Meinen Eltern

ORIGINS AND CONSEQUENCES OF PERSISTENT STAT3 ACTIVATION IN COLORECTAL CARCINOMA CELLS

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ABBREVIATIONS

Abl	Oncogenic Tyrosine Kinase from Abelson Murine Leukemia Virus
ADAM	Disintegrin-like and Metalloproteinase
Ang	Angiogenin
AP-1	Activator Protein - 1
APMA	4-Aminophenylmercuric Acetate
bcl_{xL}	B-cell $Lymphoma_{xL,}$ a gene that inhibits apoptosis
bp	base pair
BSA	Bovine Serum Albumine
CIS 1	Cytokine Inducible Src Homology 2-Domain (SH2) - containing Protein
d	days
DMSO	Dimethyl Sulfoxide
DTT	Dithiothreitol
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMSA	Electrophoretic Mobility Shift Assay
Erk	Extracellular Signal-Regulated Kinases
Ets	Erythroblast Transformation-Specific Domain Transcription Factors
FAP	Familial Adenomatous Polyposis
FGF	Fibroblast Growth Factor
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
gp130	Glycoprotein 130
GRO	Growth Regulated Oncogene
hMMP	Human Matrix Metalloproteinase
hMMP-1/luci	Human MMP-1 Promoter/Luciferase Reporter Plasmid
HNPCC	Hereditary Non-Polypous Colorectal Cancer

HGF	Hepatocyte Growth Factor
IL	Interleukin
INF	Interferon
IP-10	Interferon gamma Inducible Protein – 10
JAB	Jak-Binding Protein
Jak	Janus Kinase
MAPK	Mitogen-Activated Protein Kinase
Mcl	Myeloid Cell Leukemia
MCP	Monocyte Chemotactic Protein
MCSF	Macrophage Colony Stimulating Factor
MEK1/2	Mitogen-activated Protein Kinase Kinase 1 and 2
MIP	Macrophage Inflammatory Protein
MLH1	Human DNA Mismatch Repair Protein
MT1 - MMP	Membrane-Type MMP-1
OSM	Oncostatin M
p-	phospho-
PAI	Plasminogen Activator Inhibitor
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PDGFR	Platelet Derived Growth Factor Receptor
PEA-3	Polysaccharide Egg Antigen-3
PIAS	Protein Inhibitors of Activated STATs
ΔRn	Normalize Reporter Signal
RT–PCR	Reverse-Transcriptase-Mediated PCR
SCCHN	Squamous Cell Carcinoma of the Head and Neck
SDS	Sodiumdodecylsulfate
SDS-PAGE	Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis
SF	Scatter Factor
SH2	Src Homology
SNP	Single Nucleotide Polymorphism

SOCS	Suppressor of Cytokine Signaling
Src	Src Oncogene-Encoded Protein-Tyrosine Kinase
SSC	Sodium Chloride and Sodium Citrate Solution
SSI	Induced STAT-Inhibitor
STAT	Signal Transducer and Activator of Transcription
TAD	Transcriptional Activation Domain
TAE	TRIS/ACETATE/EDTA
TEMED	N`N`N`N-Tetramethyl-Ethylendiamin
TGF	Transforming Growth Factor
TSH	Thyroid Stimulating Hormone
TIMP	Tissue Inhibitor of MMP
UICC	*Union Internationale Contre le Cancer* - International Union Against Cancer
uPA	Urokinase-Type Plasminogen Activator
uPAR	uPA Receptor
VEGF	Vascular Endothelial Growth Factor

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1. ZUSAMMENFASSUNG

Der „Signal Transducer and Activators of Transcription“ Stat3 vermittelt Signale von Cytokinen und Wachstumsfaktoren in den Zellkern und steuert fundamentale zellphysiologische Prozesse wie Proliferation, Differenzierung und Apoptose. Seit wenigen Jahren ist bekannt, dass Stat3 ein potenzielles Oncoprotein ist. Es tritt in konstitutiv aktiver Form in vielen malignen Tumoren auf und gilt heute als vielversprechende Zielstruktur zur Tumortherapie.

In dieser Arbeitsgruppe wurde zum ersten Mal gefunden, dass Stat3 im colorectalen Carcinom (CRC) hyperaktiv ist. Ziel dieser Arbeit ist es, Ursachen für die erhöhte Stat3 -Aktivität und Auswirkungen von Stat3 auf die Malignität der Colon-Tumorzellen aufzuklären.

