STAT5 Phosphorylation in Malignant Melanoma Is Important for Survival and Is Mediated Through SRC and JAK1 Kinases

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Altered signaling pathways are key regulators of cellular functions in tumor cells. Constitutive activation of signal transducer and activator of transcription (STAT)3 and -5 may be involved in tumor formation and progression. We have investigated the role of STAT5 in cutaneous melanoma metastases using various RNA and protein techniques. In melanoma specimens, *Stat5b* transcripts were upregulated approximately 3.8-fold. In 13 of 21 (62%) human melanoma metastases, STAT5 was phosphorylated in comparison to normal human melanocytes and benign nevi. The STAT5 target gene *Bcl-2* was frequently upregulated. The investigation of the underlying mechanism revealed specific STAT5 activation by recombinant human epidermal growth factor (rEGF). rEGF-induced activation of STAT5 occurred *in vitro* through the non-receptor tyrosine kinases transforming gene (src) of Rous Sarcoma virus and Janus kinase 1. Inhibition of *Stat5b* expression by small interfering RNA strongly reduced the expression of Bcl-2 and led to decreased cell viability and increased apoptosis in the melanoma cell lines A375 and BLM. Transfection with dominant-negative *Stat5b* caused enhanced cell death and G1 arrest in A375 cells. Our study identifies phosphorylated STAT5 in melanoma and shows regulation through rEGF; STAT5 may thus act as a survival factor for growth of human melanoma and may represent a potential target for molecular therapy.

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INTRODUCTION

Signal transducers and activators of transcription (STATs) represent a family of transcription factors that are located in the cytoplasm and are activated by a variety of different stimuli such as cytokines, growth hormones, and various tumor promoters, for example, 12-*O*-tetradecanoylphorbol-13-acetate, ocadaic acid, and chrysarobin (Darnell, 1997; Chan *et al.*, 2004). Upon binding of the ligand to the corresponding receptor, receptors become phosphorylated by receptor-associated tyrosine kinases like Janus kinase (JAK) (Darnell *et al.*, 1994). Receptors with intrinsic tyrosine kinase activity such as the EGFR are able to autophosphorylate tyrosine residues and interact directly with STAT proteins (Coffer and Kruijer, 1995). Recent studies have demonstrated the activation of STATs by diverse proto-oncogenic non-receptor tyrosine kinases like v-SRC (Garcia *et al.*, 1997), LYN (Chin *et al.*, 1998), BCR/ABL (Carlesso *et al.*, 1996), and LCK (Lund *et al.*, 1999). The recruitment of STAT molecules with receptor or non-receptor tyrosine kinases occurs via a conserved SRC (transforming gene (src) of Rous Sarcoma virus)-homology-2 domain (Rosen *et al.*, 1995). Upon homo- or heterodimerization phosphorylated STATs translocate to the nucleus and bind to STAT-specific DNA-response elements of target genes and induce gene expression (Darnell, 1997). STAT target genes are involved in the control of different biological processes such as proliferation, apoptosis, survival, differentiation, and angiogenesis (Darnell, 1997; Levy and Darnell, 2002; Chan *et al.*, 2004). Constitutive activation of STAT3 has been linked to oncogenic transformation, tumor formation, and progression (Bowman *et al.*, 2000; Calo *et al.*, 2003; Yamashita *et al.*, 2003; Li *et al.*, 2004) in esophageal cancer (Leu *et al.*, 2003), gastrointestinal stroma tumors (Duensing *et al.*, 2004), melanoma (Niu *et al.*, 2002a), and multiple myeloma (Chatterjee *et al.*, 2004).

While STAT5 plays a key role in the regulation of hematopoietic differentiation and stem cell function (Darnell, 1997; Kyba *et al.*, 2003; Chan *et al.*, 2004), it has only recently been discovered to be involved in malignant transformation of hematological malignancies (Lin *et al.*, 2000), breast (Yamashita *et al.*, 2003), and prostate cancer (Li *et al.*, 2004) as well as human non-small-cell carcinoma (Xi *et al.*, 2003).

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Abbreviations: ERK, extracellular signal-regulated kinase; GFP, green fluorescent protein; JNK, Janus kinase; NHM, normal human melanocyte; rEGF, recombinant human epidermal growth factor; siRNA, small interfering RNA; STAT, signal transducers and activators of transcription

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Inhibition of STAT3 signaling in tumor cells induced apoptosis and/or growth arrest *in vitro* and *in vivo* (Niu *et al.*, 2001). For example, dominant-negative STAT3 in human myeloma cells downregulated the expression of the anti-apoptotic gene *Bcl-2*, resulting in sensitization of cells towards Fas-mediated apoptosis (Catlett-Falcone *et al.*, 1999; Grandis *et al.*, 2000). STAT3 has also been found activated in murine melanoma leading to an upregulation of Bcl-2 and c-MYC (Niu *et al.*, 1999, 2002a).

The potential impact of STAT5 as a point of convergence in melanoma has remained unexplored. In this study, we examined STAT5 in human melanoma specimens and investigated the activation of STAT5 by the EGFR tyrosine kinase as well as by the non-receptor tyrosine kinases JAK1 and SRC *in vitro*. To elucidate the functional effects of STAT5 in melanoma, *Stat5b* expression was inhibited by small interfering RNA (siRNA) experiments leading to decreased Bcl-2 and increased apoptosis. When a dominant-negative human *Stat5b* construct was expressed in melanoma cell lines, enhanced cell death and G1-arrest were also observed.

RESULTS

Activation of STAT5 in human melanoma metastases

To investigate a possible role of STAT5 in melanoma, we examined the transcription and protein expression levels as well as the activation of STAT5 in human melanoma metastases using real-time PCR, Western blot analysis, and immunohistochemistry (Figures 1 and 2). The expression of *Stat5b* mRNA was 3.8-fold upregulated compared to normal human melanocyte (NHM) (Figure 1a; $P=0.0002$). Furthermore, STAT5 protein was constitutively expressed in melanoma samples and benign nevi, but very weakly in NHM (Figure 1c and d), with STAT5 being phosphorylated in 13 of 21 (62%) analyzed melanoma specimens in contrast to NHM (Figure 1b). In addition, we investigated the tyrosine phosphorylation of STAT5 in different melanoma cell lines in cytoplasmatic as well as in nuclear fractions (Figure 1d). P-STAT5 was present in low amounts in cytoplasmatic extracts of BLM, M13, and MV3, but was absent in the A375 human melanoma cell line (Figure 1d). The immunohistochemical data are summarized together with clinical information in Table 1. Immunohistochemically, P-STAT5 was also highly abundant in 13 of 23 (56%) analyzed metastases (Figure 2c and d), whereas unphosphorylated STAT5 showed constitutive levels (Figure 2a and b). Cutaneous melanoma metastases showed nests of melanoma cells with predominant cytoplasmatic and little nuclear STAT5 staining detectable upon higher magnification (Figure 2b). A corresponding section showed more intense staining with P-STAT5, where most cells contained cytoplasmatic and pronounced nuclear staining (Figure 2d). In total, two of four primary melanoma and 11 of 19 cutaneous metastases showed activated STAT5 in histological sections (Table 1). In consecutive sections of primary melanoma, some P-STAT5-positive melanoma cells also stained with an EGFR antibody (Figure 2e and f).

