Neoplasia • Vol. 9, No. 4, April 2007, pp. 279–291 279

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Signal Transducer and Activator of Transcription 3 Activation Promotes Invasive Growth of Colon Carcinomas through Matrix Metalloproteinase Induction[1]

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Abstract

Signal transducer and activator of transcription 3 (STAT3) is aberrantly activated in colorectal carcinomas (CRCs). Here, we define the relationship between STAT3 function and the malignant properties of colon carcinoma cells. Elevated activation of STAT3 enhances invasive growth of the CRC cell lines. To address mechanisms through which STAT3 influences invasiveness, the protease mRNA expression pattern of CRC biopsies was analyzed and correlated with the STAT3 activity status. These studies revealed a striking coincidence of STAT3 activation and strong expression of matrix metalloproteinases MMP-1, -3, -7, and -9. Immunohistological examination of CRC tumor specimens showed a clear colocalization of MMP-1 and activated STAT3. Experimentally induced STAT3 activity in CRC cell lines enhanced both the level of MMP-1 mRNA and secreted MMP-1 enzymatic activity. A direct connection of STAT3 activity and transcription from the MMP-1 promoter was shown by reporter gene experiments. Moreover, high-affinity binding of STAT3 to STAT recognition elements in both the MMP-1 and MMP-3 promoter was demonstrated. Xenograft tumors arising from implantation of CRC cells into nude mice showed simultaneous appearance and colocalization of p-Y-STAT3 and MMP-1 expression. Our results link aberrant activity of STAT3 in CRC to malignant tumor progression through upregulated expression of MMPs.

Neoplasia (2007) **9,** 279–291

Keywords: Colorectal carcinoma, invasiveness, STAT3, matrix metalloproteinases, gene regulation.

Introduction

Carcinomas frequently show aberrant activity of signal transducers and activators of transcription (STATs). Increased STAT3 activity is mostly tumor promoting, whereas elevated STAT1 activity is tumor growth inhibiting [1]. Among the members of the STAT family, STAT3 mediates the most complex spectrum of cellular responses including differentiation, proliferation, survival, and apoptosis, depending on the tissue context [2]. Constitutive activity of STAT3 was observed in many malignant tumors of both hematopoietic and solid type [3]. In addition, an artificially dimerized variant of STAT3 was shown to act as a *bona fide* oncoprotein by eliciting cellular transformation of fibroblasts and tumorigenesis in nude mice [4]. We have recently investigated biopsies of colorectal carcinomas (CRCs) and analyzed the spectrum of STAT activity. Most significantly, we observed constitutive activation of STAT3α in carcinoma cells of more than 90% of the tumors [5].

Experimental inhibition of STAT3 activity in tumor cell lines and tumor tissue yielded promising results with regard to STAT3 as an interesting target for tumor therapy [6–8]. Although downstream events connecting aberrant STAT3 activity to malignant cell properties are insufficiently defined, they involve the upregulation of genes promoting cell cycle progression (cyclin D1, c-myc) and/or preventing apoptosis (bcl-x, mcl-1, survivin) [4,9,10].

Recent studies showed that STAT3 activation in tumors may be associated with metastasis and poor prognosis [8,11], indicating that inappropriate signaling by STAT3 coincides with invasive growth of cancer cells. Interestingly, blocking STAT3 activity reduced the motility of ovarian cancer cells whose aggressive clinical behavior showed a correlation with constitutively active STAT3 levels [12]. So far, how STAT3 promotes

Abbreviations: c.a., constitutively active; CRC, colorectal carcinoma; d.n., dominant negative; EMSA, electrophoretic mobility shift assay; IL, interleukin; MMP, matrix metalloproteinase; JAK, janus kinase; STAT, signal transducer and activator of transcription

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[1]Part of this work was funded by the Interdisziplinäres Zentrum für Klinische Forschung—IZKF Jena, Project B 307-01035.
Received 22 December 2006; Revised 28 February 2007; Accepted 2 March 2007.

DOI 10.1593/neo.06820

cancer development and whether it is able to control invasive cell properties are largely undefined.

Tumor invasion and metastasis require proteolytic degradation of the basement membrane and the extracellular matrix (ECM). Matrix metalloproteinases (MMPs) are chiefly involved in the dissemination of cancer cells by breaking down the ECM and creating an environment that supports the initiation and maintenance of tumor growth. The MMP family, consisting of roughly 20 members, displays a conserved structure and enzymatic mechanism [13]. MMP expression is low in most normal cells under regular physiological conditions; however, it is dramatically increased in a variety of cancer types, which is indicative of invasive disease with a poor clinical prognosis [14–16]. Interestingly, knocking out or inhibition of some MMPs results in the reduction of tumor growth. For instance, the spontaneous development of intestinal tumors in mice with adenomatous polyposis coli (Apc) gene mutations is slower in MMP-7–deficient animals [17]. Moreover, experimental metastasis is suppressed in MMP-9–deficient mice [18], and MMP-11 knockout mice show reduced chemically induced tumorigenesis [19]. Inhibitors acting on MMP-1, MMP-3, MMP-7, and MMP-9 behaved as potent antiangiogenic and antimetastatic agents in animal models [20,21].

The recognition of STAT proteins as being potential players in oncogenic cell transformation was paralleled by their involvement in the modulation of activity for both promoters of MMPs and tissue inhibitors of MMP (TIMPs) [22–26]. A recent report demonstrated that transcriptional upregulation of MMP-9 is directly involved in the oncogenic transformation of mammary epithelial cells via a constitutively active mutant of STAT3 [27]. Similar studies on other MMPs have not yet been published.

The interstitial collagenase MMP-1 degrades collagen types I, II, and III, which are the most abundant proteins of the ECM [28]. It is already well established that MMP-1 is required for invasive growth of tumor cells [29]. For instance, it plays a critical role in the development of malignancy in CRC. Levels of MMP-1 increase during CRC progression and higher levels of expression are associated with shorter disease-free survival times [30,31]. The particular importance of MMP-1 for increased invasiveness of colon carcinoma cells *in vitro* has recently been demonstrated [32].

Various stimuli such as cytokines, growth factors, cell–cell, and cell–matrix interactions influence MMP expression through transcriptional regulation [33]. Well-characterized intracellular signal transduction reactions leading to the modulation of MMP promoter activity are mostly centered around mitogen-activated protein kinases, which, upon activation, enter the nucleus and phosphorylate transcription factors such as AP-1 and Ets proteins [34,35]. The complex transcriptional regulation of MMP promoters also involves contributions from more specific transcription factors, including NFκB as a mediator of toll-like receptors [36] and Smads, which are effectors of receptors for transforming growth factor-β, activins, and related ligands [37].

Our finding that STAT3 is activated in most CRCs left us with the question as to how STAT3-driven transcription might contribute to the malignancy of CRC cells. Reports on the involvement of janus kinase (JAK)/STAT signaling in the regulation of invasion-associated proteases [23–25] prompted us to study the correlation of STAT3 activation and MMP protease expression in CRC. Here, we show for the first time a causal role of STAT3 for the elevated expression of MMP-1, MMP-3, MMP-7, and MMP-9 in CRC leading to increased invasiveness of tumor cells.

