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Original Article

Signal transducer and activator of transcription 3 upregulates interleukin-8 expression at the level of transcription in human melanoma cells

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Abstract: Many melanoma cells continuously produce interleukin-8 (IL-8). The involvement of signal transducer and activator of transcription 3 (STAT3) in the constant production of IL-8 in melanoma cells was examined. The level of IL-8 production correlated well with that of the phosphorylated (activated) STAT3 in six human melanoma cell lines. Introduction of the constitutively activated form of STAT3 (STAT3-C) into WM35 melanoma cells, that show low levels of IL-8 and phosphorylated STAT3, enhanced IL-8 production. Knockdown of STAT3 suppressed IL-8 production in WM1205Lu cells that contain a high level of IL-8 accompanied by STAT3 phosphorylation. Introduction of STAT3-C markedly increased the luciferase activity in WM1205Lu cells transfected with reporter vectors linked to the 5′-flanking region of the IL-8 gene from -546 to +44 base pair (bp) and from -272 to +44 bp, but not in cells expressing reporter plasmids from -133 to +44 bp and from -98 to +44 bp. These results indicate that the upregulation of IL-8 production is caused by constitutive STAT3 activation at the level of gene transcription in melanoma cells.

Key words: IL-8 – luciferase assay – melanoma – STAT3 – transcription

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Introduction

Malignant melanoma is one of the most lethal types of cancer and its rate of incidence has been rising for decades (1,2). The major reason for the poor prognosis of melanoma seems to be the rapid progression of the invasive and metastatic properties of the cancer cells. Although extensive studies have been carried out on the mechanisms by which melanoma cells develop the highly malignant phenotype, our knowledge on the properties of melanoma cells is still limited. One of the characteristics of the transformation from normal melanocytes to melanoma cells is that these cancer cells acquire the ability to produce their own growth factors, cytokines, and chemokines to form autocrine and/or paracrine stimulatory loops (3,4). Interleukin (IL)-8, a member of the chemokine superfamily, is one of these mediators, and has a potent chemotactic and activating effect on neutrophils and T cells, respectively, (5,6), as well as strong angiogenic activity (7,8). In normal human melanocytes, similar to other normal cell types (5), the production of IL-8 is not continuous but is induced by stimulation with proinflammatory cytokines such as IL-1 and tumor necrosis factor-α (TNF-α) (9). The 5′-flanking region of the IL-8 gene contains several potential binding sites for known transcription factors such as AP-1, NF-IL6 and NF-κB, and the induction of IL-8 by IL-1 and TNF-α can be ascribed, at least partly, to the activation of these transcription factors (10). In contrast, the continuous

Abbreviations: EMEM, Eagle's minimal essential medium; IL, interleukin; STAT3, signal transducer and activator of transcription 3; TNF-α, tumor necrosis factor-α.

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production of this chemokine has been observed in many cultured human melanoma cells (9,11–13) and melanoma specimens (14,15). Moreover, the serum IL-8 level is elevated in patients with metastatic melanoma (16). Therefore, many researchers have been investigating the role of IL-8 in melanoma cells. The mRNA level of IL-8 in melanoma cells correlates directly with their metastatic potential as evaluated by implantation in nude mice (17). Ultraviolet B irradiation of cultured SB-2 melanoma cells, which do not secrete a detectable level of IL-8 or form tumors in nude mice, promotes their tumorigenic and metastatic properties through induction of IL-8 (18). Ultraviolet B irradiation also enhances melanoma cell motility via induction of autocrine IL-8 secretion (19). Furthermore, it has been shown that overexpression of IL-8 in melanoma cells enhances their tumorigenicity and metastatic potential in mice (20,21). Thus, it appears that continuously produced IL-8 plays crucial roles in melanoma progression and metastasis. It has been reported that capsaicin, an inhibitor of NF-κB, inhibits, only partially, the continuous production of IL-8 in melanoma cells (22), suggesting that not only NF-κB but also other factor(s) participate in the regulation of the continuous expression of IL-8 in melanoma cells. Detailed mechanisms for continuous IL-8 production in melanoma cells, however, have not been fully elucidated.