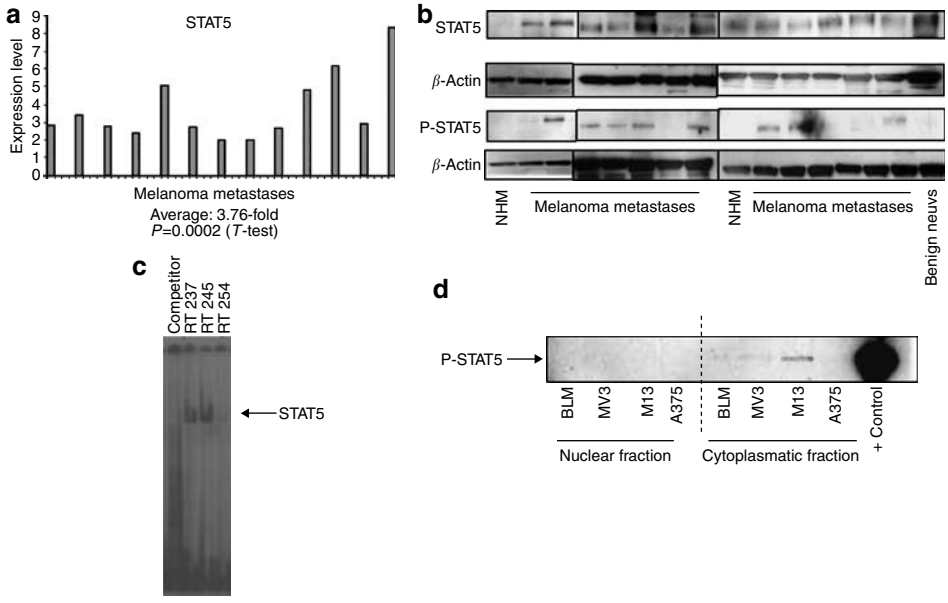

Figure 1. Expression analysis of STAT5. (**a**) Real-time PCR analysis shows upregulation of *stat5b* transcripts in 13 human melanoma specimens. Bar graph shows expression of *stat5b* in melanoma lesions relative to NHM. The expression of *stat5b* was on average 3.8 times higher compared to NHM ($n=3$; *t*-test; $P=0.0002$). Variation coefficients for C_T values for triplicate reactions were less than 1% for all gene products (data not shown). (**b**) Expression of STAT5 and P-STAT5 in melanoma specimens by Western blot analysis. P-STAT5 was present in 13 of 21 (62%) metastatic melanomas, whereas it was absent in NHM; depicted are seven representative samples. STAT5 protein was present in all melanoma samples while it was low in NHM. Equal protein loading was confirmed by staining for β-actin. (**c**) Electrophoretic mobility shift assay to demonstrate STAT-5 in two of three tested clinical samples (**d**) P-STAT5 Western blot analysis from cytoplasmatic and nuclear fractions of different melanoma cell lines (BLM, MV3, M13, and A375). P-STAT5 was absent in the nuclear fraction of all four analyzed cell lines. Basic levels of activated STAT5 were present in cytoplasmatic extracts of M13 cells and were very low in BLM, and MV3 cells. In the A375 cell line, P-STAT5 was absent in both cytoplasmatic and nuclear extracts.

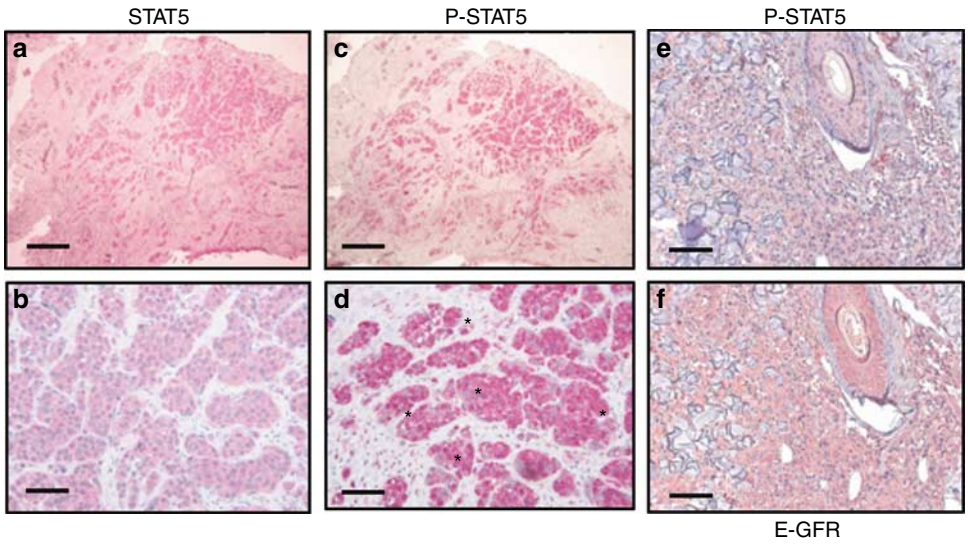

Figure 2. Immunohistochemistry of STAT5, P-STAT5 and EGF-R. Immunohistochemically, a representative sample of a (**a**) metastatic melanoma showed, upon higher magnification, (**b**) predominant cytoplasmatic STAT5 staining. (**c**) A corresponding section showed intense staining with P-STAT5 . Upon higher magnification, most cells contained cytoplasmatic and pronounced nuclear staining (* in **d**). Of note, some tumor cells were negative for P-STAT5. (**e**) Some P-STAT5-positive melanoma cells also stained with an EGFR antibody in consecutive sections of (**f**) primary melanoma. The appropriate isotype controls showed no staining (data not shown). Bar = 50 μm in (**a**) and (**c**) and 25 μm in (**b**) and (**d**)–(**f**), respectively.

Analysis of STAT5 target genes

As activated STAT5 induces antiapoptotic signals, we analyzed the regulation of several STAT5 target genes involved in apoptosis. The antiapoptotic protein Bcl-2 was consistently detectable in all tumor specimens, melanoma cell lines as well as in NHM (Figure 3).