Materials and Methods

Tumor Biopsies

Surgical tumor specimens were obtained from patients with CRC after approval of the study by the local ethical committee. Biopsies were snap-frozen in liquid nitrogen immediately after resection. For protein and RNA analysis, frozen blocks were disintegrated mechanically by using a Micro Dismembranator (Braun, Melsungen, Germany). For Western blot studies, whole-cell extracts were prepared by suspending the material in a buffer containing 20 mmol/l Hepes (pH 7.9), 400 mmol/l NaCl, 1 mmol/l EDTA, 20% glycerol, 1 mmol/l dithiothreitol, 1 mmol/l phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 0.2 U/ml aprotinin, 5 mmol/l sodium orthovanadate, 10 mmol/l NaF, and 5 mol/l β-glycerophosphate, followed by three to four freeze–thaw cycles in liquid nitrogen and on ice. Extracts were cleared at 20,000*g* for 30 minutes at 4°C. Supernatants were subjected to protein determination and SDS-PAGE. Total RNA was isolated from disintegrated biopsies by homogenization in TRIzol Reagent (Gibco BRL, Gaithersburg, MD) as described below.

Cell Lines and Cell Culture

Colon carcinoma cell line HT-29 was purchased from the American Type Culture Collection (Rockville, MD). Low passage number cell line CoGa-1 [38] was obtained from L.A. Huber, Innsbruck. All cell lines were cultured in RPMI 1640 medium containing 10% fetal calf serum, 200 mmol/l L-glutamine, 100 mmol/l sodium pyruvate, and 1 mg/ml gentamycin. Cells were grown in coated tissue culture plasticware (Greiner Labortechnik, Frickenhausen, Germany).

For cytokine stimulation, HT-29 or CoGa-1 cells were incubated with 20 ng/ml recombinant human interleukin (IL)-6 (R&D Systems GmbH, Wiesbaden, Germany) for 30 minutes (Western blotting), 18 hours (reporter gene assay) or 24 hours (expression analysis) at 37°C. Cells were harvested at 80% confluence after three PBS washes at 4°C. Whole-cell extracts were prepared from cell pellets as described above.

DNA Constructs

Construction of the retroviral expression plasmids for STAT3 mutants and generation of HT-29–derived cell lines by retrovirus infection has been described previously [5]. CoGa-1 clones stably expressing STAT3 wild type or the constitutively active STAT3 mutant (STAT3 c.a.) were generated similarly.

The MMP-1 promoter/luciferase reporter plasmid −4372 hMMP-luci (GenBank accession no. AF023338), obtained from C. E. Brinckerhoff (Dartmouth Medical School, Hanover, NH) was described previously [39].

Electrophoretic Mobility Shift Assay

Extracts from tumor biopsies and cultivated cells were prepared and subjected to band shift analysis as described [5]. Briefly, double-stranded, blunt-ended oligonucleotides corresponding to known and potential STAT binding sites were annealed and end-labeled using [^{32}P]γ-ATP and T4 polynucleotide kinase to a specific activity of 10,000 cpm/fmol as described [40,41]. Binding reactions were performed by incubating 10,000 cpm of radiolabeled probe with 20 μg of cell lysate for 30 minutes at room temperature. For supershift reactions of STAT3-containing complexes, 2 μg of anti-STAT3 antibody C-20 (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the binding reactions. Samples were separated by electrophoresis through 6% native polyacrylamide gels and analyzed by autoradiography using BioMax sensitive films (Kodak, Stuttgart, Germany). For the determination of inducible STAT3 binding to potential binding sites, 293T cells were cotransfected with eukaryotic expression vectors for STAT3 and JAK2 by the lipofectamine transfection method and further treated as described [40,41]. Cells were optionally stimulated with human IL-6 (10 ng/ml; R&D Systems) for 30 minutes before harvesting. Extracts were prepared using unstimulated [plain DMEM containing 10% fetal calf serum (FCS)] versus IL-6–stimulated extracts. The mutated "m67" STAT-binding site from the human c-fos promoter 5′-GTCGACATTTCCCGTAAATCGTCGA-3′ [41] served as a positive control for high-affinity binding of STAT3.

Immunohistology

Immunohistological detection of MMP-1 expression in tumor biopsies was performed as described previously [42]. Briefly, formalin-fixed, paraffin-embedded sections (4 μm) were deparaffinized and subsequently treated with 0.3% H_2O_2 for 30 minutes to reduce endogenous peroxidase activity. Sections were incubated at 4°C overnight with a 1:50 dilution of anti–MMP-1 (Dako Diagnostik, Hamburg, Germany) in TBS buffer containing 20% goat serum to block unspecific binding sites, followed by incubation with goat anti-mouse biotin and streptavidin–horseradish peroxidase (BioGenex, San Ramon, CA) and development with diaminobenzidine. Finally, all specimens were coverslipped using Aquatex (Merck, Darmstadt, Germany). Negative controls were performed by omission of the primary antibody.

Soft Agar Assay for Anchorage-Independent Growth

Formation of colonies in soft agar as a parameter of cell transformation was determined as described [43]. Briefly, HT-29– or CoGa-1–derived cells were assayed by seeding 10,000 cells into 500 μl of 0.6% agar (Agarose nobile, Becton Dickinson, Bedford, MA) diluted in RPMI 1640 medium containing 10% FCS. The mixture was poured into six-well plates lined previously with 500 μl of 0.5% agar in medium. The plates were incubated for 2 to 3 weeks at 37°C; 250 μl of fresh medium was added every 3 days. After this period, colonies were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 500 μg/ml) at 37°C for 2 hours before photography and counting.

In Vitro *Cell Invasion Assay*

Cell invasiveness was quantified by a modified Boyden chamber method using polycarbonate transwells (Corning Costar Corp., Cambridge, MA) as described in detail previously [44]. Briefly, 2×10^5 exponentially growing cells were seeded onto membrane filters coated with Matrigel (Becton Dickinson) and transmigration through the Matrigel layer was monitored after incubation for 24 hours at 37°C. The percentage of invasive cells was expressed by relating the total number of migrated cells to the number of cells originally applied to the top of the transwell, which was set at 100%.

Protease Expression Analysis by cDNA Arrays

cDNA macroarrays on the basis of nylon membranes were prepared as described previously [44]. Information on the sequences and the arrangement of probes as well as experimental details are available upon request.

Polymerase chain reaction (PCR) primers used to generate the probes relevant in this study were the following: MMP1 sense, 5′-AGGGTCAAGCAGACATCATG-3′; MMP1 antisense, 5′-AGCATCGATATGCTTCACAGT-3′; MMP3 sense, 5′-TTGCAGTTAGTGAACATGGA-3′; MMP3 antisense, 5′-ATCCAGCTCGTACCTCA-3′; MMP7 sense, 5′-GGCATGAGTGAGCTACAGTG-3′; MMP7 antisense, 5′-CCAGCGTTCATCCTCATCGA-3′; MMP9 sense, 5′-CTTCTACGGCCACTACTGTG-3′; MMP9 antisense, 5′-CACTGCAGGATGTCATAGGT-3′; β-actin sense, 5′-ACCACGGCCGAGCGGGAAATC-3′; and β-actin antisense, 5′-GAGCCGCCGATCCACACGGAGTA-3′.

Hybridization pattern data were processed by use of the AIDA imaging software (Raytest, Straubenhardt, Germany). The average densitometry signals of the duplicate spots, minus background, were calculated. The signals were then normalized against an average of all signals from the respective membranes and expressed as relative densitometric units.