Zur Verfügung standen mehrere CRC-Zelllinien, in denen verschiedenen Formen von Stat3 durch retrovirale Infektion überexprimiert wurden. In dieser Arbeit wird gezeigt, dass eine verstärkte Expression von Stat3 in allen untersuchten Linien die Proliferation deutlich beschleunigt. Auch eine Aktivierung von Stat3 durch das Cytokin IL-6 führte zu einer schnelleren Zellteilung. Der proliferative Effekt war am stärksten, wenn eine konstitutiv aktive Mutante von Stat3 exprimiert wurde und wurde durch eine dominant-negative Variante von Stat3 supprimiert. Der Einfluss von Stat3 auf die Zellproliferation ging einher mit der Tyrosinphosphorylierung des Proteins, die im Western-Blot untersucht wurde.

Parallel dazu wurde der Einfluss von Stat3 auf Parameter der malignen Zelltransformation studiert. Eine konstitutiv aktive Mutante von Stat3 war in der Lage, sowohl die Entstehung von Kolonien in „*Soft Agar*“ zu induzieren als auch die Zellmigration durch künstliche Basalmembranen zu verstärken.

Der zweite Teil der Arbeit widmet sich den Mechanismen, durch die Stat3 -Aktivität maligne Zelleigenschaften beeinflusst. Es wurde durch Verwendung von cDNA-Arrays und „*Real Time RT-PCR*“ gezeigt, dass in Tumorgewebe-Proben von CRC-Patienten die Expression der Matrix-Metalloproteinasen MMP-1, -3, -7 und -9 immer dann stark erhöht war, wenn gleichzeitig auch starke Stat3-Aktivierung zu beobachten war. Hieraus wurde die Hypothese abgeleitet, dass die Stat3 -Aktivität eine Ursache für die erhöhte Expression von Proteasen sein könnte, die dann das invasive Verhalten und damit die Malignität von Colorectal-Carcinomzellen fördern.

Die Untersuchungen konzentrierten sich auf die Matrix-Metalloproteinase MMP-1, in deren Promoterregion mehrere Erkennungssequenzen für STAT-Proteine identifiziert wurden. Für einen kausalen Zusammenhang zwischen Stat3 -Aktivität und MMP-1 Expression sprach der immunhistochemische Nachweis der Kolokalisation in Tumorgewebe. Reportergenexperimente in CRC-Zelllinien erbrachten den Beweis einer spezifische Induktion des MMP-1 Promoters durch Stat3-Aktivität. Des weiteren wurde auch demonstriert, dass die erhöhte Produktion der MMP-1 mRNA durch Stat3 -Vermittlung auch zu einer verstärkten MMP-1 Enzymaktivität führt.

In dieser Arbeitsgruppe wurde zuvor gezeigt, dass die konstitutive Stat3 -Aktivität in CRC-Zellen bei Kultivierung *in vitro* verloren geht. Interessanterweise kehrt sie zurück, wenn diese Zelllinien in Nacktmäuse implantiert werden und dort zu Tumoren auswachsen. Der letzte Teil dieser Arbeit umfasst Experimente zur Erklärung dieses Phänomen. Es wurde versucht, die Situation der Zellen im Tumor in Zellkultur nachzuahmen. Hierzu wurden die Zellen in nicht beschichteten Plastikgefäßen zu kultiviert, in denen sie nicht geordnet und adhärent wachsen, sondern unorganisierte Zellaggregate bilden. Es wurde gezeigt, dass unter solchen „tumorartigen“ Bedingungen Stat3 in den HT-29 Zellen durch Vermittlung eines sezernierten autokrinen Faktors aktiviert wird, bei dem es sich möglicherweise um ein Chemokin handelt.

2. SUMMARY

Signal Transducer and Activator of Transcription Stat3 is a regulator of fundamental cellular processes such as proliferation, differentiation and cell death. It possesses oncogenic properties and was found aberrantly active in various malignant tumors. Target genes of Stat3 are important for survival, proliferation, inflammation and migration. It was recently shown that Stat3 is persistently active in the great majority of malignant colorectal tumors (colorectal carcinoma, CRC). However, its role in development and progression of this disease has not been investigated so far.

One major purpose of this work is an analysis of functions of Stat3-activation in colorectal cancer cells by studying its influence on parameters such as cell proliferation, invasion properties, and anchorage-independent growth. Moreover, the project intends to correlate the expression of malignancy-associated proteinases with aberrant Stat3 activity, thereby possibly identifying novel target genes of Stat3 in the context of CRC. Another goal of this work is to investigate potential origins of persistent Stat3 activation in colorectal cancer tissue. Methods of discriminating between paracrine and autocrine mechanisms of Stat3 are to be developed with the goal of obtaining information on the nature of the factor(s) that induce Stat3 activity in CRC cells.