Correlation of STAT5 activation with EGFR expression

STAT proteins are activated by different receptors in a cell- and ligand-specific manner. As we have previously shown the responsiveness of NHM and A375 cells to epidermal growth factor (EGF) leading to phosphorylation of STAT5 (Mirmohammadsadegh *et al.*, 2005) (Figure 4a), we investigated the levels of EGF receptors on these cell lines. By Western blot analysis, EGFR levels were high on NHM, A375, and HaCaT cells, whereas they were low in BLM, MV3, and absent in M13 (Figure 4b), respectively. The expression of EGFR correlated with the activation of STAT5 induced by EGF in A375 cells (Figure 4c). Phosphorylation of STAT5 occurred after 5 minutes and lasted for up to 1 hour. As we have previously shown high levels of EGFR in melanoma metastases and primary melanoma in comparison with benign nevi (Mirmohammadsadegh *et al.*, 2005). A375 cells seemed to be the best model of clinical melanoma and were consequently chosen for further experiments.

Activation of STAT5 is mediated by SRC and JAK1 tyrosine kinases

To characterize the recombinant human epidermal growth factor (rEGF)-induced STAT5 activation, we investigated the role of SRC and JAK tyrosine kinases. As shown in Figure 4c, JAK1 and SRC were activated by rEGF after 1 minute, whereas JAK2 was weakly phosphorylated after 30 minutes. Chemical inhibition of JAKs by AG490 and SRC by peroxisome proliferator (PP) 1 and PP 2, respectively, blocked EGF-stimulated activation of STAT5 (Figure 4d), suggesting that both JAKs and SRC are required for maximal activation of STAT5 in human melanoma. In addition, immunoprecipitation with an EGFR antibody in A375 cells treated with rEGF revealed an interaction of the EGFR with P-SRC (Figure 4e). Furthermore, under the same conditions, immunoprecipitation with P-SRC showed P-STAT5 immunostaining, suggesting a direct interaction between both molecules (Figure 4f). Together, these data suggested that upon rEGF stimulation, STAT5 is directly phosphorylated by SRC.

Influence of rEGF on STAT5 DNA-binding activity

Activation of STATs induces homo- or heterodimerization and translocation to the nucleus, resulting in transcription of STAT target genes. In order to analyze this event, we stimulated A375 cells with rEGF and utilized cytoplasmatic and nuclear extracts for the detection of P-STAT5 (Figure 5a). Fifteen minutes after rEGF stimulation, P-STAT5 was present in the cytoplasm and in the nucleus (Figure 5a), whereas under non-stimulated conditions nuclear P-STAT5 was not detected (Figure 1d). To examine the role of STAT5 in the regulation of transcription induced by rEGF, we analyzed STAT5 DNA-binding activity by supershift experiments in the presence of P-STAT3, P-STAT5, extracellular signal-regulated kinase (ERK)1/2, and P-ERK1/2 antibodies (Figure 5b). The nuclear extracts were prepared and assayed for STAT5 DNA-binding activity with a γ-^{32}P-ATP labeled oligonucleotide probe containing STAT5 consensus binding sites. The rEGF-induced DNA-binding complex could be supershifted with anti-P-STAT5 (and weakly with anti-P-STAT3), but not with control antibodies against ERK1/2 and P-ERK1/2,

Table 1. Clinical characteristics

	Sex	Age (years)	Tumor type	Primary tumor site	Clark's level	P-STAT5
1	M	68	NM	Leg	IV	+
2	F	83	SSM	Head	IV	–
4	F	72	SSM	Foot	V	+
5	F	75	SSM	Arm	IV	–
3	F	77	Cutaneous metastasis	Leg		+
6	M	80	Cutaneous metastasis	Arm		–
7	M	69	Cutaneous metastasis	Leg		+
8	M	48	Cutaneous metastasis	Leg		–
9	M	62	Cutaneous metastasis	Head		–
10	F	65	Cutaneous metastasis	Leg		+
11	M	52	Cutaneous metastasis	Trunk		+
12	F	71	Cutaneous metastasis	Trunk		+
13	M	68	Cutaneous metastasis	Leg		–
14	M	67	Cutaneous metastasis	Head		+
15	M	71	Cutaneous metastasis	Head		–
16	M	62	Cutaneous metastasis	Helix		–
17	F	89	Cutaneous metastasis	Leg		+
18	F	71	Cutaneous metastasis	Trunk		+
19	M	51	Cutaneous metastasis	Arm		–
20	M	67	Cutaneous metastasis	Leg		+
21	M	85	Cutaneous metastasis	Foot		+
22	M	68	Cutaneous metastasis	Leg		–
23	F	75	Cutaneous metastasis	Arm		+

F: female; M: male; NM: primary nodular melanoma; SSM: primary superficial spreading melanoma.
Important clinical and demographic information is detailed along with the results of P-STAT5 immunohistochemistry staining. For primary tumors, the Breslow index (vertical diameter in mm) and the Clark level (depth of invasion into the skin) are also depicted. Note that 13 of 23 (56%) of melanoma specimens were positive for P-STAT5 by immunohistochemistry. The appropriate isotype controls showed no staining (data not shown).

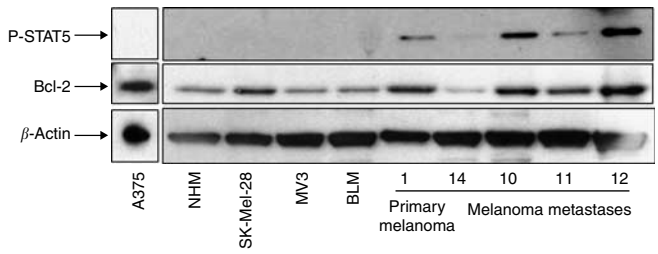

Figure 3. Expression of STAT5 target genes with relevance for apoptosis. The antiapoptotic protein BCL-2 was consistently detectable in all clinical specimens, melanoma cell lines, and in NHM, including A375.

demonstrating the presence of STAT5 protein in these complexes (Figure 5b).

Inhibition of STAT5B reduces Bcl-2 levels and enhances apoptosis

Treatment of A375 and BLM cells with siRNA-Stat5 resulted in efficient and specific inhibition of *Stat5b* transcripts (Figure 6). The siRNA-Stat5 reduced significantly the Bcl-2 and cyclin D2 transcription in A375 cells, two Stat5 target genes with antiapoptotic and proliferative function. These data correlated with the overexpression of Bcl-2 in melanoma metastases (Figure 3). The depletion of *Stat5b* by specific siRNA reduced cell viability after 48 hours as evidenced by a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay (Figure 7a and b). We observed a 21.6% reduction in cell viability by BLM cells and 13.7% by A375 cells compared to mock siRNA. This suggested that STAT5B is involved in transcriptional regulation of genes such as *Bcl-2* that are involved in cell viability. Furthermore, siRNA-Stat5 induced apoptosis in BLM (1.5-fold) and A375 (2.58-fold; $P<0.05$) cells as revealed by 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) FACS staining (Figure 7c and d), which measures the mitochondrial membrane potential, the loss of which is a hallmark of apoptosis. To confirm the JC-1 results suggesting apoptosis, annexinV staining was performed on unstained, non-target and siRNA-Stat5b-treated A375 and BLM cells (Figure 8a and b). As can be seen, upon treatment with siRNA-Stat5 more than 35% of A375 and 65% of BLM cells became annexinV