Quantification of Specific mRNAs by Real-Time PCR

Quantitation of cDNA from specific mRNA transcripts was accomplished by real-time reverse transcription-PCR (RT-PCR) using the TaqMan protocol and an ABI/Prism 7000 equipment (Applied Biosystems, Foster City, CA). Predesigned fluorogenic Assays-on-Demand TaqMan probes and primer pairs for MMP-1 (Hs00233958_m1) and 18S rRNA (Hs99999901_s1) were obtained from Applied Biosystems. The following 6-carboxy-fluorescein–labeled probes were used for PCR amplification: MMP-1, 5′-AAAGACAGATTCTACATGCGCACAA-3′; 18S rRNA, 5′-TGGAGGGCAAGTCTGGTGCCAGCAG-3′. The specific primers for MMP-1 and 18S rRNA were predicted to amplify 150-

and 120-bp DNA fragments, respectively. A total reaction volume of 25 μl contained 12.5 μl of 2 × TaqMan Universal Master Mix, No AmpErase UNG (Applied Biosystems), 3 μl cDNA (prepared as described above), 1.25 μl of 20 × Assays-on-Demand Gene Expression products (Applied Biosystems) for MMP-1, consisting of 20 × mix of unlabeled PCR primers (18 μmol/l) and 5 μmol/l TaqMan MGB probe (6-carboxy-fluorescein dye–labeled MMP-1). Forty PCR cycles of 95°C for 15 seconds and 60°C for 1 minute were run after an initial incubation at 95°C for 10 minutes.

The MMP-1 gene copy number in samples to be analyzed was quantified by simultaneously generating a standard curve for both MMP-1 and 18S rRNA as an endogenous control from serial dilutions of HT-29 cDNA (equivalent to a cDNA amount from 100 ng to 100 pg of initial total RNA). The target amounts of unknown samples were divided by the endogenous reference amount to obtain a normalized target value. Results were expressed as *n*-fold differences in MMP-1 mRNA relative to 18S rRNA. Data collection and analysis was performed with the SDS software version 1.0.1 (Applied Biosystems). Data were then exported and further processed using the Excel software (Microsoft, Redmond, WA).

Luciferase Reporter Gene Assay

Exponentially growing cells (7×10^5) of HT-29 and CoGa-1 derivatives were plated in six-well cluster plates (Greiner) in 2 ml RPMI 1640/10% FCS and grown to 80% to 90% confluency. The cells were washed, transferred into 1 ml/well fresh medium, and cotransfected with 1 μg of MMP-1 promoter/luciferase construct and 0.1 μg of the pRL-TK *Renilla* luciferase plasmid (Promega, Madison, WI) as an internal control for transfection efficiency by using PolyFect reagent (Qiagen, Hilden, Germany). Both DNAs were diluted with growth medium containing no serum, antibiotics, or proteins to a final volume of 100 μl. The solutions were mixed and 9 μl of PolyFect was added per well. The mixture was incubated at room temperature for 5 to 10 minutes to allow complex formation before 1 ml medium was added. After 14 hours incubation at 37°C, the cells were gently washed with RPMI 1640 and incubated with fresh RPMI, optionally containing IL-6 (20 ng/ml). The cells were harvested 8 hours later using reporter lysis buffer (Promega). Luciferase and *Renilla* activities were determined with the Dual-Luciferase Reporter Assay System kit from Promega and following the manufacturer's instructions. Luminescence was measured on a Micro Luminat LB 96 P Luminometer and reported as relative light units. Three independent transfections, each run in triplicate, were performed, and the results were normalized to the *Renilla* activity.

Determination of MMP-1 Activity in Cell Supernatants

Levels of enzymatically active MMP-1 were measured using an MMP-1 Fluorokine E ELISA kit (R&D Systems) according to the manufacturer's instructions. HT-29–derived cells were cultivated for 4 days in RPMI 1640/10% FCS in the presence or absence of 20 ng/ml IL-6. After centrifugation (4°C, 1000*g*, 5 minutes), 150 μl of culture supernatants from HT-29 cells was mixed with 100 μl of Assay diluent buffer (R&D Systems) in individual wells of 96-well ELISA plates. Simultaneously, standard samples containing known concentrations of active MMP-1 were treated in the same fashion. The plates were gently shaken for 3 hours at room temperature before unbound material was washed off. Subsequently, 200 μl of activation reagent (0.5 M APMA in DMSO, R&D systems) was added to each well for pro–MMP-1 activation. The plates were incubated for 2 hours at 37°C in a humidified environment. After washing, 200 μl of 1 mmol/l fluorogenic substrate in DMSO (R&D Systems) was added. After another 20 hours at 37°C, fluorescence was determined using a fluorescence plate reader set (SPECTROmax plus, Molecular Devices, Sunnyvale, CA) with an excitation wavelength set to 320 nm and emission wavelength set to 405 nm. Standard curves were generated by plotting the mean relative fluorescence units for each standard for active MMP-1 against the concentrations. Concentrations of active MMP-1 in the cell supernatant samples were determined from the standard curves.

Xenograft Experiments

A total of 10^5 cells of the HT-29 cell line was suspended in PBS and injected subcutaneously at a volume of 100 μl into the neck of 5- to 8-week-old male athymic Hsd:NMRI-nu/nu mice (Harlan, Indianapolis, IN). Mice were sacrificed after the tumors had reached a size of approximately 2000 mm^2. Tumor specimens were further treated similarly to the human biopsy samples (see above).

Statistical Analysis

Statistical analysis was performed using SPSS software version 11.0 (SPSS, Chicago, IL). To calculate significance levels, data sets were compared using the Wilcoxon test for paired samples and Mann–Whitney test for unpaired samples, respectively. Probability values of $P < .05$ were used as the generally accepted level of statistical significance.

Results

STAT3 Activity Enhances Anchorage-Independent Growth and Invasiveness of CRC Cells

We have shown that STAT3 is constitutively active in carcinoma cells from a majority of human CRC biopsies. It also drives proliferation of both the established CRC cell line HT-29 and the low passage number colon cancer cell line CoGa-1 [5]. To address the role of STAT3 in the development and maintenance of malignancy, we studied the influence of STAT3 activity on parameters of cell transformation in both cell lines.

First, the STAT3 expression–dependent formation of colonies in soft agar was determined as a measure of oncogenic cell transformation. For this purpose, we employed nontransfected HT-29 and CoGa-1 cells as well as derivatives stably overexpressing STAT3 or the mutant STAT3 c.a., respectively. STAT3 c.a. functions independently of cytokine

activation through dimerization via an artificial disulfide bridge. After confirmation of elevated STAT3 DNA-binding activity in the transfected cells compared with the parental cell lines [5] (data not shown) cells were seeded into soft agar, overlaid with medium, and incubated for 2 weeks before colonies were counted. As shown in Figure 1, in both cell lines an overexpression of wild type or, to a higher degree, of STAT3 c.a., resulted in a profoundly increased number of colonies, most notably in the absence of exogenous cytokines.

Next, we addressed the influence of STAT3 on the invasive cell properties of colon carcinoma cells as measured by their ability to cross an artificial ECM (Matrigel). Parental cells of HT-29 and CoGa-1 as well as derivatives overexpressing either STAT3 or STAT3 c.a. were seeded onto a layer of Matrigel and analyzed after 24 hours for the fraction of cells migrating through the gel and accumulating in the lower compartment of a transwell chamber (Figure 1*C*). In both cases, cells expressing STAT3 c.a. and overexpressing STAT3 had a much stronger invasive character than the parental CRC cell lines, indicating that STAT3 has an enhancing effect on invasiveness in colon carcinoma cells.

To support this notion, we measured the invasiveness of HT-29 cells dependent on STAT3 activation through IL-6 and/or reduction of STAT3 activity by means of a dominant-negative STAT3 mutant (STAT3 d.n.). As shown in Figure 1*D*, IL-6 evoked stronger cell invasiveness of HT-29 cells in addition to enhancing the invasion-promoting effect of overexpressed STAT3. On the other hand, expression of STAT3 d.n. with the critical Tyr705 mutated to Phe, which blocked receptor-mediated activation of endogenous STAT3, reduced the constitutive invasiveness of HT-29 cells and completely suppressed the effect of IL-6. These results are in good accordance with the tyrosine phosphorylation status of STAT3 in HT-29 cells under the respective conditions [5] and indicate a clear correlation of STAT3 activity with the potency of cells to grow in an invasive manner.