The potential functional involvement of Stat3 in the malignant transformation of colon epithelium cells was studied by using variants of the established colon carcinoma cell line HT-29 stably expressing different derivatives of Stat3. Heterologous overexpression of Stat3 and activation *via* the interleukin-6 resulted in accelerated cell proliferation, enhanced colony formation in soft agar and elevated invasiveness *in vitro*. All these effects were pronounced upon transfection with a constitutively active Stat3 mutant and suppressed by a dominant negative Stat3 variant.

An important determinant of cancer cell malignancy, invasiveness and metastasis is the proteolytic degradation of different components of the extracellular matrix, such as basement membrane and interstitial stroma. Stat3 is known to be activated by various proteins that also influence protease expression. Therefore possible connections between the transcriptional regulation of various proteases and the activity of Stat3 in colon cancer cells were investigated.

mRNAs from cancer samples were analyzed by means of cDNA arrays and Real-Time RT-PCR for protease expression patterns. A striking correlation of strong Stat3 activity with upregulation of matrix metalloproteinases MMP-1, MMP-3, MMP-7 and MMP-9 was observed. Notably, Stat3 was able to positively regulate transcription from the MMP-1

promoter in colon carcinoma cell lines as shown by luciferase reporter gene experiments. Moreover, Stat3 promoted the secretion of enzymatically active MMP-1. Finally, the co-localization of activated Stat3 and MMP-1 in tumor tissue could be demonstrated by immuno-histochemistry.

Previously. constitutive Stat3 activity was found abundant in de-differentiated cancer cells of CRC samples, but not in non-neoplastic colon epithelium. Unexpectedly, cell lines derived from malignant colorectal tumors lost persistent Stat3 activity in culture. Earlier work from this laboratory showed that xenograft tumors arising from implantation of colon carcinoma cells into nude mice developed restoration of Stat3 activity. In this work, evidence was obtained for the involvement of an extracellular stimulus within the tumor microenvironment as a trigger for STAT activation. Growth conditions forcing detachment of CRC cancer cells induced the secretion of a Stat3-activating autocrine factor whose abundance correlated with the level of Stat3 phosphorylation, as shown by Western blot experiment. Preliminary results suggest that the autocrine activity has the nature of a chemokine.

3. INTRODUCTION

3.1. Significance and Causes of Colorectal Carcinoma

3.1.1. Epidemiology and Prognosis of CRC

Colorectal carcinoma (CRC) is the most common malignant tumor of the alimentary tract in the Western world; it is the second most common type of cancer death for both sexes in Germany. In men, CRC ranks third after lung and prostate carcinoma with 14.2%, in women it has the second position behind the mamma carcinoma with 16.8%. Incidence grows exponentially with age, with 90% of colorectal carcinomas appearing after the age of 50. In Germany alone, 50,000 new cases of CRC are diagnosed annually, and about 50% of the patients die of the disease within 5 years. The incidence of CRC is considerably lower in Asia, Africa and South America (Klein *et al.,* 2003).

Sixty percent of the colorectal carcinomas are localized in the rectum, with 80% of these involving the rectum and sigma. At the time of diagnosis, colorectal carcinoma usually shows extensive local invasion and metastasis (Liotta, 1986). Prognosis of CRC depends on whether the tumor can be removed surgically. In the case of curative resection the prospects for the successful treatment of colorectal cancer depend critically on the stage at which the disease is diagnosed. The relative 5-year survival rates for colorectal cancer are: Stage *Union Internationale Contre le Cancer* - International Union Against Cancer (*UICC*) I: 90-100%; Stage *UICC* II: 78%; Stage *UICC* III: 54%, Stage *UICC* IV: 6%. Other than tumor stage, venous invasion, lymphogenic invasion and surgical quality are additional prognostic factors (Klein *et al.,* 2003).

The aetiology of CRC is not precisely defined, but involves a wide range of risk factors. Nutrition and life habits are believed to be of major relevance to the occurrence of gastrointestinal tumors. However, about 15% of colon and rectum carcinomas originate from genetic predisposition (Schackert *et al*., 1998). These genetically determined tumors include several syndromes that can be clinically and molecularly distinguished.

3.1.2. Molecular Aspects of CRC Formation

Colorectal tumors based on hereditary factors show a familial pattern of occurrence. Carriers of the very rare familial adenomatous polyposis (FAP) develop dozens or perhaps hundreds of intestinal polyps that, if untreated, progress to cancer in 60%-70% of cases. Most genetically determined bowel malignancies fall under the category of “hereditary

non-polypous colorectal cancer" (HNPCC). In this disease, also known as "Lynch syndrome", intestinal tumors develop at a young age, usually in the right colon, and are commonly associated with multiple neoplasms in other organs (*e.g.*, stomach, skin, uterus) (Ponz de Leon, 1994; Lynch *et al.,* 1995).