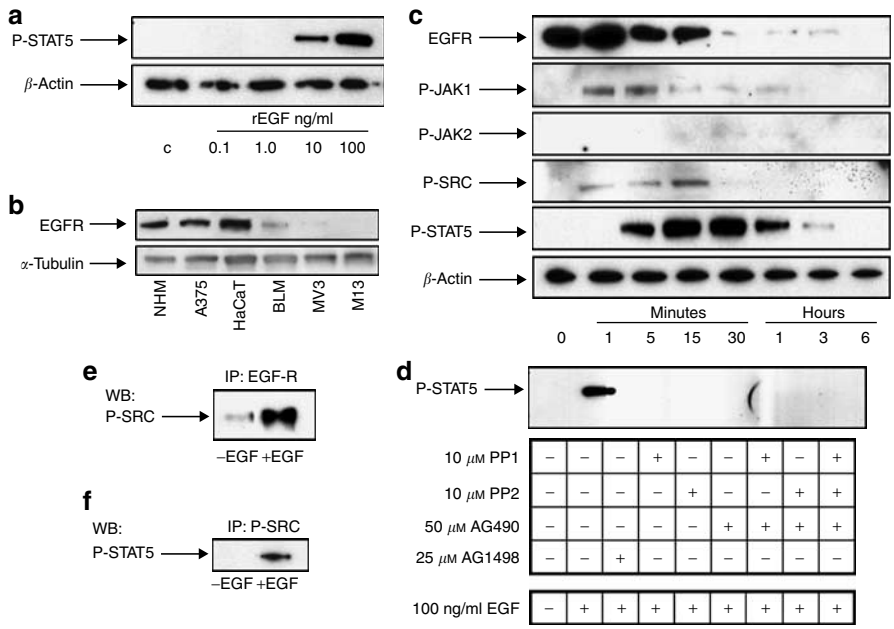

Figure 4. Effect of rEGF on melanoma cell lines. (**a**) A375 cells were treated with increasing doses of rEGF (0.1–100 ng/ml EGF) for 15 minutes leading to STAT5 phosphorylation. (**b**) NHM and various melanoma cell lines (A375, BLM, MV3, and M13) were analyzed for EGFR by Western blot analysis. NHM and A375 cells showed the highest EGFR levels, whereas BLM cells had significantly lower amounts of EGFR. HaCaT cells (immortalized epithelial cell line) were used as positive control. (**c**) Time course of treatment with 100 ng/ml rEGF showing activation of STAT5 after 5 minutes with the maximal activity occurring between 15 and 30 minutes. The activation was absent at 6 hours. The STAT-interacting proteins JAK1 and SRC were phosphorylated after 1 minute. JAK1 activation continued until 1 hour, whereas SRC phosphorylation was absent after 30 minutes. The JAK2 signal was very weak. (**d**) Preincubation of A375 cells with the SRC inhibitors PP1 and PP2 (each 10 μM, 15 minutes before stimulation with 100 ng/ml rEGF) abolished STAT5 phosphorylation. AG490, a JAK inhibitor (50 μM), and AG1478, an EGFR inhibitor (25 μM), were added 3 hours prior to EGF stimulation, respectively. Activation of STAT5 was inhibited by SRC as well as by JAK and EGFR inhibitors, respectively. (**e**) Immunoprecipitation (IP) with the EGFR antibody and Western blot (WB) analysis with a P-SRC antibody before and after rEGF treatment showing a direct interaction. (**f**) IP with a P-SRC antibody followed by immunostaining with a P-STAT5 antibody. Upon rEGF treatment, SRC directly interacts with STAT5.

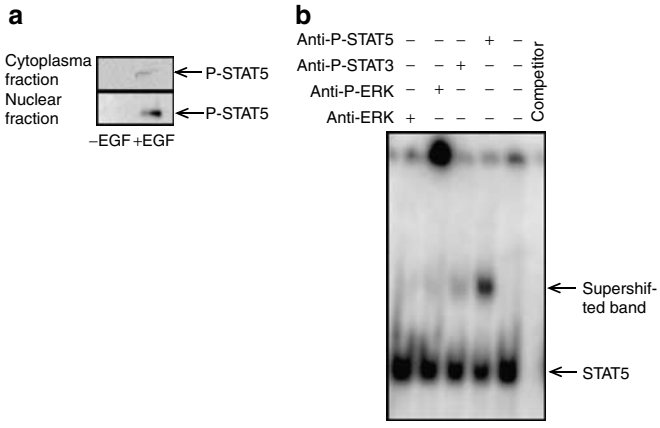

Figure 5. Activation of STAT5 by rEGF and supershift analysis. A375 melanoma cells were stimulated with 100 ng/ml rEGF for 15 minutes when nuclear extraction was performed. (**a**) Western blot analysis of nuclear and cytoplasmatic extracts before and after rEGF treatment confirming the phosphorylation of STAT5. Within 15 minutes, P-STAT5 translocated from the cytoplasm to the nucleus. (**b**) Supershift analysis was performed using nuclear extracts of A375 cells stimulated with rEGF incubated with a γ-^{32}P-labeled oligonucleotide probe containing consensus binding sites for STAT5 in the presence of antibodies against P-STAT5, P-STAT3, ERK, and P-ERK as indicated. A supershift was only observed with P-STAT5 and, to a lesser extent, with P-STAT3 antibodies.

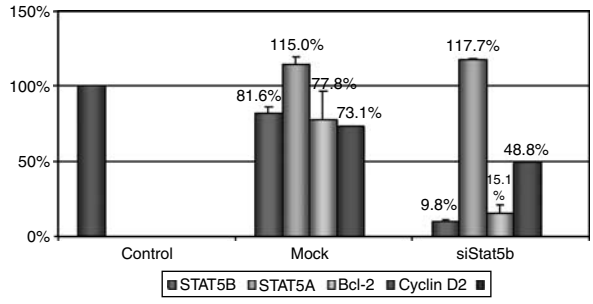

Figure 6. Treatment with siRNA-Stat5 reduced expression of target genes Bcl-2 and cyclin D2. siRNA-Stat5 transfection of A375 cells showed a more than 90% reduction of *Stat5b* transcript levels, whereas *Stat5a* gene expression was unaffected. Real-time PCR also revealed a significant reduction of Bcl-2 ($P<0.005$) and cyclin D2 transcripts ($P<0.05$).

positive (Figure 8a and b). These results are consistent with the finding that depletion of Stat5b reduced the levels of the antiapoptotic gene *Bcl-2* (Figure 6). Despite the multiple pleiotrophic effects of siRNA, our data support the importance of STAT5 for survival of melanoma cells.