Elevated Expression of MMPs in Colorectal Tumor Biopsies Coincides with Constitutive Activity of STAT3

Our results initiated the question of the mode of action of STAT3 in enhancing the invasiveness of colon carcinoma cells. Proteinases, in particular collagenases, are known to be key lytic enzymes required for the invasion process because their inhibition totally prevented amnion or Matrigel invasion [45]. To explore the molecular mechanisms responsible for STAT3-mediated tumor invasion, we

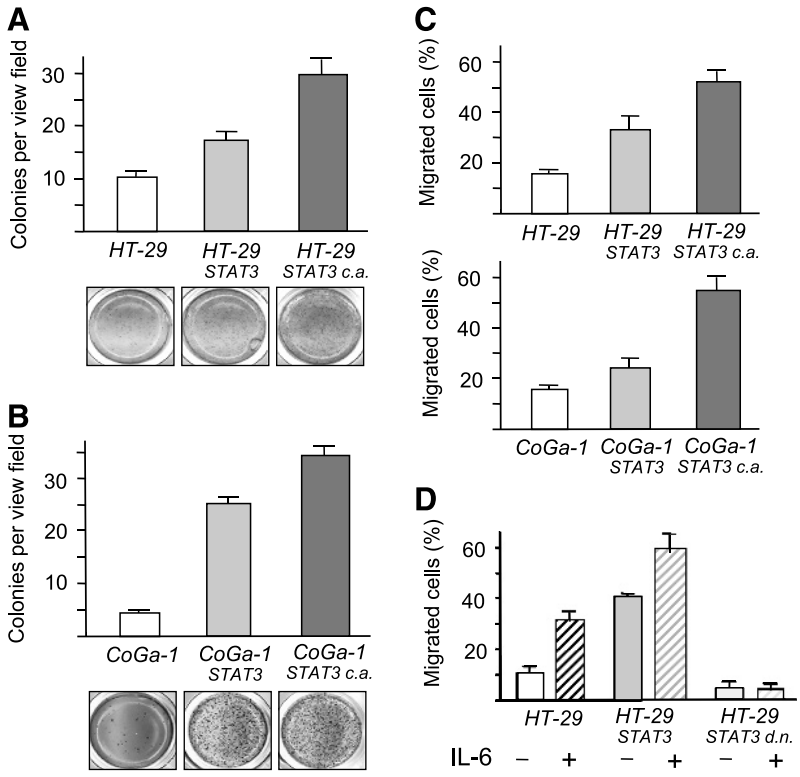

Figure 1. *Influence of STAT3 expression and activity on soft agar colony formation and invasiveness of colon carcinoma cells. (A and B) Dependence of colony formation of HT-29 and CoGa-1 cells on STAT3 expression and activity. Cell lines overexpressing STAT3 or STAT3 c.a. as well as nontransfected control cells were grown in soft agar for 2 weeks. Colonies formed were visualized by staining with MTT (500 μg/ml) (bottom) and counted (top). The values represent triplicate experiments. (C) STAT3 expression– and activity-dependent invasiveness* in vitro *of HT-29–derived cell lines. HT-29, HT-29-STAT3, and HT-29-STAT3 c.a. cells were incubated in invasion chambers containing Matrigel-coated membranes. After 24 hours, cells that had migrated through both Matrigel and membrane to the lower compartment of the system were counted. Invasiveness was expressed as the percentage of cells that had reached the lower chamber at the end of the experiment. The assay was repeated four times in triplicate. (D) IL-6–induced invasiveness of HT-29 cells and derivatives. HT-29, HT-29-STAT3, and HT-29-STAT3 d.n. were grown on Matrigel-coated membranes as in (C). IL-6 was optionally present in the medium (+; striped columns) at a concentration of 20 ng/ml 24 hours before the cell lines were tested and throughout the entire experiment. The experiment was repeated three times. All treatments were performed in triplicate.*

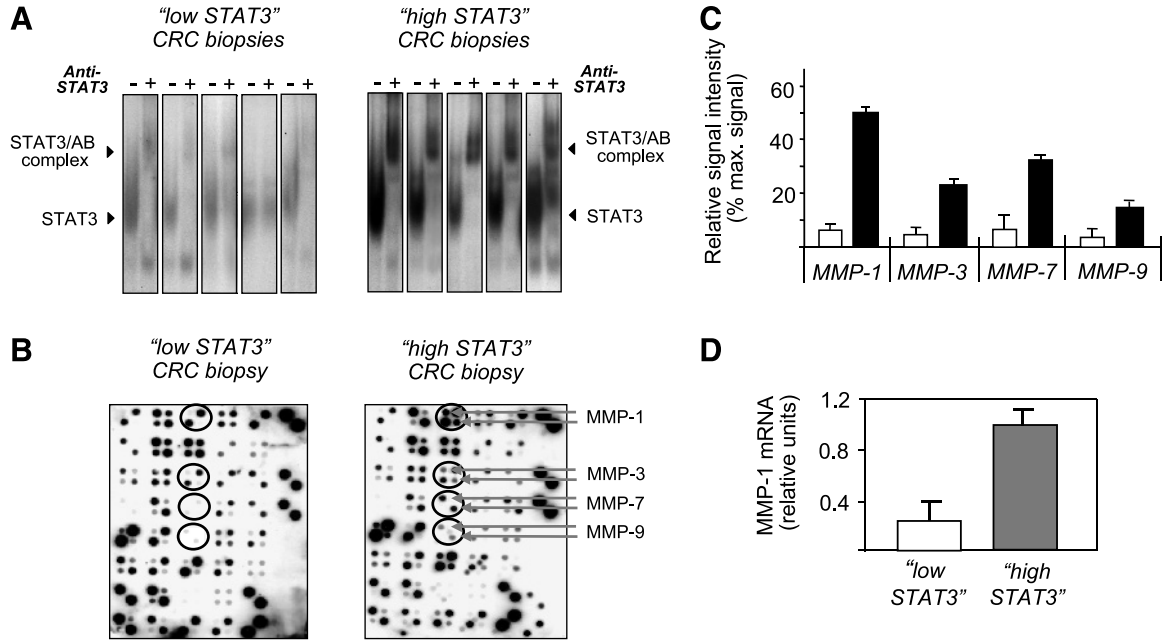

Figure 2. *Correlation of STAT3 activation and protease expression in CRC biopsies. (A) EMSA analysis of STAT3 activation in CRC biopsies scored as low STAT3 or high STAT3 according to previous determination of STAT3 tyrosine phosphorylation [5]. Extracts were incubated with the double-stranded ^{32}P-labelled SIE m67 STAT binding site. Complexes were resolved on 6% native polyacrylamide gels and visualized by autoradiography. The identity of STAT3-containing complexes was verified by including an antibody to STAT3 in the binding reactions as indicated. Positions of the specific STAT3-containing complexes are marked by arrows. (B) Comparative analysis of protease expression in a respective CRC sample with high and low STAT3 activity using protease cDNA macroarrays. Total RNA isolated from tumor samples was reverse transcribed into digoxigenin-labeled cDNA samples, which were hybridized to cDNA arrays covering probes for 50 different human proteases. (C) The relative expression levels of MMP-1, -3, -7, and -9 in 5 tumor samples of each group determined as in (B) were quantified by densitometry and normalized to four "housekeeping" mRNAs (GAPDH, β-actin, ubiquitin, and R-13a). (D) Quantification of MMP-1 mRNA expression levels in CRC biopsies of the high-STAT3 and low-STAT3 type. Three independent experiments, each run in triplicate, were performed; results were normalized to 18S rRNA expression and expressed as the means ± S.D. Relative MMP-1 expression was calculated by arbitrarily setting the expression level of high-STAT3 samples as 1.*

correlated STAT3 activation in colorectal tumors with protease expression.

