Phosphorylation of STAT3 in Head and Neck Cancer Requires p38 MAPKinase, whereas Phosphorylation of STAT1 Occurs *via* a Different Signaling Pathway

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Abstract. *STAT proteins work as signal transducers as well as transcription and activator proteins. In head and neck cancer both STAT1 and STAT3 are overexpressed. STAT3 contributes to malignant transformation and regulates tumor promoting cytokines, whereas STAT1 is purported to act antagonistically as a tumor suppressor. Since our previous data determined p38 MAPK to be a potent regulator of interleukin-6 (IL-6) and IL-8 expression in permanent head and neck squamous cell carcinoma (HNSCC) cell lines, we investigated the influence of this pathway on STAT3 and STAT1. Down-regulation of p38 MAPK expression levels by siRNA strongly reduces phosphorylation of STAT3 tyrosine 705 without any effects on phosphorylation of STAT3 serine 727 and STAT1. Analyzing the effect of silencing of ERK1/2 MAPK revealed that this MAPK strongly influences IL-6 and IL-8 expression, but is not involved in either activation of STAT1 or STAT3. Our data indicate STAT3 as a potent promoter of HNSCC progression.*

Head and neck squamous cell carcinoma (HNSCC) is one of the most common tumors (1). Its frequency is rising continuously in several western countries (1-6) and over the last 40 years, standard treatment has only negligibly improved the 5-year survival rate of patients with HNSCC. Head and neck cancer cells are known to practice molecular strategies to evade antitumor immune responses (7-9). It is supposed that tumor production of various immunosuppressive mediators contributes to massive effects on immune functions, but the molecular mechanisms remain mostly unknown (10, 11).

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Signal transducers and activators of transcription (STAT) proteins act as transcription factors, as well as transducers of cytoplasmic signals from extracellular stimuli, such as cytokines and growth factors (12). In mammals, seven functionally and structurally related STAT proteins are known (13). Since cytokine receptors do not possess intrinsic tyrosine kinase activity, they require associated tyrosine kinases such as Janus-kinase family members (JAK). JAKs are activated after ligand binding and receptor dimerization and phosphorylate STATs on a single tyrosine residue. Upon activation by tyrosine phosphorylation STAT3 can either homodimerize and form the SIF-A complex or heterodimerize with STAT1 to form the SIF-B complex (14). Both complexes translocate into the nucleus where they bind a variety of target genes (13). Growth factor receptors such as epidermal growth factor receptor (EGFR), with intrinsic tyrosine kinase activity, also activate STAT proteins. Phosphorylation of tyrosine residues of STAT proteins by JAK or intrinsic kinases is thought to be essential for dimer formation, whereas phosphorylation of serine residues is considered to maintain transcription factor activity of STAT proteins in the nucleus (15, 16).

STAT proteins are known to be highly expressed in different types of cancers and play a crucial role in cancer inflammation and immunosuppression (17). The expression of cytokines, growth factors and angiogenic factors are induced by STAT3, and the associated receptors in turn activate STAT3, creating a feedforward loop between tumor and cells of the microenvironment, including immune cells, which propagates the tumor-promoting inflammation.

In HNSCC, the aberrant activity of nuclear factor kappa B (NF-κB) and STAT3 is due to growth factors and cytokines, and promotes the tumor cell survival and proliferation. For example, NF-κB and STAT3 are both activated by EGFR; NF-κB induces IL-6 expression which in turn also leads to STAT3 activation (18). Both proteins modulate the BAX/BCLXL ratio in HNSCC. Targeting STAT3 leads to growth inhibition and increases apoptosis and down-modulation of BCLXL expression (14).

STAT1 and STAT3 are known to form heterodimers and in head and neck cancer STAT1 expression is well established (19). In general, STAT1 is associated with Th1 type immunostimulation and pro-apoptotic functions (13). Therefore STAT1 shows tumor-suppressor activities and counteracts the effects of STAT3. This was demonstrated in HNSCC as well as in fibroblasts (20, 21). Patients with oesophageal cancers with activated EGF-STAT1 pathway show better prognosis than patients lacking this pathway (22, 23). In contrast, Lui *et al*. have detected that STAT3 blockade using a transcription factor decoy is independent of STAT1 activation in HNSCC (19). For this antagonism the role of STAT1 and STAT3 in head and neck cancer needs to be further investigated.