For research, both FAP and HNPCC syndromes represent models of carcinogenesis also for sporadic colorectal carcinomas. In this context, FAP is considered an equivalent of the more frequent (85%) "tumor suppressor" phenotype, whereas HNPCC mirrors the less frequent "mutator" phenotype (Reviewed in Reinacher-Schick and Schmiegel, 2002). Fundamental to the tumor suppressor phenotype is a multi-step accumulation of chromosomal and genomic aberrations and the development of a deoxyribonucleic acid (DNA) aneuploidy. It is associated with stepwise changes in phenotype, which represent the „classical" adenom carcinom sequence. This process involves both oncogenes and tumor suppressor genes (Vogelstein and Kinzler, 2004).

Activation of protooncogenes to yield oncogenes can happen through mutation, amplification or rearrangement. For gastrointestinal malignancies, multiple genetic aberrations have been described which basically concern the following groups: Oncogene-encoding receptor tyrosine kinases (*e.g.*, c-erbB-2), growth factors and their receptors (*e.g.*, EGF receptor, cripto), mediators of intracellular signal transduction (*e.g.,* K-ras, H-ras, N-ras, c-Src), cell cycle regulators (*e.g.,* pic-1), and transcription factors (*e.g.*, c-fos and c-jun). An additional issue is the phenomenon of genetic instability (Reviewed in Allgayer *et al*., 2001). Functional impairment of tumor suppressor genes results from mutations or loss of alleles. Important tumor suppressors in colorectal carcinogenesis are APC, genes on chromosome 18q (dcc, smad4 und smad2) and p53.

c-erbB-2 is probably of prognostic value for gastrointestinal carcinoma. It codes for a transmembrane tyrosine kinase of 185 kD in size, and over-expression was found to be associated with an unfavourable prognosis (Tsugawa *et al*., 1998; Lee *et al*., 2002).

Malfunction of intracellular signal transduction regulating has been associated with erroneous cell proliferation and differentiation. Here, much attention has been focused on the ras oncogenes (N-ras, K-ras, H-ras). They encode G-proteins localized at the intracellular face of the plasma membrane which transmit proliferative signals emanating preferentially from receptor tyrosine kinases. Mutations within in ras genes can lead to an elimination of the Ras proteins' intrinsic GTPase activity, resulting in a state of persistent activation (*e.g.*, Yan *et al*., 2002; Johnson *et al*., 2003). In gastrointestinal cancers, ras

mutations are most frequently found in carcinomas of the colon, rectum and pancreas. C-ras is discussed as a potential prognostic factor in CRC (Tsuda *et al*., 1995).

Elevated activity of the non-receptor tyrosine kinase Src in colon carcinoma compared to normal mucosa was reported in various studies (*e.g.*, Aligayer *et al.,* 2002). Src is involved in cell proliferation, rearrangement of the cytoskeleton and cell transformation. Aberrant activation can be caused by mutations, but is mainly the result of de-regulation of upstream signal cascades. Recent investigations point to an association of Src activity with enhanced invasiveness in colon carcinoma (Pories *et al*., 1998).

With regard to tumor suppressors, many clinical studies have centered around the role of p53. The wild-type form of this multifunctional protein induces negative regulators of the cell cycle such as Pic1, p21 and p27, and leads to an arrest in the G1 phase. After irreparable DNA damage, it leads to apoptosis, thus protecting the organism against malignantly transformed cells. These functions are impaired in p53 mutants associated with malignant tumors (Shiohara *et al*., 1994). In colon carcinoma, p53 malfunction is related to lymph node metastasis and shortened survival (Campo *et al*., 1994; Hamelin *et al*., 1994; Gervaz *et al*., 2002).

The tumor suppressor gene nm23 encodes a nucleotide diphosphate kinase and is involved in the suppression of tumor occurrence and metastasis (Boyd, 1989). Reduced expression of nm23 in colon carcinoma appears to be associated with the development of lymph node metastases (Brenner *et al*., 2003). It also seems to be of prognostic value in CRC (Bertucci *et al*., 2004).

Bcl-2 encodes an anti-apoptotic protein associated with the mitochondrial membrane. Ayhan *et al*. (1994) observed frequent loss of heterocygocity (LOH) of the bcl-2 locus in gastric carcinomas.

Mutations in the APC (adenomatosis polyposis coli) gene are essential in colorectal carcinogenesis. Since they occur in an early stage of the “adenoma carcinoma sequence”, APC is believed to have a “gatekeeper” function. APC mutations influence catenin-mediated gene regulation and lead to intra- and intercellular malfunctions (Reviewed in Karim *et al*., 2004). They can be detected in hyperproliferative colon mucosa prior to the occurrence of an adenoma; loss of the second APC allele usually happens in the early phase of adenoma development. Additional genetic alterations (K-ras, p53) subsequently result in the stepwise progression towards carcinoma formation (Heinzlmann *et al*., 2001).