First, we verified the previously determined status of 32 CRC biopsies with regard to STAT3 tyrosine phosphorylation [5] by electrophoretic mobility shift assay (EMSA) analysis of specific DNA-binding activity (data not shown) and categorized them into a "high STAT3" and a "low STAT3" group. A total of 13 samples with strikingly strong STAT3 activity in both assays were considered high STAT3, and 9 biopsy samples with very low STAT3 activity were considered low STAT3. Figure 2*A* shows five representative examples from each group. Seven samples with intermediate activity and three samples completely negative for STAT3 activity were excluded from further analysis. The remaining samples were tested in a comparative fashion for the respective abundance of protease mRNAs by subjecting them to the isolation of total RNA and the synthesis of digoxigenin-labeled cDNAs. cDNAs were hybridized to arrays covering 50 different human protease probes (arrangement of probes on the arrays is available upon request). Figure 2*B* shows in one representative biopsy for each group that in particular the mRNAs for MMP-1, MMP-3, MMP-7, and MMP-9 were much more abundant in colorectal tumor tissue with a high degree of STAT3 activity. Figure 2*C* summarizes expression data for the MMPs obtained from a larger population of tumors (22 patients in total) quantitated by densitometric determination of signal intensities of the arrays. MMP-1 was given special attention because its expression appeared to correlate with STAT3 activity in a particularly evident fashion and because of its important role in the invasiveness of colon carcinoma cells. As an independent criterion for MMP-1 expression, we determined MMP-1 mRNA in 13 specimens of high STAT3 and 9 specimens of low STAT3 colorectal tumor biopsies by real-time PCR. Figure 2*D* shows that the expression of MMP-1 was strikingly higher in tumors of strong STAT3 activity, confirming the results previously determined by using cDNA arrays.

MMP-1 Expression in CRC Is Colocalized with STAT3 Activity

Previously, we have shown that phosphorylated STAT3 in colorectal tumor tissue is predominantly localized to dedifferentiated epithelial cells [5]. To further elucidate possible functional connections between persistent STAT3 activity and elevated MMP expression, we studied the spatial distribution of MMP-1 expression in CRC tissue using immunohistochemical methods. Sections from biopsies, classified high or low STAT3 according to both biochemical and histological testing, were stained with an antibody to MMP-1. Figure 3 shows that no significant MMP-1 expression was detected in and around normal crypts of both high- and low-STAT3 specimens. In contrast, the two tumor types showed a strikingly diverse picture with regard to MMP-1 abundance in

tumor tissue. Whereas tumor cells in the STAT3 low sample were virtually negative for MMP-1 expression, tumorous epithelium in the high-STAT3 specimens displayed strong MMP-1 staining. There was no significant MMP-1 expression in stromal cells (i.e., fibroblasts, inflammatory cells) around the cancerous tissue.

These findings indicate that an abundance of MMP-1 mRNA in CRC specimens of the high-STAT3 type results in considerable levels of immunoreactive MMP-1 protein within the malignant areas of the tumors.

STAT3 Drives Expression of Active MMP-1 in CRC Cells

To investigate a possible causal link between STAT3 activity and expression of MMP-1 in colon carcinoma cells, we examined MMP-1 gene transcription upon experimental activation of STAT3 in the HT-29 colon carcinoma cell line. Previously, we have shown that although quiescent HT-29 cells are negative for STAT3 activity, STAT3 is activated by IL-6. In addition, we generated HT-29 derivatives that either overexpress STAT3 (HT-29 STAT3) or stably express a constitutively active (HT-29 STAT3 c.a.) or a dominant-negative STAT3 variant (HT-29 STAT3 d.n.), respectively [5].

STAT3 activity in HT-29 cells was manipulated via IL-6 stimulation or through protein expression of the variants, and the resulting changes in both MMP-1 mRNA and MMP-1 enzyme activity were determined (Figure 5). MMP-1 mRNA was quantified by real-time PCR, whereas MMP-1 activity was measured by the turnover of a fluorogenic substrate. Figure 4*A* shows that incubating HT-29 cells with 20 ng/ml IL-6 leads to a clear increase in MMP-1 mRNA. An overexpression of STAT3 in HT-29 STAT3 cells, which coincides with a threshold activity of STAT3 tyrosine phosphorylation [5], results in an increase of MMP-1 mRNA compared with parental HT-29 cells. IL-6 stimulation of HT-29 STAT3 cells evokes a further strong increase in the concentration of MMP-1 mRNA. A stable expression of the constitutively active STAT3 variant in HT-29 STAT3 c.a. cells resulted in a more than threefold increase of MMP-1 mRNA compared with nontransfected cells.

The analysis of MMP-1 enzyme activity in HT-29 cells under an experimentally altered status of STAT3 activation gave similar results (Figure 4*B*). Stable expression of STAT3 as well as acute IL-6 stimulation evoked a more than 10-fold induction of MMP-1 enzyme activity in HT-29 cells. Stimulation of both HT-29 cells and HT-29 STAT3 cells led to enhanced MMP-1 activity compared with nonstimulated cells. Notably, the expression of a dominant-negative STAT3 variant (STAT3 Y705F) completely suppressed both basal and IL-6–induced MMP-1 activity in HT-29 cells.

These results clearly suggest that STAT3 is essential for increased expression of MMP-1 in CRC. More importantly, tyrosine phosphorylation analysis shows that the respective degrees of MMP-1 induction in HT-29 observed under varied conditions mirror the respective levels of STAT3 activation [5].