Signal transducer and activator of transcription (STAT) 3 was originally identified as a DNA-binding protein that responds to stimulation by epidermal growth factor and IL-6 (23,24), and it has been established that STAT3 has an important role in the signalling of these physiologically active substances (24,25). Similar to other STATs, STAT3 is activated by a phosphorylation reaction at a critical tyrosine residue (Tyr705) by JAK2 in response to cytokine stimulation. This is followed by dimerization via a reciprocal interaction between its Src homology 2 domain and the phosphorylated tyrosine residue (26). The dimeric STAT3 translocates to the nucleus, where it binds to defined DNA element(s) within the promoter region of the target genes to activate their transcription (26–28). In normal cells, the duration of STAT3 activation is transient, and usually lasts from a few minutes to several hours (29). In these cells, STAT3 plays crucial roles in the development of various organs and in cell proliferation (30). In contrast, constitutive activation of STAT3 has been observed in many kinds of tumors (31) including melanoma (32), and this constitutively activated STAT3 is thought to contribute to oncogenesis by modulating the expression of a variety of genes (31).

In this study, we examined the involvement of constitutively activated STAT3 in IL-8 production in human melanoma cells, and found that IL-8 production is directly regulated by STAT3 at the transcription level in melanoma cells. Furthermore, we determined the 5′-flanking promoter region of the human IL-8 gene responsible for its production, which contains a putative STAT3-binding site.

Methods

Cells and cell culture

Human melanoma cell lines from primary lesions (WM35, WM98-1 and WM115) and metastatic lesions (WM164, WM239A and WM1205Lu) were kindly provided by Dr Meenhard Herlyn (The Wistar Institute, Philadelphia, PA, USA). The detailed character of these melanoma cells has been described previously (33). The melanoma cells were cultured in Eagle's minimal essential medium (EMEM) containing 5% FCS (Biowest, Miami, FL, USA), 100-U/ml penicillin, 100-μg/ml streptomycin and 250-ng/ml fungizone in a humidified atmosphere containing 5% CO_2 at 37°C. Where indicated, melanoma cells were infected with adenovirus vectors, introduced by small interfering RNA (siRNA), or transfected with reporter plasmids, and then further cultured as described under each experiment.

Adenovirus vectors

The recombinant adenovirus vector encoding the constitutively active form of STAT3 (STAT3-C) was prepared as described previously (34) and was designated AxSTAT3-C. The adenovirus carrying the β-galactosidase gene (LacZ) from *Escherichia coli* designated AxLacZ was provided by Dr Izumu Saito (University of Tokyo, Tokyo, Japan) and used as a control.

STAT3 siRNA

STAT3 siRNA experiments were performed with double-stranded siRNAs against STAT3 (si-1: 5′-AAC UUC AGA CCC GUC AAC AAA dTdT-3′; 3′-dTdT GAA GUC UGG GCA GUU GUU U-5′ and si-2: AAC AUC UGC CUA GAU CGG CUA dTdT-3′ 3′-dTdT GUA GAC GGA UCU AGC CGA U-5′) and the scrambled siRNA for negative control (TaKaRa, Shiga, Japan) using TransIT-TKO transfection reagent (Mirus, Madison, WI, USA) according to the manufacturer's instructions.

Quantitative real-time PCR

Total cellular RNA isolation using Trizol (Invitrogen, Carlsbad, CA, USA) and cDNA synthesis were performed as described previously (35). Quantitative real-time PCR (qRT-PCR) was carried out using the SYBR Premix Ex Taq^{TM} II kit (Takara Bio Inc., Kyoto, Japan) with the Thermal Cycler Dice Real Time System (Takara Bio Inc.). Relative mRNA levels were determined using the comparative Ct method followed by normalizing to the β-actin mRNA level in each cDNA sample. The sequence of the primers were 5′-CAAGAGCCAGGA-AGAAACCA-3′ and 5′-GATAAATTTGGGGTGGAAAG-3′

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for IL-8 and 5′-ATGAAGATCAAGATCATTGCTCCTC-3′ and 5′-ACATCTGCTGGAAGGTGGACAG-3′ for β-actin.