The “mutator” phenotype is associated with defects in DNA repair processes. In the course of carcinogenesis, mutations or (inactivating) promoter hypermethylation in mismatch

repair genes cause multiple mutations in genes that directly or indirectly influence cell transformation, such as those encoding the receptor for transforming growth factor (TGF)-β or the anti-apoptotic protein Bax (Schalhorn *et al*., 2001). In hereditary non-polypous colon carcinomas (HNPCC), four different mismatch repair genes have been identified (hMLH1, hMSH2, hMSH6, PMS2) in which mutations are causal for malignant transformation (Liu *et al*., 2003; de Jong *et al*. 2004). Impaired DNA replication results in DNA microsatellite instability (MSI), the presence of repetitive mono-, di-, tri- or tetranucleotide sequences (Parker *et al*., 2004). However, MSI is not specific for HNPCC, but is also observed in 15% of sporadic tumors (Vogelsang *et al.,* 2001).

Altogether, carcinogenesis is a multistage process that involves multiple genetic and epigenetic events (Brown and Balmain, 1995; Balmain and Harris, 2000). These events are divided into three independent stages: initiation, transformation, and progression. The rate-limiting events in multistage carcinogenesis are believed to occur during transformation and progression. Thus, identifying the specific signalling pathways in the rate-limiting step is important to prevent cell transformation, as well as to inhibit tumor growth. In recent years several novel potential tumor markers and targets for therapy have been identified. Among them, the STAT family of transcription factors received much attention.

3.2. Stat3 in Tumor Formation

3.1.1. The Stat Family of Transcription Factors

Signal transducer and activator of transcription (STAT) proteins are a family of transcription factors that transmit signals, usually generated at cell surface receptors, to the nucleus where STATs bind to specific DNA promoter sequences, thereby regulating gene expression. Since discovery of the first STATs as key mediators of IFN signalling, a total of seven different STAT family members (Stat1, Stat2, Stat3, Stat4, Stat5A, Stat5B, and Stat6) encoded in distinct genes have been identified in mammalian cells (Darnell, 1997). STATs are normally inactive within the cytoplasm of cells and become activated by tyrosine phosphorylation in response to cytokines and growth factors. Signalling pathways mediated by STATs are critical for many normal cellular functions, including embryonic development, organogenesis, immunological interaction, growth, differentiation and survival (Buettner *et al*., 2002). In summary, Stat1-deficient mice are unable to respond to IFNs and are subsequently susceptible to bacterial and viral pathogens. Likewise,

disruption of Stat2 gives rise to animals unable to respond to type 1 IFNs, with increased susceptibility to viral infections (Candotti *et al*., 2002). Stat4- and Stat6-deficient animals reveal a requirement for interleukin (IL)-12– or IL-4–mediated proliferation of T cells, respectively (Decker *et al*., 2002). The phenotypes of Stat5A and Stat5B individual knockout mice reveal the importance of Stat5A in breast development and lactation and the importance of Stat5B in the development of sexually dimorphic patterns of gene expression within the liver. In addition to these phenotypes, Stat5A/5B double knockout mice are abnormal in their T cell and B cell development. Because Stat3-deficient animals die early in embryogenesis, the role of this protein in a number of biological functions had to be determined in conditional knockouts (see 2.2.2.).

The activation duration of STATs under physiological conditions is temporary and usually lasts anywhere from a few minutes (min) to several hours (h), and is tightly regulated by a number of cellular mechanisms such as tyrosine dephosphorylation, ubiquitin/ proteasome-mediated degradation, a negative feedback loop mediated by the CIS/SOCS/JAB/SSI family of proteins, or inhibition of STAT DNA-binding activity through association with a protein inhibitor of activated STAT (PIAS) proteins (Reviewed in Lin and Leonard, 2000). However, constitutively activated STATs, especially Stat1, Stat3 and Stat5, have been found in a variety of human tumors and cancer cell lines **(Tab. 1)**, including blood malignancies and solid tumors (Turkson and Jove, 2000). In various tumor cell lines, persistent signalling of specific STATs, in particular Stat3 and Stat5, has been shown to stimulate cell proliferation and prevent apoptosis through up-regulating a number of target genes encoding apoptosis inhibitors (Bcl-$x_{L,}$ Mcl-1), cell cycle regulators (cyclins D1/D2, c-Myc), and inducers of angiogenesis (Vascular Endothelial Growth Factor (VEGF)). Not surprisingly, inhibition of constitutively activated Stat3 or Stat5 leads to growth suppression or apoptosis. Although Stat1 activation is elevated in some tumors and cell lines, the function of this molecule has been associated with growth suppression rather than malignant transformation and thus can be considered a potential tumor suppressor (Buettner *et al*, 2002).