In this work, we investigated the effect of down-regulation of p38 MAPK with specific siRNA on STAT3 tyrosine 705 phosphorylation and STAT1 expression.

Materials and Methods

Cell culture, siRNA transfection and preparation of supernatants. Permanent HNSCC cell lines PCI-1 and PCI-13 (hypopharyngeal cancer; Pittsburgh Cancer Institute, Pittsburg, PA, USA) were cultured in Dulbecco's modified eagle medium (DMEM; PAA, Pasching, Austria) supplemented with 10% fetal bovine serum (FBS; PAA, Pasching, Austria), non essential amino acids (1×; PAA) and 0.1 mM sodium pyruvate (PAA) at 37˚C with 5% CO_2.

Post-transcriptional gene silencing was performed using pools of at least two target-specific siRNAs to reduce off-target effects with the SignalSilence p38 MAPK siRNA Kit (New England Biolabs, Germany). Cells were incubated in 6-well plates with DMEM at a density of 2×10^5 cells per well for 24 h to reach a cell density of 60%. Transfection was performed in serum-free medium with Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA) using 25 nM p38 siRNA. Exchange of transfection medium to DMEM was performed after 15 h. After 48 h or 72 h, cells were stimulated if applicable for 15 min to 24 h with 200 ng per ml phorbol-12-myristate-13-acetate (PMA; Sigma-Aldrich, St. Louis, MO, USA). The applied concentration of siRNAs was confirmed by titration of siRNA to determine the level of knockdown required for specific phenotype. Cells transfected with BLOCK-iT™ Fluorescent Oligo (Invitrogen) were analysed by flow cytometry to examine transfection efficiency. These cells were also used as negative control for all RNAi experiments. Cell-free supernatants were collected by centrifugation after the indicated time of cell cultivation and kept at –80˚C until needed for cytokine analysis.

Protein analysis. Crude extracts of treated cells were prepared using RIPA-buffer (1× PBS, 1% Igepal CA-630, 0.5% sodium-deoxycholat, 0.1% SDS) with proteinase inhibitors (30% aprotinin, 10% PMSF, 10% sodium-orthovanodate, 20% sodium fluoride, 10%; Sigma-Aldrich). Protein concentrations were determined using the Quick Start Bradford Dye Reagent (Bio-Rad, Munich, Germany) with bovine serum albumin (BSA) as a standard. Aliquots of protein extracts (30 μg) were applied to 10% SDS-polyacrylamide gel. After electrophoresis the gel was transferred to a nitrocellulose membrane. Blots were incubated with antibodies against p38α, phospho-p38α, STAT3, phospho-STAT3 Tyr705 (Invitrogen), phospho-STAT3 Ser727 (Acris, Herford, Germany), STAT1, phospho-STAT1 Tyr 701 and phospho-STAT1 Ser 727 (Invitrogen).

Cytokine analysis. Cytokines (IL-6, IL-8) in cell culture supernatants were determined using the Cytometric Bead Array Flex Set system (CBA; BD Bioscience, Sanjoje, CA, USA) according to the instructions provided by the manufacturer. The CBA human Flex Set is a bead-based immunoassay capable of analysing multiple cytokines in a small volume of cell culture supernatant using spectrally addressed polystyrene beads coated with the corresponding antibodies. Similar to a sandwich ELISA, cytokines bind to specific beads and a PE-coated detection antibody binds to the cytokines. The cytokine assay was performed using a FACSCanto instrument (BD Biosciences) and the data analyzed by FCAP Array Software (BD Biosciences).

Phospho-p38α MAP (T180/Y182) K ELISA. To analyze phosphorylation levels of p38α MAPK in cell lysates, a sandwich ELISA (DuoSet IC, RnD Systems, Wiesbaden, Germany) was performed. The HNSCC cell lines PCI-1 and PCI-13 were incubated and stimulated with 200 ng/ml PMA for 15 min as described previously, ELISA was performed according to the instructions provided by the manufacturer.