Immunoblot analysis

Immunoblot analysis was carried out essentially as described (36). Briefly, the cells were washed with phosphate buffered saline and lysed in 20-mM Tris–HCl (pH 7.5) containing 1-mM EDTA, 1-mM EGTA, 10-mM 2-mercaptoethanol, 1% Triton X-100, 150-mM NaCl, 10-mM NaF, 1 mM sodium orthovanadate and 50-μg/ml phenylmethylsulfonyl fluoride for 30 min. Cell lysates were then centrifuged at 17 000 ***g*** for 10 min. Protein concentrations of the supernatants were measured using Coomassie plus protein assay reagent (Pierce, Rockford, IL, USA). Equal amounts of protein samples were separated by SDS/PAGE and transferred onto an Immobilon P membrane (Millipore, Bedford, MA, USA). The membranes were blocked with phosphate buffered saline containing 5% skim milk and 0.05% Tween 20 for 30 min at room temperature, probed with each primary antibody against STAT3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phosphorylated STAT3 (phospho-STAT3, Cell Signalling Technology, Beverly, MA, USA), and actin (Santa Cruz), and then followed by incubation with peroxidase-conjugated anti-rabbit antibody (Promega, Madison, WI, USA). The oxidative reaction by peroxidase was detected using Enhanced Chemiluminescence Western Blotting Detection Reagents (Amersham Bioscience, Piscataway, NJ, USA).

IL-8 determination

The IL-8 protein contents in the culture medium were measured using the Human IL-8 ELISA Kit (Biosource, Camarillo, CA, USA) according to the manufacturer's instruction.

Luciferase assay

Luciferase reporter plasmids containing serially deleted 5′-flanking regions of the human IL-8 promoter spanning from -546 base pair (bp), -272 bp, -133 bp, and -98 bp to +44 bp were subcloned into the luciferase expression vector as described (37), and designated as -546-luc, -272-luc, -133-luc and -98-luc, respectively. WM1205Lu cells were seeded into 24-well plates at a density of 4×10^4 cells/well and cultured overnight. The cells were transfected with 0.2 μg of each reporter plasmid DNA along with the β-galactosidase expression vector as an internal control (38) using the FuGENE 6 transfection reagent (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instruction. Transfected cells were incubated for 24 h, and then infected with or without 10 plaque-forming units/cell of AxSTAT3-C. Thereafter, the cells were further cultured for 24 h. The luciferase activity in cell lysates was determined using a Pica Gene kit (Toyo Ink Co., Tokyo, Japan) and a Fluoroskan Ascent FL fluorometer (Labsystems Inc., Franklin, MA, USA) (37).

Results

Correlation of the levels of IL-8 secretion and STAT3 activity in human melanoma cells

As an initial step in the study of the involvement of STAT3 in IL-8 production in melanoma cells, the expression of tyrosine-phosphorylated (activated) STAT3 and the secretion of IL-8 in six human melanoma cell lines, including WM35, WM98-1, WM115, WM164, WM239A and WM1205Lu, were examined by immunoblot analysis and ELISA, respectively (Fig. 1). All of the cells contained similar amounts of the STAT3 protein, although the level of tyrosine phosphorylation of this protein was different among the melanoma cell lines (Fig. 1a). The level of tyrosine-phosphorylated STAT3 as well as the relative ratio of tyrosine-phosphorylated STAT3/total STAT3 in WM35 cells were markedly lower than in the other five cell lines (Fig. 1a,b). The amount of IL-8 produced varied among these melanoma cell lines (Fig. 1c). Specifically, WM98-1 and WM1205Lu cells produced high levels of IL-8, whereas WM35 and WM115 cells expressed low levels of this chemokine. Importantly, the relative ratio of tyrosine-phosphorylated STAT3/total STAT3 correlated well with that of the IL-8 secretion. These data led us to investigate whether STAT3 regulates IL-8 production in the melanoma cells.

STAT3 activity regulates IL-8 secretion in melanoma cells

To directly evaluate the role of STAT3 in IL-8 production, the effects of the constitutively active form (34) and siRNAs of STAT3 on IL-8 production were examined in WM35 and WM1205Lu cells, as representatives of the cells producing low and high levels of IL-8, respectively (Fig. 2). The IL-8 secretion in WM35 cells was potentiated approximately nine times by infection with AxSTAT3-C, the recombinant adenovirus vector encoding the STAT3-C, compared with that with AxLacZ, the adenovirus carrying the β-galactosidase gene (LacZ) from *E. coli* (Fig. 2a, top panel). IL-8 mRNA was markedly upregulated by infection with AxSTAT3-C (Fig. 2a, second panel). siRNA-mediated knockdown of STAT3 resulted in a marked reduction in IL-8 production (Fig. 2b, top panel) as well as IL-8 mRNA (Fig. 2b, second panel). These results suggest that IL-8 production is regulated at the level of transcription by STAT3 activity in the melanoma cells.