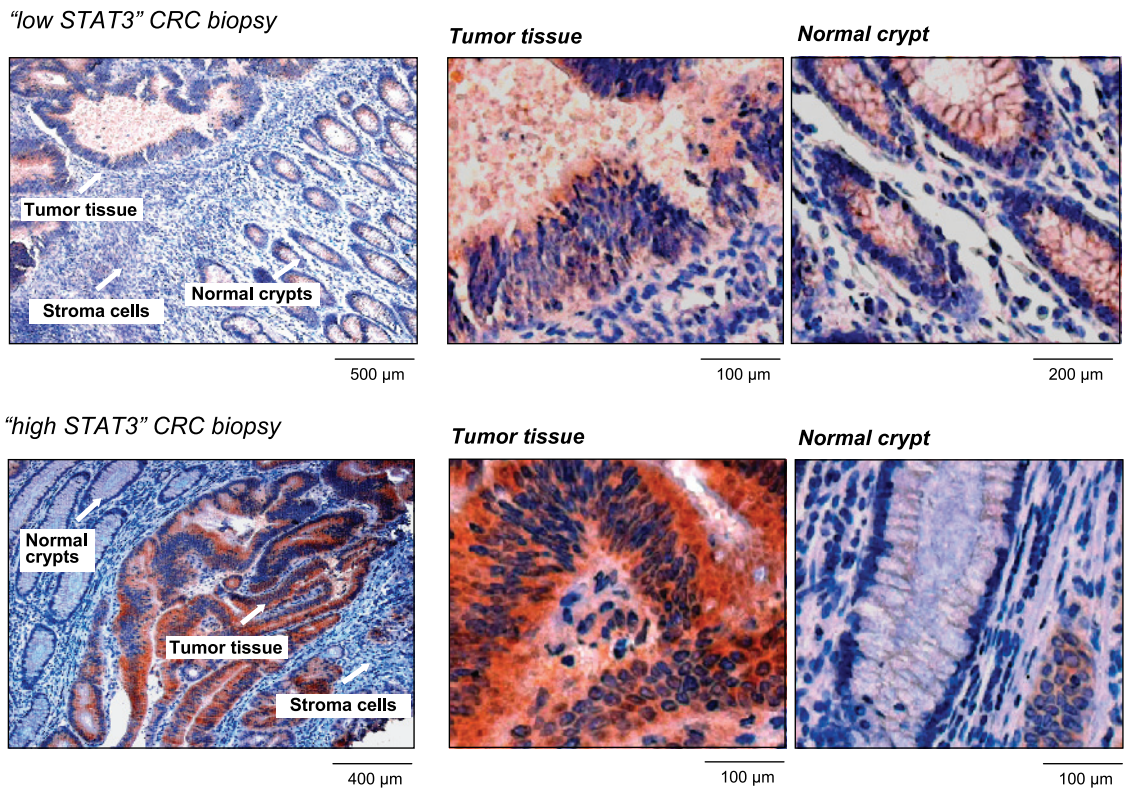

Figure 3. *Immunohistochemical staining for MMP-1 in paraffin-embedded specimens of high-STAT3 and low-STAT3 primary CRC biopsies. Sections from representative tumor samples positive (top) and negative (bottom) for tyrosine-phosphorylated STAT3, showing both differentiated normal cells in crypt structures and de-differentiated tumor tissue, were treated with an antibody specific for MMP-1. Sites of differentiated normal cells in crypt regions, of tumor tissue, and of stromal cells are indicated by arrows in the left-hand pictures, center, and right-hand pictures show enlargements of tumor tissue and normal crypt structures.*

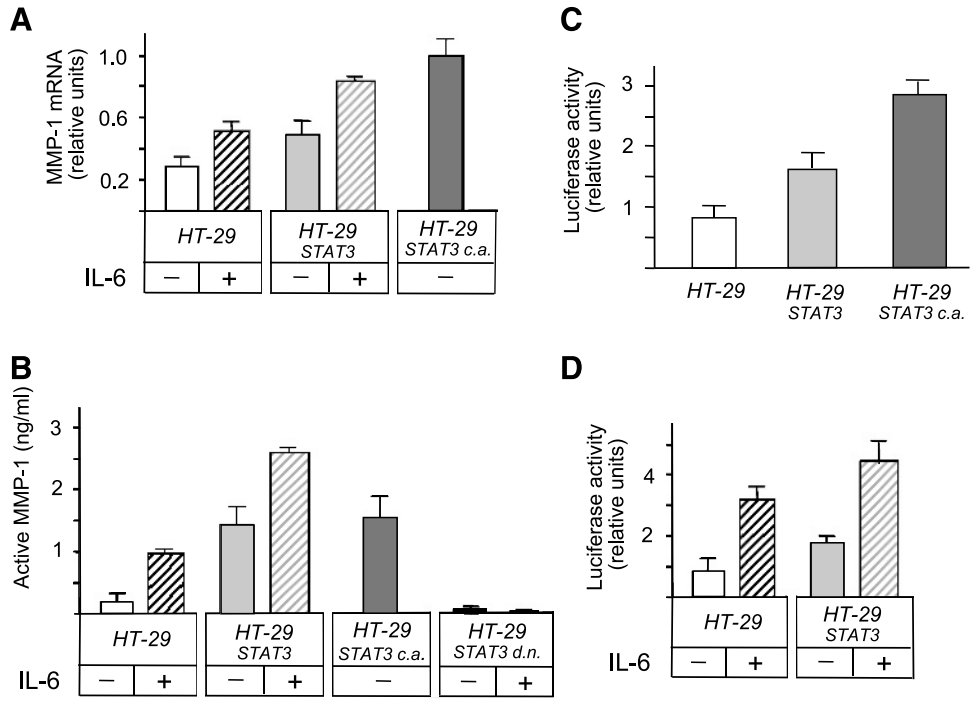

Figure 4. *Determination of MMP-1 expression in HT-29 colon carcinoma cells dependent on STAT3 activation. (A) Influence of STAT3 activation by cell stimulation with IL-6 and/or (over)expression of STAT3 variants. Parental HT-29 cells and derivatives stably expressing STAT3 or STAT3 c.a. were grown for 24 hours in the absence or presence of 20 ng/ml IL-6 as indicated. Total RNA was isolated, transcribed into cDNA, and subjected to quantitative RT-PCR with specific primers and probes for MMP-1 as described in Materials and Methods. Three independent experiments, each run in triplicate, were performed, and the results were normalized to 18S rRNA expression. Relative MMP-1 expression was calculated by arbitrarily setting the expressions from samples with STAT3 c.a. as 1 (100%). (B) Influence of STAT3 activation on enzymatic MMP-1 activity. HT-29 cells (over)expressing STAT3, the constitutively active variant STAT3 c.a. or the dominant-negative STAT3 mutant Y705F (STAT3 d.n.) were cultured for 4 days in the absence or presence of 20 ng/ml IL-6 as indicated. Culture supernatants were assayed for active MMP-1 by the turnover of a fluorogenic substrate as described in Materials and Methods. Data represent the means of three independent experiments. (C) Influence of STAT3 expression in HT-29 cells on MMP-1 promoter activity. HT-29 cells or HT-29 cells stably (over)expressing STAT3 or STAT3 c.a., respectively, were transiently transfected with 1 μg of the −4372 hMMP-luci plasmid together with 0.1 μg of* Renilla *plasmid as an internal control. After 14 hours, cells were harvested and protein lysates were assayed for luciferase and renilla luciferase activities as described in Materials and Methods. (D) Influence of IL-6–induced STAT3 activation on MMP-1 promoter activity in HT-29 cells. The experiment was performed as described in (C), but after 14 hours cells were washed and, before lysis, incubated for 8 hours with fresh medium with or without 20 ng/ml IL-6 as indicated. Results in (C) and (D) represent the means of three independent transfections, each run in triplicate.*

To determine whether activated STAT3 enhanced transcription from the MMP-1 promoter in colon carcinoma cells, we employed the reporter gene construct −4372 hMMP-luci in which the 4.3-kb human MMP-1 promoter was placed upstream of a luciferase reporter gene [39]. First, the reporter gene construct was transiently transfected into parental HT-29 cells and into HT-29 derivatives stably (over) expressing either STAT3 or STAT3 c.a. (Figure 4*C*). We observed that enhanced STAT3 expression and activity in both cell lines resulted in a two- to threefold increase in transcription driven by the MMP-1 promoter compared with nontransfected cells.

Figure 4*D* shows that IL-6 treatment strongly induced the activity of the MMP-1 promoter in HT-29 cells as well as in HT-29 cells overexpressing STAT3. In HT-29 STAT3 c.a. cells, IL-6 did not induce a further increase of MMP-1 transcription compared with nonstimulated cells (data not shown), probably due to a saturation of the respective mechanisms.

These data demonstrate that STAT3 activation promotes transcriptional activity of the MMP-1 promoter in HT-29 cells. They also point to a functional relation between STAT3 activity and expression of a protease with particular relevance for tumor cell malignancy by tissue invasion.

Xenografts of HT-29 Cells Develop STAT3 Activity and MMP-1 Expression Simultaneously and in a Colocalized Fashion

We have previously shown that HT-29 cells are negative for tyrosine-phosphorylated STAT3 activity in cell culture but develop STAT3 activity when growing out into tumors in nude mice [5]. Here, we demonstrated a transcriptional connection between STAT3 activation and induction of MMP-1 expression in colon carcinoma cells. We wanted to further substantiate these results by demonstrating that the development of STAT3 activation in HT-29 xenograft tumors is accompanied by an increase in MMP-1 abundance. First, by quantitative RT-PCR, we compared the levels of MMP-1 mRNA in cultured HT-29 cells with those in xenograft tumors arising 2 weeks after injection into nude mice. Figure 5*A* shows that the relative quantity of MMP-1 mRNA in HT-29–derived tumors was over fivefold higher compared with HT-29 cells cultivated *in vitro*.