Results

p38 MAPK induces IL-6 and IL-8 secretion. We investigated the activation of p38 MAPK by phospho-p38α ELISA after incubation of cells with PMA for 15, 30, 45 min, 1 h, 24 h and 48 h. As shown in Figure 1A, the highest stimulatory effect of PMA on p38 MAPK occurred within the first 30 min of incubation. Phosphorylation of p38 MAPK decreased after 45 min, with the lowest level occurring at the 48 h time point. Our data demonstrate strongly increased secretion levels of IL-6 and IL-8 in response to 24 h of PMA stimulation (Figure 1B).

Inhibition of p38 activity was achieved using specific siRNA approaches with grenn fluorescent protein (GFP)-labelled dsRNA oligomer as an internal control (Figure 1). 48 h after transfection, cells were stimulated with PMA for 24 h. Preparation of whole protein extracts and collection of cell culture supernatants followed 72 h after transfection. Western blot analysis showed a distinct decrease of p38 protein expression due to siRNA silencing (Figure 2A). Analysis of the cell culture supernatants revealed reduced secretion levels of IL-6 and IL-8 in response to the siRNA transfection (Figure 2B).

Previous investigations showed constitutive expression of ERK1/2 MAPK in HNSCC cell lines. We therefore investigated the role of ERK1/2 MAPK, especially in connection with cytokines and STAT proteins. The HNSCC cell lines PCI-1 and PCI-13 were transfected with siRNA against ERK1/2 MAPK. As shown in Figure 3A, the silencing of ERK1/2 MAPK resulted in down-regulation of IL-6 and IL-8, to a much greater extent than down-regulation after p38 MAPK silencing.