Transactivation of IL-8 promoter by STAT3

To investigate whether STAT3 transcriptionally regulates IL-8 expression, the luciferase assay was carried out using serially deleted 5′-flanking regions of the human IL-8 gene

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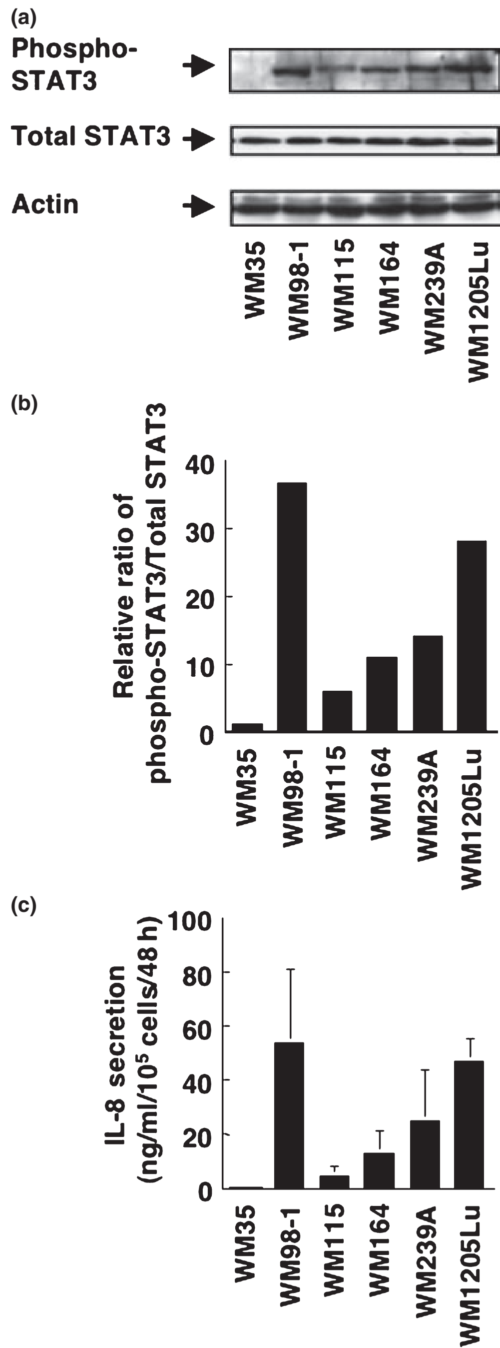

Figure 1. Activation of STAT3 and secretion of IL-8 in human melanoma cell lines. (a) Whole-cell extracts from six melanoma cell lines were prepared, and the phosphorylation and expression of STAT3 were determined by immunoblot analysis using anti-phospho-STAT3 (upper panel) and anti-STAT3 (middle panel) antibodies, respectively. Expression of actin was also examined (lower panel). (b) The relative ratio of phospho-STAT3/total STAT3 was calculated using the value from WM35 as 1. The results shown are representative of three independent experiments. (c) The culture media were recovered after 48-h culture of six melanoma cell lines, and the IL-8 protein was quantitated by ELISA. Data were obtained in three independent experiments and are shown as mean ± SD.