Tab. 1: *STAT activation in human cancer cell lines and primary tumors*[a]

	Activated STAT
Blood tumors	
Multiple myeloma	Stat1, **Stat3**
Leukemias	
HTLV-I-dependent	**Stat3**, Stat5
Erythroleukemia	Stat1, Stat5
Acute lymphocytic leukemia	Stat1, Stat5
Chronic lymphocytic leukemia	Stat1, **Stat3**
Acute myelogenous leukemia	Stat1, **Stat3**, Stat5
Chronic myelogenous leukemia	Stat5
Megakaryotic leukemia	Stat5
Large granular lymphocyte leukemia	**Stat3**
Lymphomas	
EBV-related/Burkitt's	**Stat3**
Mycosis fungoides	**Stat3**
HSV saimiri-dependent (T-cell)	**Stat3**
Cutaneous T-cell lymphoma	**Stat3**
Hodgkin's disease	**Stat3**
Solid tumors	
Breast cancer	Stat1, **Stat3,** Stat5
Squamous cell carcinoma of the head and neck (SCCHN)	Stat1, **Stat3**
Renal cell carcinoma	**Stat3**
Melanoma	**Stat3**
Ovarian carcinoma	**Stat3**
Lung cancer	Stat1, **Stat3**
Prostate carcinoma	**Stat3**
Pancreatic cancer	**Stat3**
Brain tumors	Stat1, **Stat3**

[a] Modified after Buettner *et al*., 2002

The origins of STAT activation are diverse, both in healthy and tumor tissue. Many different polypeptides (cytokines, hormones, growth factors) can activate STAT proteins, either through kinases of the janus kinase (Jak) family, which associate with receptors that are devoid of tyrosine kinase activity (*e.g.*, receptors of the glycoprotein (gp) 130 family), or by receptor tyrosine kinases carrying their own kinase domain (*e.g.*, EGF-R, PDGF-R (Bowman *et al*., 2001), IGF-1R (Zong *et al*., 2000)). It has also been shown in several studies, that STAT proteins are activated by some non-receptor tyrosine kinases (*e.g.*, v-Src (Garcia *et al.,* 2001)) or through G-protein-coupled receptors (macrophage inflammatory protein (MIP)-1, RANTES) **(Tab. 2)**. Stat2, Stat4 and Stat6 are activated

primarily by one or two single extracellular factors, whereas Stat1, Stat3 and Stat5 can be activated by a wide variety of factors (Levy and Gilliland, 2000; Bromberg, 2001) or oncoproteins (Migone *et al*., 1995; Garcia and Jove, 1998). Stat1α, Stat5, and Stat6 are indirectly linked to the malignancy of fibroblasts that are transformed by oncoproteins (Garcia *et al*., 1997). Stat3 is required specifically for v-Src induced transformation (Yu *et al*., 1995; Bromberg *et al*., 1998; Turkson and Jove, 2000). However, unlike the other STATs, Stat3 can act as an oncoprotein by itself (Bromberg, *et al.* 1999).

Tab. 2: *Cytokines, growth factors and oncogenes that activate signal transducers and activators of transcription (STAT) proteins relevant for oncogenesis*[a]

Stat1	Stat3	Stat5
IFNα, IFNγ EGF, PDGF, FGF c/v-Eyk ACRII	IL-6, IL-11, OSM, CNTF, LIF, IL-10, GM-CSF, leptin EGF, PDGF, v-Sis Insulin, TSH MIP-1, RANTES, MCP-1 Galphao Npm-alk $G_{\alpha}o$ c-Met, Ret, Ros Middle T antigen c/v-Src, v-Eyk, v-Abl, v-Fps c-Fes, Lck, Tel-Jak, Etk	IL-2, IL-7, IL-9, IL-15, IL-3, IL-5, GM-CSF EGF v-Abl Growth hormone, prolactin, erythropoetin, thrombopoetin

IFN, interferon ; IL, interleukin; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; CNTF, ciliary neurotrophic factor; LIF, leukaemia inhibitory factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; MIP-1, macrophage inflammatory protein; MCP-1, monocyte chemotactic protein-1; OSM, oncostatin M.
[a] Modified after Bromberg, 2000 and Bromberg, 2001

Dimerisation through reciprocal SH2-phospho-tyrosine interactions of tyrosine-phosphorylated STATs can occur either as homodimers, as seen with all STATs except Stat2, or in some cases as heterodimers, as seen with Stat1/2, Stat1/3, and Stat5A/5B. This leads to their accumulation in the nucleus where they interact with other transcriptional modulators bound to specific promoter sequences (cognate sequences of the type **TTC**NNN**GAA**) and directly regulate gene expression **(Fig. 1)**. STAT dimers are dephosphorylated within the nucleus and transported back to the cytoplasm (Darnell, 1997). In normal cells, STAT activation is transient, whereas in a large number of primary tumors and cancer-derived cell lines, STAT proteins (in particular Stat3) remain activated by persistently activated tyrosine kinases and/or a decrease in the negative regulators of STAT dephosphorylation (Bromberg, 2002).