Expression of dominant-negative STAT5B

To confirm the functional effects of siRNA directed against *Stat5b*, we performed transfection assays expressing

dominant-negative STAT5B fused to green fluorescent protein (GFP). Analysis of DNA content in propidium iodide-stained A375 cells transfected with wild-type and dominant-negative human STAT5B (transfection efficiency: 22.2±5.7%; $n=3$) showed a 16.9-fold increase in the sub-G1-peak fraction (indicative of apoptosis) for the dominant-negative mutant (3.72±2.04%) in relation to wild-type STAT5 ($P=0.04$) (Figure 9a). In the presence of rEGF, the difference was less pronounced (4.3-fold ±1.6) (Figure 9b), consistent with the hypothesis that STAT5B activation inhibits apoptosis. STAT5B-induced apoptosis was not seen in BLM cells in accordance with the low EGFR levels and limited STAT5B activation (Figure 9c and Figure 1d). Furthermore, dominant-negative STAT5B inhibited the expression of bcl-2 in A375 cells (Figure 9c). Thus, inhibition of STAT5B signaling using dominant-negative mutants led to G1 arrest and enhanced cell death in rEGF-sensitive cells.

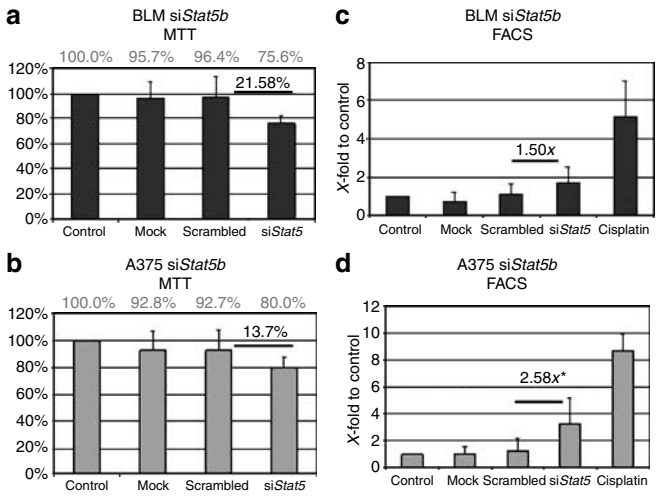

Figure 7. siRNA-Stat5 transfection leads to decreased cell viability and increased apoptosis. Reduced expression of *Stat5b* led to a 21.6% (NS) and 13.7% (NS) reduction in viability of BLM (**a**) and A375 (**b**) cells, respectively, as evidenced by a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay. Blocking *Stat5b* expression led to a 1.5-fold apoptosis increase in (**c**) BLM and a 2.58-fold increase in (**d**) A375 cells as assessed by the JC-1 FACS assay. As positive control, cisplatin (100 nM)-treated cells are shown (*coefficient of variation: 109.39%).

DISCUSSION

Since their discovery as key mediators of cytokine signaling, considerable progress has been made in defining the structure–function relationships of STATs (Darnell, 1997). In addition to their central role in normal cell signaling, recent studies have demonstrated that constitutively activated STAT signaling directly contributes to oncogenesis and angiogenesis (Bowman *et al.*, 2000; Niu *et al.*, 2002b). Extensive studies of primary tumors and tumor-derived cell lines revealed that inappropriate activation of specific STATs occurs with high frequency in a wide variety of human cancers including leukemias (Lin *et al.*, 2000), breast cancer (Yamashita *et al.*, 2003), prostate cancer (Li *et al.*, 2004), human non-small-cell carcinoma (Grandis *et al.*, 2000; Xi *et al.*, 2003), and the lung cancer (Song *et al.*, 2003).

In the present work, we have demonstrated an abundance of *stat5b* transcripts and activation of STAT5 protein in

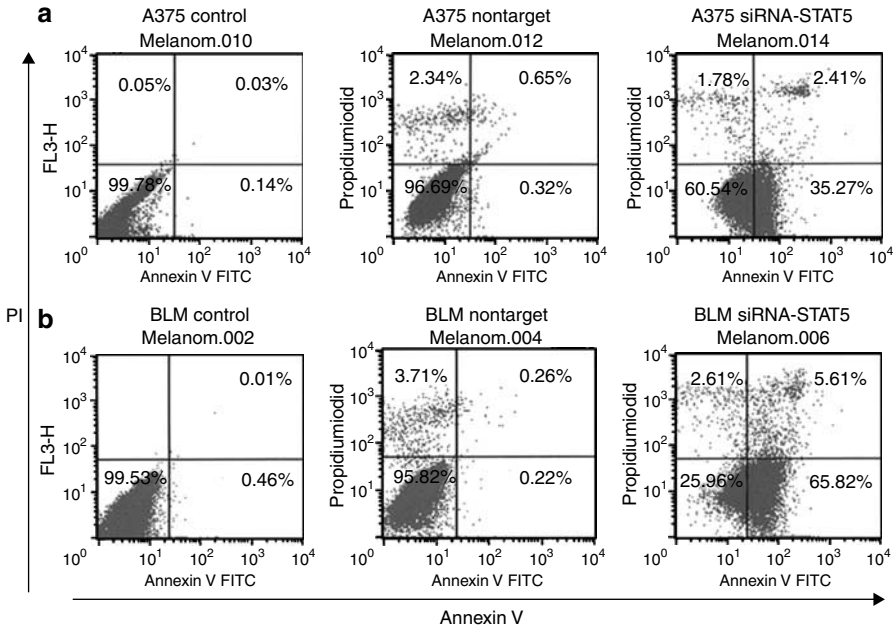

Figure 8. siRNA-Stat5 transfection leads to increased apoptosis. To confirm the JC-1 results suggesting apoptosis, annexinV staining was performed on unstained, non-target and siRNA-Stat5b-treated (**a**) A375 and (**b**) BLM cells. As can be seen, upon treatment with siRNA-Stat5 a significant percentage of (**a**) A375 and (**b**) BLM cells became annexinV positive.

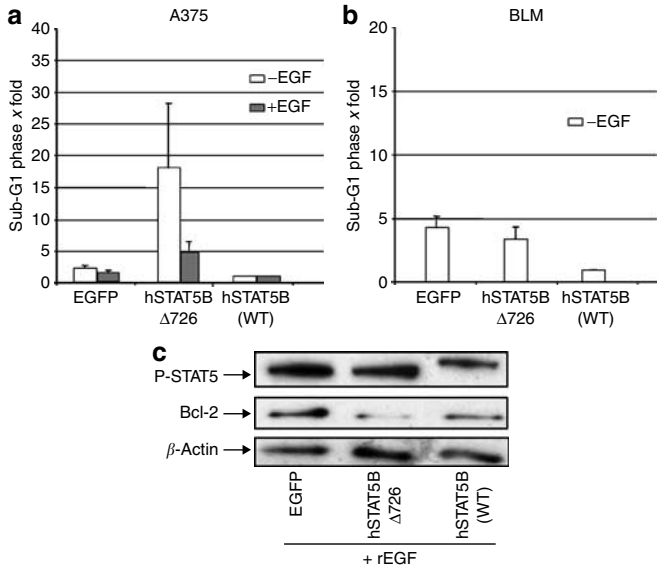

Figure 9. FACS analysis of DNA content of A375 and BLM cells after transfection with different STAT5B vectors. Depicted are fold changes of sub-G1 peaks at 72 hours relative to transfection with human STAT5B wild-type (**a**) without or with rEGF treatment ($n=3$). (**b**) BLM cells gated for the sub-G1 phase without rEGF treatment ($n=3$). The standard deviation of three independent experiments is shown. Gray columns: all cells were analyzed; white columns: only the transfected GFP-expressing cells were analyzed; GFP: control transfection with GFP vector; hSTAT5BΔ726: transfection with dominant-negative human STAT5B in which the 61 carboxy-terminal amino acids were deleted; and hSTAT5(WT): transfection with human STAT5B wild-type. (**d**) Western blot analysis with Bcl-2 and P-STAT5 antibodies with lysates of A375 transfected cells in presence of rEGF.