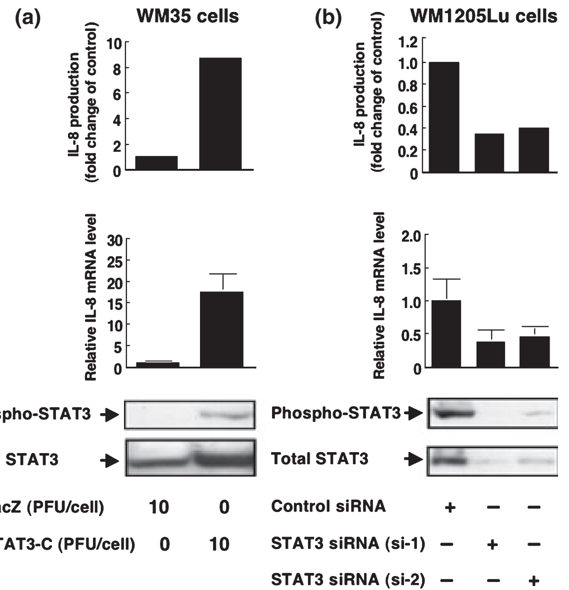

Figure 2. Effects of overexpression of STAT3-C by the adenovirus vector or knockdown of STAT3 with siRNA on IL-8 production. (a) WM35 melanoma cells were infected with 10 PFU/cell of either AxSTAT3-C or AxLacZ, and the cells were harvested after incubation for 72 h. The IL-8 protein in the culture supernatants (top panel) was quantitated by ELISA, and the IL-8 mRNA level (second panel) was quantitated by qRT-PCR. The results are expressed as the fold change using the value of the uninfected cells as 1. Whole-cell extracts from melanoma cells were prepared, and the expression of phosphorylated STAT3 protein (third panel) as well as of total STAT3 protein (fourth panel) was determined by immunoblot analysis as in Fig. 1. (b) Transfection of control siRNA (40 nM) or STAT3 siRNAs (40 nM) was performed in WM1205Lu cells. Cells were harvested 72 h after transfection. IL-8 protein (top panel), IL-8 mRNA (second panel), phosphorylated STAT3 (third panel) and total STAT3 (fourth panel) were quantitated as in (a). The results of IL-8 secretion and STAT3 are representative of three independent experiments.

ligated to the luciferase expression plasmid in WM1205Lu cells (Fig. 3). The 5′-flanking region of the IL-8 gene contains several potential binding sites for known nuclear factors such as AP-1, NF-IL-6 and NF-κB (10) (Fig. 3a). In addition, a putative STAT3-binding site sequence, TTCACCAAA (-245 to -237 bp), is also present in the 5′-flanking region of the IL-8 gene. Infection of AxSTAT3-C markedly increased the luciferase activity in WM1205Lu cells transfected with the -546-luc or -272-luc vector, but not with the -133-luc or -98-luc vector (Fig. 3b). This indicates that the induction of the IL-8 production by STAT3 occurs at the transcriptional level and that the minimally essential elements for IL-8 gene regulation by STAT3 activation are present from -272 to -133 bp of the promoter region.

Discussion

In the present study, we have demonstrated that STAT3 plays a crucial role in the continuous production of IL-8

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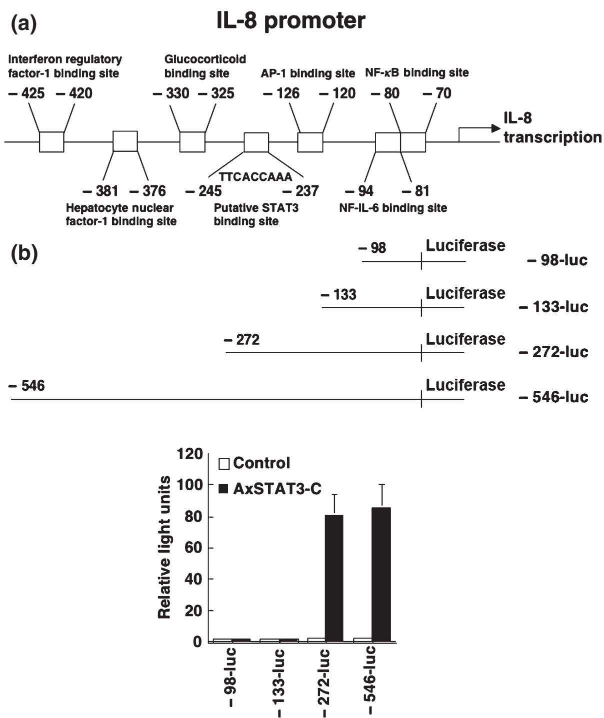

Figure 3. STAT3-induced IL-8 transcription. (a) Potential binding sites for known nuclear factors in the IL-8 promoter (-1481 to +44 bp) as well as the putative STAT3-binding site (- 245 to -237 bp) in the IL-8 promoter region are shown. (b) -546-luc, -272-luc, -133-luc and -98-luc luciferase expression vectors were constructed as shown and co-transfected with β-galactosidase serving as an internal control into WM1205Lu cells. Twenty-four hours later, the cells were infected with AxSTAT3-C and incubated for 24 h. The relative promoter activity in the cell lysates of the infected and control cells was determined using the luciferase assay. The results shown are representative of three independent experiments and are shown as mean ± sd.

