot and immunofluorescence. GP220 cells were treated with 20 ng/ml IL-6 or 40 ng/ml IL-6 for 20 hours and cell lysates were subjected to Western blot. No

MUC4 apomucin was observed in GP220 untreated cells, but its expression was induced after treating the cells with IL-6 (Figure 2A). MUC4 expression was also analysed by immunofluorescence (Figure 2B), and GP220 untreated cells did not present MUC4, whereas in GP220 IL-6 treated cells (40 ng/ml IL-6 for 20 or 40 hours) MUC4 was detected.

### *3.2. MUC2 expression is not induced by IL-6*

MUC2 expression was also analysed in the six gastric cancer cell lines after 20 hours IL-6 treatment (20 ng/ml and 40 ng/ml). No changes on MUC2 mRNA levels were detected in GP220, St2957, St3051 and St23132 that express MUC2, and no induction on its expression was observed in MKN45 and NUG-4 cells that do not express MUC2 (Figure 3).

### *3.3. gp130, STAT3 and p-STAT3 levels after IL-6 treatment*

The presence of gp130 associated to the cell membrane was analysed by flow cytometry in untreated and 20 hours 40 ng/ml IL-6 treated GP220 and MKN45 cells. No differences were detected when comparing control cells and IL-6 treated cells: 22.9% vs. 27.06%, and 49.44% vs. 45.35% positive cells for GP220 and MKN45, respectively (Figure 4A). The expression levels of gp130 in other gastric cancer cell lines were: 56.83% for St2957, 35.24% for St3051 and 51.90% for St23132.

The transmembrane IL-6 receptor gp130 (130-140 KDa) was expressed at similar levels in GP220 and MKN45, detected by Western blot. After treating the cells with 20 ng/ml or 40 ng/ml IL-6 for 20 hours, no differences in gp130 protein levels were observed (Figure 4B).

The protein levels of the inactive STAT3 transcription factor and its phosphorylated active form p-STAT3, implicated in the IL-6/gp130 pathway, were analysed by Western blot in two cell lines that express high levels of MUC4 mRNA (GP220 and St2957) and in a cell line expressing low levels of MUC4 (MKN45) after IL-6 treatment (Figure 4C). In GP220 and St2957 untreated cells STAT3 was detected but p-STAT3 was not present. p-STAT3 can be already detected after 10 and 30 minutes IL-6 treatment (data not shown). In GP220 cells, maximum levels of p-STAT3 were observed after 2 hours of IL-6 treatment. STAT3 activation was IL-6 dose-dependent, and higher levels of p-STAT3 were detected when IL-6 concentration (40 ng/ml) was increased. p-STAT3 expression decreased in a time-dependent fashion and after 20 hours treatment it was not detected. In St2957, p-STAT3 was detected after 2 hours IL-6 treatment and its levels did not change with time.

In MKN45 cells, STAT3 was constitutively active and IL-6 treatment at different times (2, 5, 10 and 20 hours) and doses (20 and 40 ng/ml) did not induce remarkable differences in p-STAT3 expression levels.

#### *3.4. Anti-gp130 and AG490 incubation decrease STAT3 activation and MUC4 expression*

To determine the direct involvement of the IL-6/gp130/STAT3 pathway in regulating MUC4 transcription, GP220 and St2957 cells were selected because they do not express p-STAT3 constitutively. This pathway was blocked at two different levels: at the level of the gp130 receptor and at the level of STAT3 activation.

To block the IL-6 binding to its receptor, GP220 and St2957 cells were incubated with a specific antibody against the transmembrane type I cytokine receptor  $\beta$ -subunit gp130 for 3 hours previously to IL-6 stimulation (20 ng/ml and 40 ng/ml) and MUC4 mRNA levels were analysed by quantitative RT-PCR after 2 and 20 hours. In GP220 cells incubated with the antibody lower levels of MUC4 mRNA were detected. (Figure 5A). The same results were obtained with St2957 cells (data not shown). Regarding STAT3 activation, decreased levels of p-STAT3 were observed in GP220 cells (Figure 5B) and St2957 cells (data not shown) incubated with the antibody.

To block STAT3 activation, cells were incubated with the potent inhibitor of the Jak tyrosine kinase family AG490 [27]. When GP220 cells were treated with a combination of 60  $\mu$ M AG490 and 40 ng/ml IL-6 for 2 or 5 hours, there was a detectable decrease in MUC4 mRNA levels (Figure 5C). p-STAT3 protein levels were also analysed after 2 hours AG490-IL6 treatment. A significant decrease in STAT3 activation was observed in AG490 GP220 treated cells (Figure 5D). In MKN45 untreated cells that express p-STAT3 constitutively the same effect of AG490 was detected (data not shown).

### *3.5. p-STAT3 activates MUC4 expression through its binding to MUC4 promoter*

To assess the binding of the active form of STAT3 to MUC4 promoter, which contains two putative STAT-binding sites (Figure 6A), electrophoretic mobility shift assays, chromatin immunoprecipitation and luciferase reporter experiments were performed.