approximately 71% of patients with cutaneous melanoma metastases of different localizations. Activated STAT5 signaling seems to play an important role in the malignant transformation of melanocytes and growth of melanoma in fish (Wellbrock *et al.*, 1998).

The activation of STAT5 is ligand-, receptor-, and cell type-specific and may also depend on activating stimuli within the tumor microenvironment. In melanoma, STAT5 signaling may be induced by rEGF and is mediated by the EGFR tyrosine kinase and by intracellular non-receptor tyrosine kinases such as SRC and JAK1 (Coffer and Kruijer, 1995). Immunoprecipitation experiments with EGFR indicated that SRC and not STAT5 or JAK1 directly interacts with EGFR in melanoma cells. As SRC kinase and STAT5 were associated in immunoprecipitates from A375 cell lysates only after treatment with rEGF, this raises the possibility that upon dissociation from the EGFR, SRC may directly phosphorylate STAT5 when exogenous rEGF is present.

Furthermore, the inhibition of SRC abrogated the phosphorylation of STAT5. EGFR-mediated phosphorylation of STAT5 may also occur through JAK1, whereas JAK2 seems not to be generally involved in melanoma, in contrast to prostate (Yamashita *et al.*, 2003; Li *et al.*, 2004) and breast cancer (Canbay *et al.*, 1997), where STAT5 is activated in response to prolactin through JAK2. Therefore, the role of the JAK family and SRC kinases depends on the particular receptor–ligand interaction in a given cell type. In human squamous cell carcinoma, it has been demonstrated that STAT3 and STAT5 are activated through EGFR and SRC family kinases (Chan *et al.*, 2004), which corresponds to our data of STAT5 activation through SRC in melanoma. In the human squamous cell carcinoma xenograft model using antisense oligonucleotides, it has been shown that STAT5B and not STAT5A resulted in tumor growth inhibition and abrogation of STAT5 target genes (Grandis *et al.*, 2000). This finding is in agreement with our results from transfection assays with C-terminal-truncated dominant-negative STATΔ5B constructs, which significantly enhanced cell death and G1 cell arrest. These findings support a role for STAT5 in cell cycle regulation and cell survival in melanoma. Recent functional studies have also demonstrated that dominant-negative STAT5 blocked cell proliferation and tissue invasion and had a profound inhibitory effect on tumor progression (Morcinek *et al.*, 2002).

Furthermore, activation of STAT5 promoted cell cycle progression in erythroid precursors and protection from apoptosis through the upregulation of the STAT5 target gene BCL-x_L (Socolovsky *et al.*, 1999; Krasilnikov *et al.*, 2003). Consistent with this finding, we investigated the expression of antiapoptotic Bcl-2 and demonstrated strong expression in clinical samples where STAT5 was active and in the melanoma cell line A375 upon treatment with rEGF. siRNA and transfection experiments with dominant-negative *Stat5b* showed that induction of apoptosis in A375 cells correlated with decreased expression of Bcl-2. This antiapoptotic effect of dominant-negative *Stat5b* may act through the abrogation of STAT5 target genes such as Bcl-2 (Morcinek *et al.*, 2002; Krasilnikov *et al.*, 2003).

The participation of STAT signaling in tumor promotion seems to be evolutionarily conserved, as constitutive activation of STAT5 by the EGFR-related Xmrk has been first demonstrated in *Xiphophorus* melanoma cells, which resulted in cell proliferation and prevention of apoptosis upon stimulation with rEGF (Wellbrock *et al.*, 1998; Morcinek *et al.*, 2002). Reduced expression of the product of the *marelle* gene, a fly STAT homolog, has also been shown to inhibit hematopoietic neoplasia (Hou *et al.*, 1996).

The activation of STAT5 signaling in a variety of cancers including melanoma (Wellbrock *et al.*, 2005) point to the importance of STAT5 signaling in cancer development. In addition, our findings provide evidence that aberrant STAT5 signaling contributes to progression of melanoma by preventing apoptosis and identifies SRC and STAT5 as potential targets for therapeutic intervention.

MATERIALS AND METHODS

Cell culture

NHMs were cultured in phorbol 12-myristate 13-acetate-free melanocyte medium (both obtained from PromoCell, Germany). The human melanoma cell lines BLM, MV3, M13, A375 were cultured in DMEM (Invitrogen-Gibco, Germany) under standard conditions. Inhibitors of mitogen-activated protein kinase kinase -1/2 (U0126), of the EGFR (AG1478), JAK kinase (AG490), SRC kinase

(PP1, PP2) as well as cisplatin were obtained from Sigma-Aldrich, Germany.

Tumor specimens

The melanoma metastases were from different biopsy sites (Table 1). Biopsies were snap-frozen in liquid nitrogen and homogenized using a dismembrator (Braun-Melsungen, Germany). One-half of the powder was immediately transferred into Trizol™ reagent (Invitrogen, Germany) for total RNA isolation according to the manufacturer's instruction and the other half transferred into ice-cold lysis buffer containing 25 mM hydroxyethylpiperazine ethanesulfonic acid, pH 7.9, 50 mM NaF, 1% Triton X-100, 5 mM EDTA, 100 mM NaCl, and one tablet protease inhibitor cocktail per 10 ml buffer (Roche, Germany) for Western blot analysis. Participants gave their written informed consent. The study was conducted according to the Declaration of Helsinki Principles.

Quantitative real-time PCR

The primers and the probe used for the detection of *stat5b* and 18S were ordered as TaqMan® gene expression assay (Applied Biosystems Inc.). PCR was monitored in real time using the PRISM 7300 Sequence Detector (Applied Biosystems Inc., Foster City, CA). All samples were amplified simultaneously in triplicate in one assay run. Variation coefficients of C_T values for triplicate reactions were less than 1% for all gene products. The endogenous control showed low interindividual coefficients of variation (8.5%).