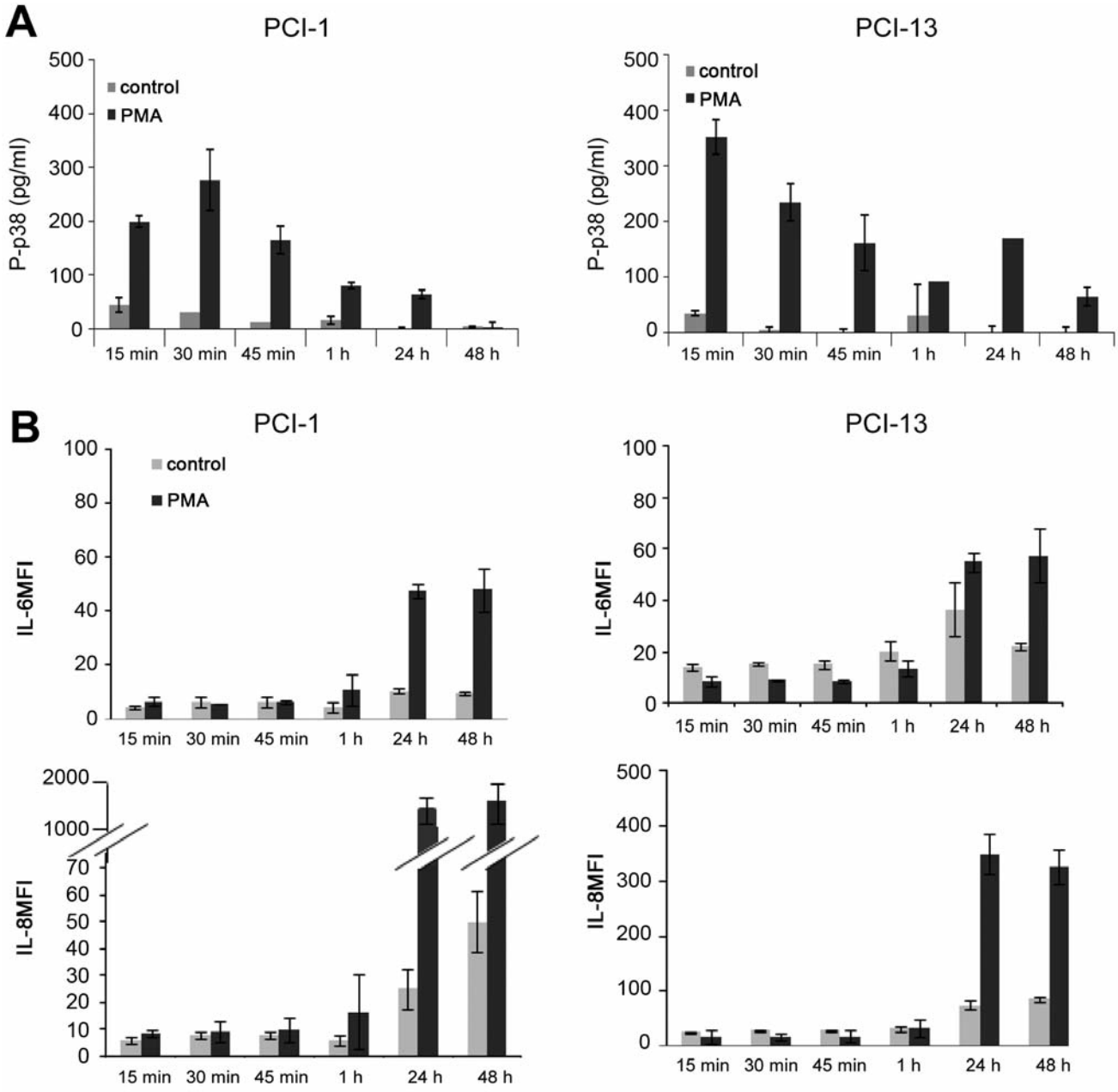

Figure 1. *PMA-induced IL-6 and IL-8 secretion occurs in a delayed time-frame. A: Phosphorylation status of p38 MAPK in HNSCC cell lines was analyzed by phospho-p38α ELISA after treatment with 200 ng/ml PMA at the indicated time points. Non-stimulated cells served as negative control. The highest expression of phosphorylated p38 MAPK was determined within the first 30 min of PMA treatment and decreased after 1 h dependent on the cell line. Data show the average of three independent experiments. B: Interleukin 6 (IL-6) and IL-8 expression levels of cell culture supernatants after incubation with PMA. IL-6 and IL-8 concentrations were analyzed by cytometric bead array. Compared with non-stimulated cells (control), the secretion of IL-6 and IL-8 was increased after 24 h of PMA incubation. This data indicate a delayed time-frame from p38 MAPK activation to its potential outcome. MFI: Mean fluorescent intensity.*

Western blot analysis revealed no effect on STAT1 and STAT3 expression levels in response to ERK1/2 silencing. The phosphorylation of tyrosine 705 and serine 727 residues of both proteins was also not affected (Figure 3B).

Phosphorylation of STAT3 in head and neck cancer requires p38 MAPK whereas phosphorylation of STAT1 uses a different signaling pathway. Next we investigated the influence of p38 silencing on STAT1 and STAT3 phosphorylation levels in