by transcriptional regulation in human melanoma cells. Various types of cells can rapidly produce IL-8 upon stimulation with inflammatory stimuli such as lipopolysaccharide, IL-1 and TNF-α (5). However, the IL-8 production by these stimuli is tightly regulated primarily at the transcriptional level to prevent its aberrant production (10,39). The 5′-flanking region of the IL-8 gene contains DNA-binding sites for several transcription factors such as AP-1 (-126 to -120 bp), NF-IL-6 (-94 to -81 bp) and NF-κB (-80 to -70 bp) (10) (Fig. 3a). Besides these three binding sites, additional potential binding sites have been identified for known nuclear factors such as interferon regulatory factor-1 (-425 to -420 bp) and hepatocyte nuclear factor-1 (-381 to -376 bp) as well as for glucocorticoid-responsive element (-330 to -325 bp) (40). Upregulation of IL-8 gene expression by external stimuli is mediated through activation of NF-κB alone or cooperative activation of NF-κB with other transcription factors depending on the type of cells and stimuli (10). In addition, it has been shown recently that small fragments of the extracellular matrix component hyaluronic acid increases IL-8 expression through toll-like receptor 4 signalling (41).

Although the IL-8 expression induced by external signals has been extensively studied as described above, the regulation mechanism of the continuous production of IL-8 in cells has not been fully elucidated. Our present study demonstrated that STAT3 is an important regulator of the continuous production of IL-8, and indicated that STAT3 regulates IL-8 production at the transcriptional level presumably through its direct interaction with the IL-8 promoter region spanning from -272 to -133 bp. A putative STAT3-binding site sequence TTCACCAAA, which fits with the consensus sequences TT(N4/N5)AA (42), was identified from -245 to -237 bp in the 5′-flanking region of the human IL-8 gene (Fig. 3a). It would be interesting to know whether this TTCACCAAA sequence in the IL-8 promoter is the functional site for STAT3-dependent IL-8 production. It has been reported that NF-κB participates in the regulation of the continuous expression of IL-8 in melanoma cells (22). We postulate that continuous IL-8 production is regulated by the cooperative activation of NF-κB and other transcription factors such as STAT3 in melanoma cells in a manner similar to the case of external stimuli-induced IL-8 production.

Our data support recent reports showing that IL-8 is a target gene of STAT3. Trevino et al. (43) indicated that STAT3 activation by c-Src is required for IL-8 expression in PANC-1 pancreatic cells. Gharavi et al. (44) demonstrated that JAK2 activation by oxidized phospholipids induces IL-8 transcription through STAT3 activation in human endothelial cells. In addition, they suggest that either STAT3 alone is sufficient or a different co-activator such as NF-κB is required with STAT3 to stimulate IL-8 expression as discussed above. However, these reports did not examine whether STAT3 regulates IL-8 production directly at the transcriptional level. Here, we provide evidence that IL-8 production is directly regulated at the transcriptional level by STAT3. In contrast to these reports and the present data, indicating that IL-8 production is positively regulated by STAT3, de la Iglesia et al. (45) reported negative regulation of IL-8 production by STAT3. They show that activated STAT3 occupies the endogenous IL-8 promoter and directly represses IL-8 transcription in U87 glioblastoma cells, and that the deficiency of tumor suppressor phosphatase and tensin homolog, which is often observed in glioblastoma, induces IL-8 upregulation through inactivation of STAT3. Analyses employing different cells are needed to clarify the role of STAT3 in the transcriptional regulation of IL-8.

As IL-8 plays crucial roles in the progression and metastasis of melanoma, the treatment of melanoma using anti-IL-8 antibody has been proposed (46). Furthermore, it has

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been revealed that, in addition to IL-8, the expression of matrix metalloproteinase-2, basic fibroblast growth factor and vascular endothelial growth factor, all of which contribute to tumor invasion and metastasis, is upregulated by STAT3 in melanoma cells (47,48). Namely, STAT3 appears to be at the crossroads of the signalling pathway of many growth factors and chemokines which contribute to aggressive melanoma behaviour. Therefore, it is conceivable that STAT3 is an attractive target for future melanoma therapies. Elucidation of the exact regulation mechanism of constitutive activation of STAT3 will help such strategies.

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