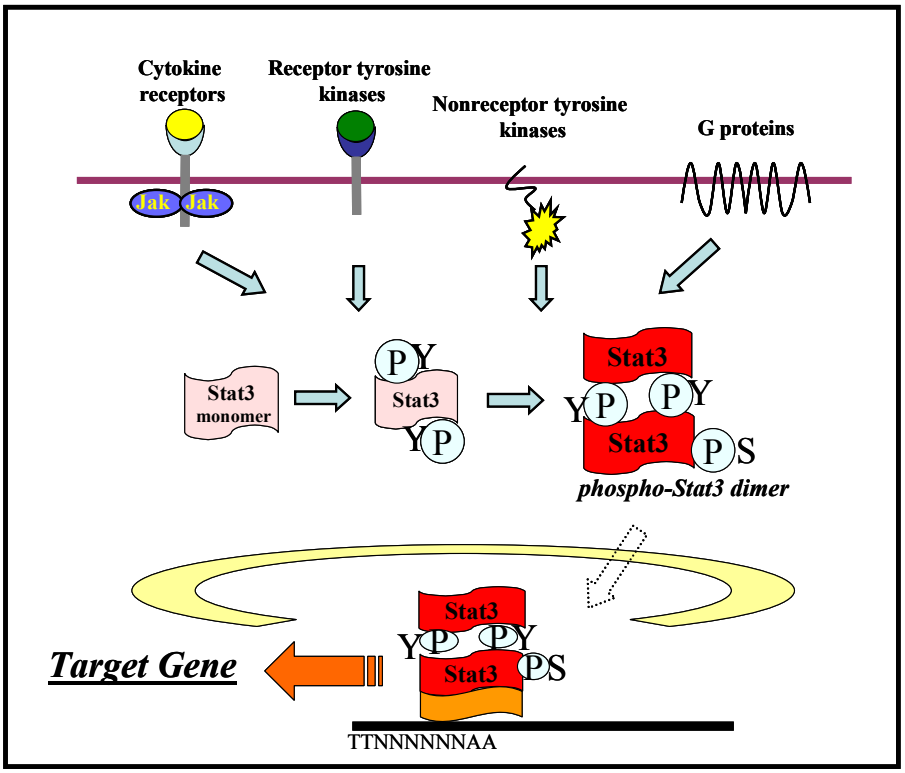

Fig. 1: A simplified overview of the STAT activation and function an example of the Stat3. *STAT proteins become tyrosine-phosphorylated by at least four different mechanisms: (1) Cytokine-mediated activation of the Jaks (e.g., IL-6, IFN); (2) Receptor tyrosine kinase mediated activation (e.g., EGFR, PDGFR); (3) Non-receptor tyrosine kinase (Src, Abl, Ros) and (4) G-protein-mediated activation (chemokines, TSH). Two tyrosine phosphorylated STAT proteins form dimers, and translocate into the nucleus. Once in the nucleus, STAT dimers bind specific promoter sequences, regulating the transcription of target genes.*

The basic molecular topology of STAT proteins is illustrated in **Fig. 2** using Stat3 as the prototype. The protein interaction site is required for dimer-dimer interactions to form tetrameric STAT molecules. Tetramerisation of STATs contributes to stabilized DNA-binding activity on weak promoters (John *et al*., 1999). The adjacent coiled-coil domain is another protein-protein interaction site that provides potential contacts for transcription factors and other regulatory proteins (Bromberg and Darnell, 2000). The DNA-binding domain in the center of the molecule determines DNA sequence specificity of individual STATs (Becker *et al.,* 1998). A linker region that participates in DNA-binding leads to the COOH-terminal SH2 domain, which is required for the recruitment of STATs to phosphorylated receptors and for reciprocal SH2-phosphotyrosine interactions between monomeric STATs to form dimers (Shuai *et al.,* 1994). The critical tyrosine (Y) residue required for SH2-phosphotyrosine interaction and thus STAT activation is located near the SH2 domain.