Next, we studied the effect of altered STAT3 activity in HT-29 cells on MMP-1 expression upon development of xenograft tumors. Tumors grown from HT-29 cells as well as from HT-29 derivatives expressing constitutively active STAT3 (HT-29 STAT3 c.a.) or dominant-negative STAT3 Y705F

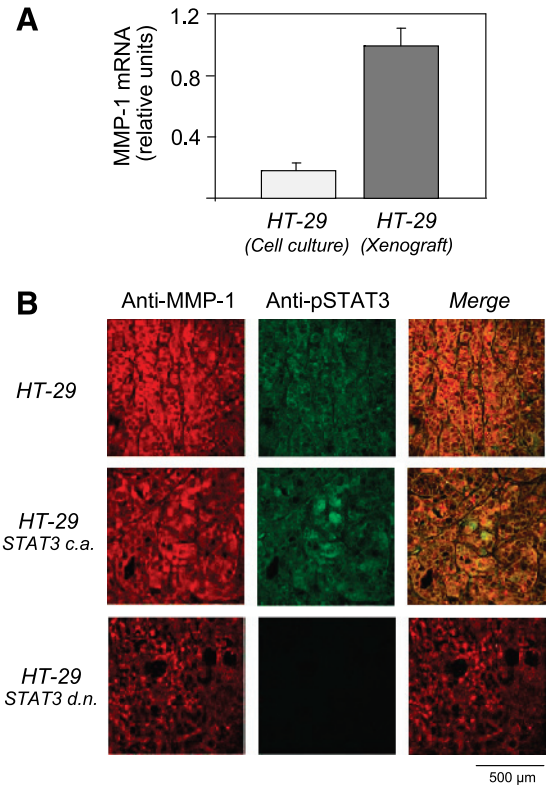

Figure 5. *Determination of MMP-1 expression in HT-29 xenograft tumors in correlation with STAT3 activity. (A) Comparative quantification of MMP-1 mRNA in cultured HT-29 cells and HT-29–derived xenograft tumors. Total RNA was isolated from confluent cultures of HT-29 cells or from a representative xenograft tumor explanted 2 weeks after subcutaneous injection of cells into a nude mouse. RNA was transcribed into cDNA, and quantitative RT-PCR with specific primers and probes for MMP-1 was performed as described in Materials and Methods. Results were normalized to 18S rRNA expression. Relative MMP-1 abundance was expressed as fraction of the value for the xenograft tumor, which was arbitrarily set 1. The figure represents the means of three independent experiments. (B) Immunohistochemical examination of HT-29–derived xenograft tumors for activated STAT3 and MMP-1. Representative sections from a tumor induced by injection of HT-29 (top), HT-29 STAT3 c.a. (center), and HT-29 STAT3 d.n. (bottom) into nude mice were reacted with an antibody specific for MMP-1 (left) or STAT3 pY705 (center) or with a combination of both antibodies (right).*

(HT-29 STAT3 d.n.) were subjected to immunohistochemical examination using antibodies to tyrosine-phosphorylated STAT3 and MMP-1 (Figure 5*B*). Both antibodies stained carcinoma cells in HT-29 tumors, and costaining with both antibodies revealed a colocalization of the two antigens. Levels of both p-Y-STAT3 and MMP-1 were clearly enhanced in tumors derived from HT-29 STAT3 c.a. cells and hugely diminished in tumors of HT-29 STAT3 d.n. cells.

These data further support our notion that STAT3 activation is directly involved in the control of expression and activity of MMP-1, and, thus, malignancy, of colon carcinoma cells.

High-Affinity Binding Sites for STAT Transcription Factors Exist within the Promoters for Human MMP-1 and MMP-3

Finally, we wished to demonstrate that STAT3 directly interacts with human MMP promoters and to identify relevant binding sites. To this end, the sequences of the human promoters for MMP-1 and MMP-3 were analyzed for DNA elements matching the known STAT-binding consensus sequence as given by Ehret et al. [46]. Using the MEME search blast algorithm [47], we identified six potential high-affinity STAT3 binding within the human MMP-1 promoter (GenBank accession no. AF023338) and four within the human MMP-3 promoter (GenBank accession no. U43511) and ordered them within the respective groups by decreasing similarity with the consensus STAT3 binding site defined by Ehret et al. (Figure 6*A*).

Oligonucleotide hybrids representing these sequences and a wild-type and a mutant (m67) STAT3-binding site from the human c-fos promoter [41] as positive controls were subjected to EMSA analysis for specific binding of activated STAT3. Upon expression in 293T cells, STAT3 was activated by IL-6 stimulation. Appearance of STAT3–DNA complexes was tested and verified by supershifting. As shown in Figure 6*B*, one site from each MMP-1 and MMP-3 promoter, notably the ones with the highest similarity to the consensus according to the MEME search blast, specifically interacted

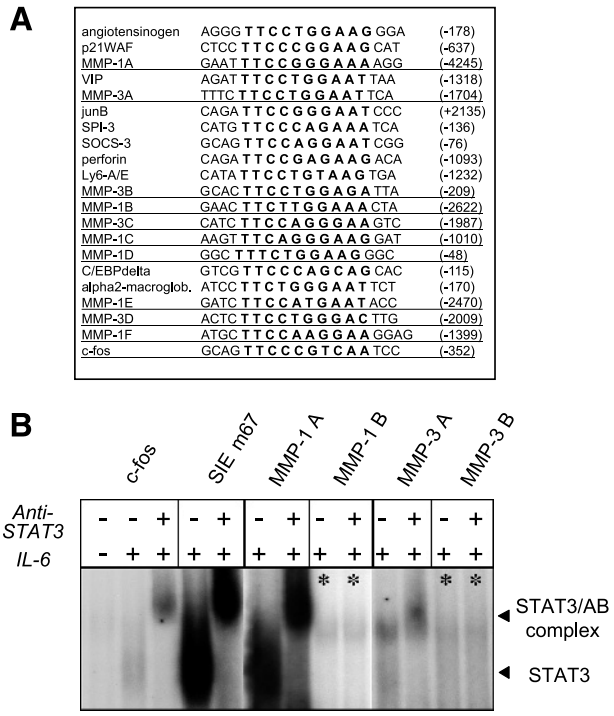

Figure 6. *Specific binding of STAT3 to cognate sequence elements within the human MMP-1 and MMP-3 promoters. (A) Sequences of potential STAT binding sites in the human MMP-1 and MMP-3 promoter as well as known STAT3 recognition elements employed as controls. Sites were identified using the MEME search blast algorithm [47] and ordered by decreasing similarity with the consensus STAT3 binding site defined by Ehret et al. [46]. Positions relative to the respective transcriptional start sites are given in brackets. (B) Analysis of STAT3 DNA binding to four potential STAT binding elements from the human promoters for MMP-1 and MMP-3 (sequences in A) and to two known STAT3 recognition sequences (wild-type and mutant SIE m67 STAT binding site from the human c-fos promoter). Extracts were either unstimulated or stimulated with IL-6 for 30 minutes as indicated before complex formation was assessed by EMSA. The specificity of STAT3 DNA binding was confirmed by supershifting using a STAT3-specific antibody. Positions of STAT3-containing DNA–protein complexes are marked by arrows. The asterisk (*) indicates background activity of unspecific DNA-binding complexes.*

with STAT3. MMP-1A represents the sequence element GAATTTCCGGGAAAAGG at position −4245 of the MMP-1 promoter, MMP-3A is the sequence element TTTCTTCCTGGAATTCA at position −1704 of the MMP-3 promoter. Neither the second best (MMP-1B and MMP-3B) nor any other putative STAT binding element from Figure 6*B* displayed significant complex formation with STAT3 in this assay (results not shown).