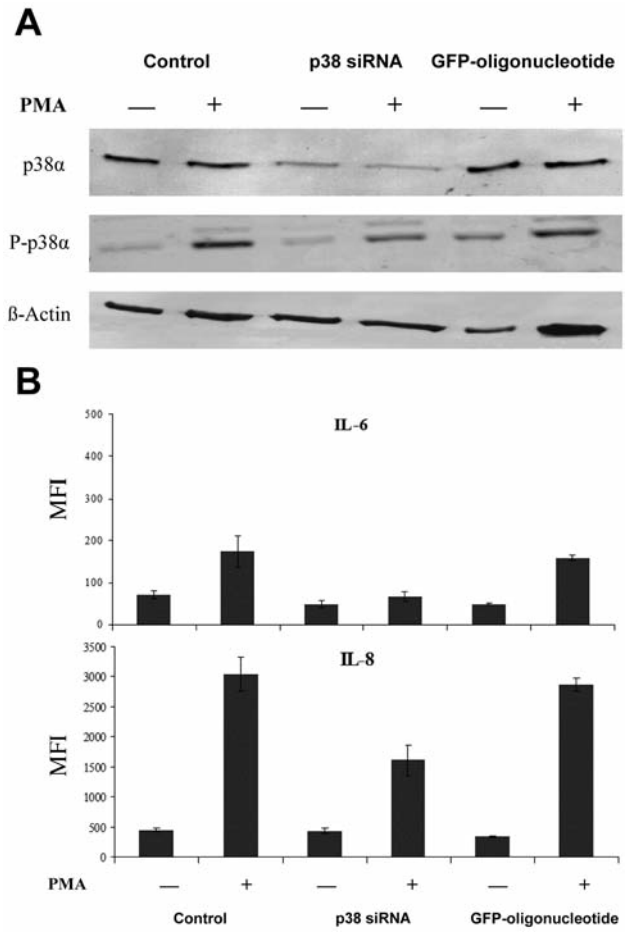

Figure 2. *p38 MAPK is an important regulator of pro-carcinogenic cytokines in HNSCC. A: Decreased expression of p38 MAPK after transfection with specific siRNA (25 nM). After 48 h of transfection cells were treated with 200 ng/ml PMA for 24 h followed by protein preparation and Western blot analysis. Non-transfected cells (control) and transfection with a non-specific green fluorescence protein GFP-oligonucleotide were used as positive and negative controls, respectively. Data shown are representative of three independent experiments. B: Silencing of p38 MAPK strongly reduced IL-6 and IL-8 secretion of HNSCC cell lines giving evidence for the important role of p38 MAPK in pro-carcinogenic mechanisms in HNSCC.*

HNSCC. As shown in Figure 4, silencing of p38 MAPK resulted in reduced STAT3 expression levels at tyrosine 705 residues while phosphorylation of serine 727 was not affected. In contrast, no influence was observed regarding the phosphorylation of STAT1. Interestingly, STAT1 phosphorylation can be induced by PMA whereas STAT3 is constitutively activated in HNSCC cell lines with no increase due to PMA stimulation. These data suggest that the activation pathways leading to STAT1 and STAT3 phosphorylation work in converse. Phosphorylation of STAT3 is dependent on p38 MAPK whereas STAT1 needs a different pathway for its activation.

Discussion

Our data demonstrate that PMA-induced secretion of IL-6 and IL-8 requires signal transduction *via* p38 MAPK. PMA is a potent stimulus, with a widespread influence on various biosynthetic pathways, such as the ERK1/2 MAPK dependent signal transduction. This MAPK is highly expressed in HNSCC, as well as being involved in regulation of IL-6 and IL-8 secretion. Thus, our results reveal two independent pathways for regulating cytokine secretion in HNSCC.

Phosphorylation of STAT proteins occurs on a *C*-terminal tyrosine residue after cytokine stimulation mostly by receptor-associated JAKs, leading to the dimerization of STAT proteins. Activated STAT dimers translocate into the nucleus where they bind at specific enhancer elements (13). In addition, most STAT proteins are phosphorylated on conserved serine residues such as serine 727 in the case of STAT1 and STAT3 (24-29). Phosphorylation of serine residues is required for the regulation of the transcriptional activity of STAT proteins but without increasing the DNA-binding capacity (16). Some studies suggest that depending on the stimulus, phosphorylation of tyrosine and serine residues occurs independently (30, 31). It is important to mention that most studies concerning p38 MAPK and serine 727 phosphorylation were carried out in the context of interferon signaling. Furthermore, it was shown that serine phosphorylation negatively regulates tyrosine phosphorylation of STAT3, both dependent on and independent of ERK1/2 MAPK. In contrast, our results show a p38 MAPK dependent tyrosine 705 phosphorylation of STAT3 without any effects on the phosphorylation status of the serine 727 residue. In many cancer entities as well as in HNSCC, aberrant signaling pathways are known which might also lead to the different functions of p38 MAPK in STAT1 and STAT3 regulation, such as in Wilms' tumor, a pediatric solid cancer of the kidney (32).