Western blot analysis, immunoprecipitation, immunohistochemistry, nuclear extraction, electrophoretic mobility shift assay, and supershift assay

Total cell extracts were prepared in lysis buffer (see above) and pretreated with ultrasound 10 pulses on ice. Immunostaining was performed with using monoclonal antibodies against STAT5, P-STAT5, Bcl-2 (each BD Biosciences Pharmingen, Becton Dickinson, GmbH, Heidelberg, Germany), β-actin (Oncogene Research Products, San Diego, CA), P-JAK1 (Y1022/1023), P-JAK2 (Y1007/1008) (BioSource, Solingen, Germany) and P-SRC (Y215) (Sigma-Aldrich, Taufkirchen, Germany). The EGFR (ErbB-1) antibody was obtained from Cell Signaling, New England Biolabs, Germany. As a positive control, the HaCaT epithelial cell line was used. For immunoprecipitation samples in Western blot buffer were incubated with EGFR or P-SRC antibodies overnight at 4°C with constant rotation followed by incubation with immobilized protein A/G (Pierce, Bonn, Germany) for 2 hours. Immunohistochemistry of melanoma sections was prepared as described (Mirmohammadsadegh *et al.*, 2003). Nuclear extraction and supershift assays were prepared as described in Hassan *et al.* (2004). A375 cells were stimulated with rEGF (100 ng/ml; BioSource) for 15 minutes. STAT5 double-stranded oligonucleotides were from Santa Cruz Biotechnologies (Santa Cruz, CA). The supershift assay was performed by preincubation of the nuclear extracts with antibodies against P-STAT5, P-STAT3, phosphor-p44/42 mitogen-activated protein kinase (T202/Y204), and p44/42 mitogen-activated protein kinase (each from Cell Signaling, New England Biolabs, Germany).

RNA interference and apoptosis assay

siRNAs were provided by Dharmacon (Solingen, Germany). Cells (2×10^5) were incubated with 100 nM siRNA transfection solution without fetal calf serum and antibiotics for 4 hours. For real-time PCR analysis, the incubation with siRNA lasted 24 hours and for FACS analysis JC-1 apoptosis assay) 48 hours, respectively. Apoptosis was measured by analyzing the change of the mitochondrial membrane potential, using the membrane potential-sensitive dye JC-1. JC-1 (5 μg/ml) in DMEM media containing 10% fetal calf serum were added to the cells for 15 minutes in a 5% CO_2 incubator at 37°C. Upon washes, cells were detached with trypsin, and centrifuged for 5 minutes at $500 \times g$. Before suspension in 500 μl phosphate-buffered saline for flow cytometric analysis. AnnexinV staining (Vybrant Apoptosis Assay kit no. 3, Invitrogen, Germany) was assessed according to the manufacturer's recommendations.

STAT5B vector construction

Human STAT5B wild-type cDNA was PCR-amplified from human kidney cDNA (superscript III RT, Invitrogen). Primers for STAT5B amplification were: 5′-GCTAGCGATTGTAAACCATGGCTGTGT GG-3′ with an *Nhe*I site and 5′-GAGCTCCGATTGTGCGTGCGG GATCC-3′ containing an *Sac*I site instead of the stop codon. The PCR product was ligated into pUC18 and sequenced (GATC company, Konstanz, Germany). The internal *Sac*I site was removed using QuikChange® XL site-directed mutagenesis kit (Stratagene, CA) using primers: 5′-CGCGGCGTGTGGAGGAGCTGCTGGGCCGGC CAATGGACA-3′ and 5′-TGTCCATTGGCCGGCCCAGCAGCTCCT CCACACGCCGCG-3′ conserving the amino-acid sequence. *Nhe*I/*Sac*I-STAT5B cDNA was ligated into *Nhe*I- and *Sac*I-cut pEGFP-N1 (BD Biosciences, Pharmingen, Germany) resulting in pSTAT5B(WT)-fus-GFP allowing the expression of a 119 kDa/787 amino acids STAT5B-GFP fusion protein under the control of the cytomegalovirus promotor. Construction of pSTAT5B(Δ726)fus-GFP was performed accordingly using the primers 5′-GCTAGCGATTGTAAACCATGG CTGTGTGG-3′ and 5′-GAGCTCCGATTGTGCGTGCGGGATCC-3′ allowing the expression of a 112 kDa protein.

Transfection assays

For transfections Polyfect® (Qiagen, Germany) was used for A375 and JetPEI® (Q-BIOgene) for BLM cells, respectively. Transfection of 9×10^5 cells was performed according to the manufacturer's recommendations using 27 μg of DNA and 162 μl of Polyfect® for A375 and 27 μg of DNA and 45 μl of JetPEI® for BLM cells, respectively.

FACS analysis

Cells were harvested and fixed in 3.7% paraformaldehyde (in phosphate-buffered saline) for 1 hour. For permeabilization, 1 vol of ice-cold 0.2% sodium citrate and 0.2% Triton X-100 (prepared in phosphate-buffered saline) was added and incubated on ice for 2 minutes. After two phosphate-buffered saline washes, pellets were resuspended in propidium iodide-staining solution (BD Biosciences Pharmingen). DNA content was analyzed with a FACScalibur (BD Biosciences Pharmingen) using Cell Quest.®

CONFLICT OF INTEREST

The authors state no conflict of interest. The authors have no competing financial interest to declare.

REFERENCES

Bowman T, Garcia R, Turkson J, Jove R (2000) STATs in oncogenesis. *Oncogene* 19:2474–8

Calo V, Migliavacca M, Bazan V, Macaluso M, Buscemi M, Gebbia N *et al.* (2003) STAT proteins: from normal control of cellular events to tumorigenesis. *J Cell Physiol* 197:157–68

Canbay E, Norman M, Kilic E, Goffin V, Zachary I (1997) Prolactin stimulates the JAK2 and focal adhesion kinase pathways in human breast carcinoma T47-D cells. *Biochem J* 324:231–6

Carlesso N, Frank DA, Griffin JD (1996) Tyrosyl phosphorylation and DNA binding activity of signal transducers and activators of transcription (STAT) proteins in hematopoietic cell lines transformed by Bcr/Abl. *J Exp Med* 183:811–20

Catlett-Falcone R, Landowski TH, Oshiro MM, Turkson J, Levitzki A, Savino R *et al.* (1999) Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. *Immunity* 10:105–15

Chan KS, Carbajal S, Kiguchi K, Clifford J, Sano S, DiGiovanni J (2004) Epidermal growth factor receptor-mediated activation of Stat3 during multistage skin carcinogenesis. *Cancer Res* 64:2382–9

Chatterjee M, Stuhmer T, Herrmann P, Bommert K, Dorken B, Bargou RC (2004) Combined disruption of both the MEK/ERK and the IL-6R/STAT3 pathways is required to induce apoptosis of multiple myeloma cells in the presence of bone marrow stromal cells. *Blood* 104:3712–21