We infer from these results that activated STAT3 immediately interacts with the human MMP-1 and MMP-3 promoter.

Discussion

There is accumulating evidence for the importance of oncogenic signaling by STAT3 in the pathogenesis of various human cancers. Thus, we focused on the contribution of STAT3 to the onset and development of colorectal cancer. Our previous work shows that STAT3 may contribute to oncogenesis in the colon epithelium by exerting a promoting effect on cell proliferation [5], whereas this paper demonstrates its critical involvement in the enhancement of invasive cell behavior.

One of the prerequisites for the invasiveness of tumor cells is increased motility, and, of metastasis, a common feature of late-stage CRC. The pleiotropic protein STAT3 is known to exert control on cell motility in various settings. STAT3 is crucial, e.g., for cell motility during gastrulation [48], wound healing, and blood vessel formation [49]. We recently observed that the invasiveness of trophoblast cells in pregnancy is directly influenced through STAT3 activation [5,50].

More importantly, inadequate STAT3 activity was found correlated with cancer-associated motility of breast [27,51] and ovarian cancer [12] as well as of choriocarcinoma cells [44]. In mesenchymal-like and colon carcinoma cells, STAT3 plays a critical role in the transmission of invasiveness-promoting signaling by the c-Met receptor [52,53].

Altered invasiveness in transformed cells is a consequence of changes in the status of cell adhesion, the cytoskeleton, and gene expression. STAT3 has been shown to act on all these aspects: In addition to its well-established role as a transcription factor, STAT3 may also function as a signaling adaptor at focal adhesions and, presumably, as a persistent scaffold factor in cytokine/growth factor receptor assemblies [12]. As recently reported, STAT3 can signal in an as yet unknown way through Rho GTPases, thus regulating multiple cellular functions including actin cytoskeleton reorganization and cell migration [54].

Transcriptional regulation of target genes provides an obvious route through which STAT3 enhances cellular malignancy and, in particular, invasiveness. A central aspect of invasiveness is the expression of proteases that render the tumor cells capable of digesting constituents of the ECM. Various reports point to a role of STAT3 in promoting invasive cell behavior by exerting influence on the transcription of protease genes. In metastatic melanoma cells, a link was found between the expression of MMP-2, invasiveness, and constitutive STAT3 activity. More importantly, inhibition of STAT3 by a dominant-negative mutant reduced MMP-2 expression and invasiveness and blocked metastasis in nude mice [55]. In skin epithelial cells, STAT3 was shown to mediate the IL-6–provoked induction of MMP-1 and MMP-3 [56]. However, STAT3 is involved in controlling the expression of MMP-7 in prostate carcinoma cells [26] and of MMP-9 in cervix carcinoma cells [57].

We showed that STAT3 directly drives transcription from the MMP-1 promoter in colon carcinoma cells and also defined a binding site for STAT3 in the MMP-1 as well as in the MMP-3 promoter. However, the precise role of STAT factors in MMP gene regulation in colon cancer cells requires further elucidation. Despite the presence of various additional consensus sequences, we identified only one sequence element in both the MMP-1 and the MMP-3 promoter that specifically binds to activated STAT3. This finding leaves open the possibility that the other elements interact with STAT1 or STAT5, both of which are also frequently active in CRC biopsies [5]. Furthermore, those sequences showing no affinity for STAT3 in our assay may require additional binding sites for the interaction with STAT3 tetramers, as recently observed for other promoters [58,59].

Transcription of the MMP-1 gene is regulated in a complex manner. The process appears to be influenced by the orchestrated action of various transcription factors and seems also to depend on the type of cells involved. Apart from STAT cognate elements, other regulatory elements within the MMP-1 promoter region include various transcription factor binding sites for AP-1, GATA binding, and ETS proteins [60,61]. The cooperative contribution of AP-1 and ETS transcription factors toward control of MMP-1 expression was demonstrated by the observation that a single nucleotide polymorphism creating an additional ETS binding site close to the AP-1 recognition element at −1602 bp results in greater transcriptional activity [39]. However, we found that, starting from different "basal" levels, STAT3 activation profoundly enhanced transcription from both variants of the MMP-1 promoter in colon carcinoma cells (data not shown). The relative importance of AP-1– and STAT-related signaling pathways in the control of MMP-1 expression apparently depends on the cell type. For instance, a specific inhibitor of JAK3, a kinase operative in the activation of STATs, almost completely inhibited MMP-1 mRNA induction and protein abundance in human chondrocytes. In contrast, inhibition of ERK1/2 kinases signaling via AP-1 had little effect on the expression of MMP-1 in these cells [62]. We conclude that, along with its importance in CRC proliferation and transformation, the STAT3 pathway is one driving force responsible for overexpression and activity of MMP-1 in invasive colonic cancer cells. The prognostic significance of MMP-1 expression in colorectal cancer has already been reported. High levels of MMP-1 expression have been correlated with metastatic spread of tumors and poor prognosis of colorectal cancer [63–66]. Interestingly, MMP-1 inhibitors such as batimastat blocked peritoneal carcinomatosis and liver metastasis development in experimental colon carcinoma [67]. Of all the proteases addressed in study, MMP-1 is one whose expression level is particularly linked with STAT3 activation in colorectal cancer. There are, however, other

proteases whose expression level was also found to follow the degree of STAT3 phosphorylation during the course of this study. Induction of MMP-7 (matrilysin) expression in prostate carcinoma cells by fibroblast growth factor was shown to involve STAT3 through a probable interaction with STAT binding sites within the matrilysin promoter [26]. A recent study reported a correlation between tyrosine-phosphorylated STAT3 and expression of MMP-9 in breast carcinoma cells and demonstrated a direct influence of STAT3 on MMP-9 promoter activity [27].

The enzymatic activity of MMPs is specifically inhibited by TIMPs, and high levels of TIMP-1 and TIMP-2 are associated with aggressive cancers [62]. STAT3 has been described as contributing to the downregulation of TIMP-1 expression in synovial lining cells [22]. We recently found that STAT3 activation through leukemia inhibitory factor enhances invasiveness and coincides with a decrease of TIMP-1 expression in choriocarcinoma cells [44]. Interestingly, in the course of this study we observed that TIMP-1 mRNA was significantly less abundant in colorectal cancer biopsies with high STAT3 activity (data not shown).

In conclusion, STAT3 activation can contribute to the malignancy of many different types of cancer. One such contribution involves the promotion of cell invasiveness through (cell type specific) alteration in the pattern of protease activity. Our data suggest that STAT3-controlled proteolysis via MMPs is a major determinant of local tumor progression and metastasis of colorectal cancer and, thus, is an attractive potential target for therapeutic intervention. Moreover, p-Y-STAT3 and MMP-1 are likely to be of prognostic value or be of diagnostic importance with regard to CRC progression.

Recent reports point to an important role of aberrant STAT3 activity in metastasis of various malignant tumor entities such as cutaneous squamous cell carcinoma [68], melanoma [69], or hepatocellular carcinoma [70]. To investigate the underlying molecular mechanisms, our future work will include the analysis of patient CRC biopsy samples and will take advantage of mouse models that closely mimic stages of human CRC.

Acknowledgements

We thank Constanze E. Brinckerhoff for the MMP-1 reporter construct and Edith Pfitzner for the HT-29– and CoGa-1–derived cell lines.

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