STAT3 is known as a potent tumor promoter by altering apoptosis and the cell cycle, or by mediating proliferation, whereas STAT1 is thought to function as a tumor suppressor (33-35). Our data show a constitutive expression of both proteins in the cell lines analyzed with higher expression levels of STAT3. Since STAT1 activation is increased by PMA stimulation in contrast to STAT3, there is evidence that the tumor promoter STAT3 is more potent compared to STAT1.

Our results give evidence for a regulatory mechanism of p38 MAPK in STAT3- induced IL-6 and IL-8 expression and confirms the tumor promoting function of this MAPK in HNSCC. These data indicate that phosphorylation of STAT3 tyrosine and serine residues proceeds independently. Phosphorylation of tyrosine 705 might be the crucial factor for a full activation of STAT3 in HNSCC, as silencing of p38 MAPK resulted in decreased tyrosine 705 phosphorylation and IL-6 and IL-8 secretion. Our data confirm previous studies of STAT3 as a potential target in tumor therapy (36).

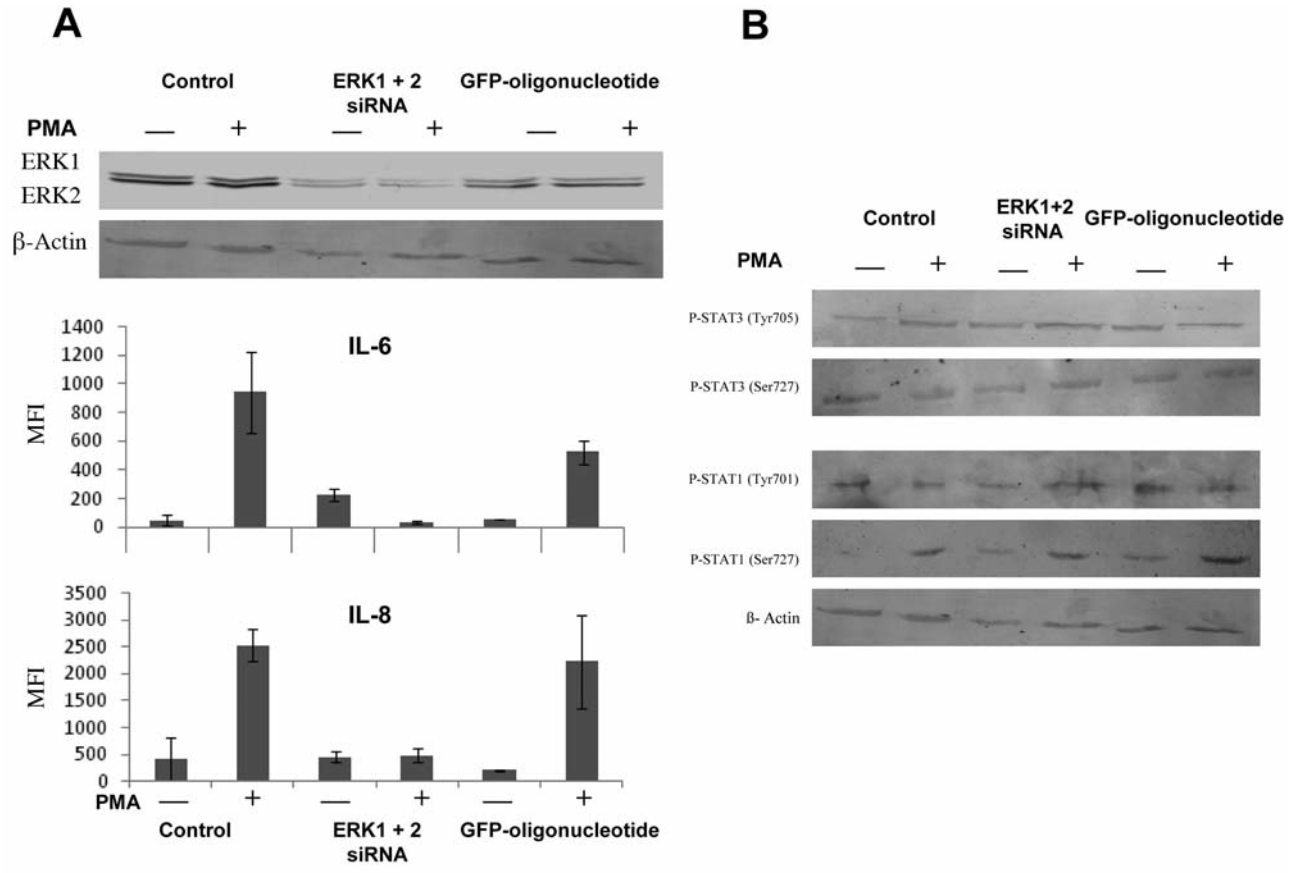