Chin H, Arai A, Wakao H, Kamiyama R, Miyasaka N, Miura O (1998) Lyn physically associates with the erythropoietin receptor and may play a role in activation of the Stat5 pathway. *Blood* 91:3734–45

Coffer PJ, Kruijer W (1995) EGF receptor deletions define a region specifically mediating STAT transcription factor activation. *Biochem Biophys Res Commun* 210:74–81

Darnell JE Jr (1997) STATs and gene regulation. *Science* 277:1630–5

Darnell JE Jr, Kerr IM, Stark GR (1994) Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* 264:1415–21

Duensing A, Medeiros F, McConarty B, Joseph NE, Panigrahy D, Singer S *et al.* (2004) Mechanisms of oncogenic KIT signal transduction in primary gastrointestinal stromal tumors (GISTs). *Oncogene* 23: 3999–4006

Garcia R, Yu CL, Hudnall A, Catlett R, Nelson KL, Smithgall T *et al.* (1997) Constitutive activation of Stat3 in fibroblasts transformed by diverse oncoproteins and in breast carcinoma cells. *Cell Growth Differ* 8:1267–76

Grandis JR, Drenning SD, Zeng Q, Watkins SC, Melhem MF, Endo S *et al.* (2000) Constitutive activation of Stat3 signaling abrogates apoptosis in squamous cell carcinogenesis *in vivo*. *Proc Natl Acad Sci USA* 97:4227–32

Hassan M, Ghozlan H, Abdel-Kader O (2004) Activation of RB/E2F signaling pathway is required for the modulation of hepatitis C virus (HC) core protein-induced cell growth in liver and non-liver cells. *Cell Signal* 16:1375–85

Hou XS, Melnick MB, Perrimon N (1996) Marelle acts downstream of the *Drosophila* HOP/JAK kinase and encodes a protein similar to the mammalian STATs. *Cell* 84:411–9

Krasilnikov M, Ivanov VN, Dong J, Ronai Z (2003) ERK and PI3K negatively regulate STAT-transcriptional activities in human melanoma cells: implications towards sensitization to apoptosis. *Oncogene* 22:4092–101

Kyba M, Perlingeiro RC, Hoover RR, Lu CW, Pierce J, Daley GQ (2003) Enhanced hematopoietic differentiation of embryonic stem cells conditionally expressing Stat5. *Proc Natl Acad Sci USA* 100:11904–10

Leu CM, Wong FH, Chang C, Huang SF, Hu CP (2003) Interleukin-6 acts as an antiapoptotic factor in human esophageal carcinoma cells through the activation of both STAT3 and mitogen-activated protein kinase pathways. *Oncogene* 22:7809–18

Levy DE, Darnell JE Jr (2002) Stats: transcriptional control and biological impact. *Nat Rev Mol Cell Biol* 3:651–6

Li H, Ahonen TJ, Alanen K, Xie J, LeBaron MJ, Pretlow TG *et al.* (2004) Activation of signal transducer and activator of transcription 5 in human prostate cancer is associated with high histological grade. *Cancer Res* 64:4774–82

Lin TS, Mahajan S, Frank DA (2000) STAT signaling in the pathogenesis and treatment of leukemias. *Oncogene* 19:2496–504

Lund TC, Coleman C, Horvath E, Sefton BM, Jove R, Medveczky MM *et al.* (1999) The Src-family kinase Lck can induce STAT3 phosphorylation and DNA binding activity. *Cell Signal* 11:789–96

Mirmohammadsadegh A, Hassan M, Gustrau A, Doroudi R, Schmittner N, Nambiar S *et al.* (2005) Constitutive expression of epidermal growth factor receptors on normal human melanocytes. *J Invest Dermatol* 125:392–4

Mirmohammadsadegh A, Tartler U, Michel G, Baer A, Walz M, Wolf R *et al.* (2003) HAX-1, identified by differential display reverse transcription polymerase chain reaction, is overexpressed in lesional psoriasis. *J Invest Dermatol* 120:1045–51

Morcinek JC, Weisser C, Geissinger E, Schartl M, Wellbrock C (2002) Activation of STAT5 triggers proliferation and contributes to anti-apoptotic signalling mediated by the oncogenic Xmrk kinase. *Oncogene* 21:1668–78

Niu G, Bowman T, Huang M, Shivers S, Reintgen D, Daud A *et al.* (2002a) Roles of activated Src and Stat3 signaling in melanoma tumor cell growth. *Oncogene* 21:7001–10

Niu G, Heller R, Catlett-Falcone R, Coppola D, Jaroszeski M, Dalton W *et al.* (1999) Gene therapy with dominant-negative Stat3 suppresses growth of the murine melanoma B16 tumor *in vivo*. *Cancer Res* 59:5059–63

Niu G, Shain KH, Huang M, Ravi R, Bedi A, Dalton WS *et al.* (2001) Overexpression of a dominant-negative signal transducer and activator of transcription 3 variant in tumor cells leads to production of soluble factors that induce apoptosis and cell cycle arrest. *Cancer Res* 61:3276–80

Niu G, Wright KL, Huang M, Song L, Haura E, Turkson J *et al.* (2002b) Constitutive Stat3 activity up-regulates VEGF expression and tumor angiogenesis. *Oncogene* 21:2000–8

Rosen MK, Yamazaki T, Gish GD, Kay CM, Pawson T, Kay LE (1995) Direct demonstration of an intramolecular SH2-phosphotyrosine interaction in the Crk protein. *Nature* 374:477–9

Socolovsky M, Fallon AE, Wang S, Brugnara C, Lodish HF (1999) Fetal anemia and apoptosis of red cell progenitors in Stat5a−/−5b−/− mice: a direct role for Stat5 in Bcl-X(L) induction. *Cell* 98:181–91

Song L, Turkson J, Karras JG, Jove R, Haura EB (2003) Activation of Stat3 by receptor tyrosine kinases and cytokines regulates survival in human non-small cell carcinoma cells. *Oncogene* 22:4150–65

Wellbrock C, Geissinger E, Gomez A, Fischer P, Friedrich K, Schartl M (1998) Signalling by the oncogenic receptor tyrosine kinase Xmrk leads to activation of STAT5 in Xiphophorus melanoma. *Oncogene* 16:3047–56

Wellbrock C, Weisser C, Hassel JC, Fischer P, Becker J, Vetter CS *et al.* (2005) STAT5 contributes to interferon resistance of melanoma cells. *Curr Biol* 15:1629–39

Xi S, Zhang Q, Dyer KF, Lerner EC, Smithgall TE, Gooding WE *et al.* (2003) Src kinases mediate STAT growth pathways in squamous cell carcinoma of the head and neck. *J Biol Chem* 278:31574–83

Yamashita H, Iwase H, Toyama T, Fujii Y (2003) Naturally occurring dominant-negative Stat5 suppresses transcriptional activity of estrogen receptors and induces apoptosis in T47D breast cancer cells. *Oncogene* 22:1638–52