EMSA assays were performed using two different radiolabeled probes (T63\* and T51\*), including the two STAT binding sites in MUC4 promoter (Figure 6A). No retardation complexes were detected when incubating nuclear extracts from GP220 untreated and GP220 IL-6 treated cells (20 ng/ml for 2 hours) with T51\* probe (data not shown). Protein-DNA interaction was observed when the extracts were incubated with T63\* probe (Figure 6B). This interaction disappeared when samples were incubated with an excess of cold unlabeled oligonucleotides or with the mutated T63 probe. As expected, a supershift band was only detected in GP220 IL-6 treated cells.

In vivo binding of p-STAT3 to MUC4 promoter was confirmed by ChIP experiments. Two different sets of specific primers, to include each of the two STAT binding sites in MUC4 promoter, were designed (Figure 6A). No binding was detected when Primer set 1 was used for quantitative PCR. In contrast, a three-fold increase in p-STAT3 binding to MUC4 promoter was observed in GP220 cells treated with 20 ng/ml of IL-6 for 2 hours, when using Primer set 2 (Figure 6C)

To confirm that T63 STAT-binding site of MUC4 promoter is required to its activation after IL-6 treatment, reporter luciferase assays were performed. GP220 cells transfected with the MUC4 wild type promoter (2150) showed increased promoter activity after IL-6 stimulation, that was not detected in IL-6 treated cells transfected with the T63 mutant construct. (Figure 6D).

Together, these data demonstrate that the active form of STAT3 (p-STAT3) can regulate MUC4 transcription through its direct binding to MUC4 promoter.

#### 4. Discussion

The activation of intestinal genes in the sequential process that leads to gastric cancer has been well reported, but the mechanisms involved in their activation remain not fully identified. The intestinal mucin genes, MUC2 and MUC4, have been detected in gastric tumors and in the early stages of the gastric carcinogenesis process [9,10,28]. The activation of MUC2 has been associated to the intestinal specific transcription factors CDX1 and CDX2, that are co-expressed with MUC2 in intestinal metaplasia and gastric carcinomas [29,30]. Moreover, the ectopic expression of Cdx2 in the gastric mucosa induces intestinal metaplasia in mice [31], suggesting that MUC2 can be activated by CDX2. Our observation that no changes in MUC2 expression were induced in the gastric cancer cell lines treated with IL-6 is consistent with the fact that no STAT boxes have been described in the MUC2 promoter [32].

No data regarding the activation of MUC4 have been reported in gastric models. However, the induction of MUC4 expression by IFN- $\gamma$  through STAT1 up-regulation in pancreatic cancer cells by the binding of STAT1 to various  $\gamma$ -interferon-activated sequences (GAS) in the MUC4 promoter has been recently described [33]. Here we report the activation of MUC4 expression by the pro-inflammatory cytokine IL-6 through the gp130/STAT3 pathway. Two putative STAT-binding sites in the 3' – end of MUC4 5' – UTR have been described [23], indicating a role for STATs transcription factors in the transcriptional regulation of MUC4. By EMSA, CHIP and reporter luciferase assays, we demonstrate that p-STAT3 can bind directly to one of these two sites, located at the promoter

region 3582 – 3604 (NCBI Sequence), modulating MUC4 expression after IL-6 stimulation in GP220 gastric cancer cells.

The role of MUC4 in the neoplastic transformation of the gastric mucosa must be associated to signalling pathways controlling cell growth through the traffic and membrane localization of specific growth factor receptors [34], but the implication of inflammatory cytokines in the activation of specific intestinal markers during the sequential process of stomach carcinogenesis has not been carefully analysed. In gastric carcinoma increased serum levels of IL-6 have been correlated with the disease status [35], and in AGS gastric cancer cells, IL-6 promotes cell motility and invasiveness by the activation of the Src/Rho/ROCK signalling pathway, suggesting an important role in tumor progression in gastric cancer [36]. High levels of IL-6 results in the persistent activation of STAT3 that is present in several mouse and human malignancies suggesting a relation between hyperactivated STAT3 and tumorigenesis [37]. In a mouse model of gp130<sup>ΔSTAT</sup> that abolishes the STAT1/3 signalling pathway, it has been suggested that the maintenance of the integrity of the gastric mucosa depends on the relation between the STAT1/3 and SHP2-Ras-ERK pathways activated through the binding of IL-6 to the gp130 receptor [38]. Moreover, hyperactivation of Stat3 in gp130 mutant mice promotes tumor initiation and growth, and contributes to inflammation, angiogenesis and cell proliferation in gastric epithelium [39],[40]. In human cancer, it has been suggested that STAT factors contribute to tumorigenesis by their implication in angiogenesis and apoptosis through the growth factor signalling [41]. In gastric cancer, the angiogenic phenotype and the VEGF overexpression correlated with elevated STAT3 expression, and abnormally activated STAT3 has been postulated as a

marker for poor prognosis [42]. Moreover, in a recent report it has been described that human gastric cancer progression is accompanied by increased gp-130 mediated cytokine signalling [43].

The activation of MUC4 by the direct binding of STAT3 to the STAT-binding sites in the MUC4 promoter after IL-6 stimulation has not been previously reported. This novel mechanism suggests that the inflammatory response detected in the stomach mucosa may play a role in the activation of genes implicated in gastric carcinogenesis.

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