Figure 3. *The role of the ERK1/2 MAPK pathway in HNSCC. A: In HNSCC cell lines, the expression of ERK1/2 was down-regulated by siRNA. Analysis of cell culture supernatants revealed suppression of interleukin 6 (IL-6) and IL-8 expression due to ERK1/2 silencing. B: Western blot analysis of proteins after ERK1/2 silencing show no effect of ERK1/2 silencing on levels of STAT3 and STAT1 phosphorylation.*

STAT1 expression levels were not affected in response to p38 and ERK1/2 silencing. Therefore a different pathway results in STAT1 overexpression and further investigations will have to elucidate the function of STAT1 in HNSCC.

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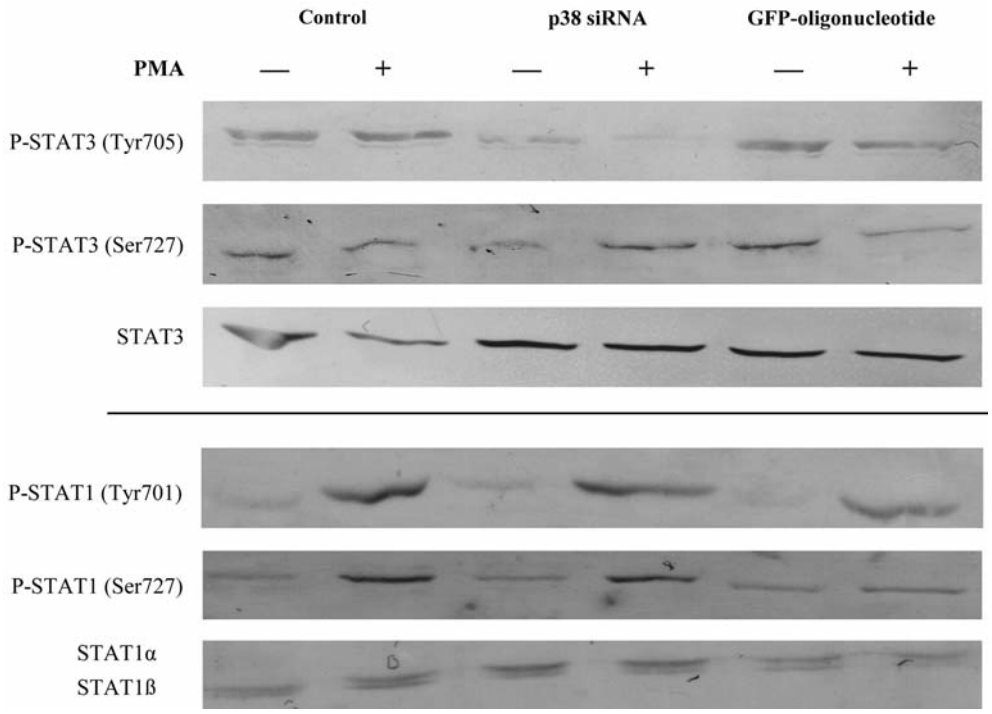

Figure 4. *STAT3 tyrosine phosphorylation is regulated by p38 MAPK signalling. Western blot analysis show a distinct down-regulation of phosphorylated tyrosine 705 residue of STAT3 after silencing of p38 MAPK. In contrast, STAT3 serine phosphorylation of STAT3 as well as STAT1 phosphorylation, is not influenced by this signaling pathway. In HNSCC cell lines, STAT1 is activated by PMA (200 ng/ml) where STAT3 is constitutively expressed.*

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