Fig. 2: The domain structure of full-length STAT proteins illustrated using Stat3 as the prototype. *Color scheme: Y, critical phosphotyrosine (amino acid 705); S, critical phosphoserine (amino acid 727); TAD, transactivation domain. See text for more details on the STAT functional domains.*

The transactivation domain (TAD) at the COOH-terminal end of the molecule is involved in communication with transcription complexes and, in case of Stat1 and Stat3, contains a serine (S) phosphorylation site (Wen *et al*., 1995). Serine phosphorylation has been shown to be required for maximal transcriptional activation of STATs. The primary amino acid sequence of the TAD varies between individual STATs but is essential for STAT function as shown by deletion experiments. Cell lines mutated in the critical residue in Stat1 retain only 20% of the activity of the wild-type Stat1 and are defective in response to interferon (IFN). TAD-deficient STATs still bind to DNA but usually function as dominant-negative counterparts of the full-length forms (Buettner *et al.,* 2002). Such truncated forms, for example Stat3-β (Caldenhoven *et al*., 1996), have been identified as naturally-occurring splice variants, but their normal physiological functions are not yet clear. Dominant-negative STATs, as well as activated mutants of STATs (such as "Stat3 c.a.", which is constitutively activated by forced dimerisation) (Bromberg *et al*., 1999), have been used to unravel the role of STATs in malignant transformation. Candidate serine kinases for the phosphorylation of STATs include the various mitogen-activated protein kinase family members (Turkson *et al*., 1999; Decker and Kovarik, 2000). The significance of STAT serine phosphorylation is not completely understood. However, the discovery that STATs cross-talk with members of other pathways, such as mitogen-activated protein kinase, indicates that STATs are embedded in complex signalling networks.

Besides serine kinases, many other (co)activators of STAT signalling have been identified thus far, including the histone acetyl transferases p300/CBP (E1A-binding protein 300), the transcription factors c-Jun, Sp1, and glucocorticoid receptor, the coactivators Nmi (N-Myc interactor) and MCM (minichromosome maintenance), the nuclear translocation protein NPI-1 (nucleoprotein interactor-1), and p48 (IFN regulatory factor family protein) (Bromberg and Darnell, 2000; Shuai, 2001). The physiological importance of the above-mentioned modulators of STAT signalling remains to be determined.

3.1.2. Roles of Stat3 in Oncogenesis

Stat3 is a SH2 domain-containing transcription factor that is latent in the cytoplasm of cells until stimulation with cytokines, growth factors and oncogenic tyrosine kinases with a pattern of cell-type dependence (*e.g.*, Sadowski *et al.,* 1993; Johnston *et al*., 1995; Stancato *et al.,* 1996; Takeda *et al*., 1998) (see **Tab. 2**). Stat3 was discovered in 1994 by three independent groups as an acute-phase response factor produced by liver cells (Lütticken *et al*., 1994; Akira *et al.,* 1994) or by cloning from a murine cDNA bank (Zhong *et al.*, 1994), and named Stat3 by Zhong and co-workers. Three main Stat3 isoforms have been described: Stat3-α with 92kDa, the shorter splice isoform Stat3-β of 83kDa and Stat3-γ, a short 72 kDa isoform of unknown physiological function, which is generated through proteolytic cleavage of Stat3-α (Akira *et al*., 1994; Hevehan *et al*., 2002).

Targeted disruption of Stat3 revealed that it is essential for early embryonic development, because mice lacking Stat3 die before birth (Takeda *et al.,* 1997). Tissue-specific gene deletions have demonstrated a critical role of Stat3 in the regulation of epithelial cell apoptosis, involution in the post-lactating mammary gland, skin remodelling, keratinocyte migration, macrophage inactivation, and down-regulation of inflammatory cytokines in T-helper cell responses (Chapman *et al*., 1999; Sano *et al*., 1999; Takeda *et al*., 1999). Furthermore, many different studies investigating STAT signalling (using established cell lines, primary cell cultures, and animal models as well as clinical tumor samples with matched adjacent normal tissues) suggest that Stat3 is highly activated in many types of cancer cells (*e.g.*,Garcia *et al*., 1997; Bromberg *et al*., 1999; Bowman *et al.,* 2000), and constitutively activated Stat3 actively participates in tumor development and progression (see **Tab. 1**) (*e.g.*,Catlett-Falcone *et al.,* 1999; Bromberg and Darnell, 2000; Turkson and Jove, 2000; Bowman *et al.,* 2000; Chan *et al.,* 2004; Nakanishi *et al*., 2004).

A critical role for Stat3 in malignant transformation was first proposed after initial studies showed that Stat3 is constitutively activated during *v-Src* transformation (Yu *et al.,* 1995). More recent studies have confirmed this view and have demonstrated that Stat3 signalling is required for oncogenic transformation by *v-Src* (Bromberg *et al*., 1998; Turkson *et al*., 1998). In addition to *v-Src*, many other transforming tyrosine kinases, such as *v-Eyk* (east lansing tyrosine kinase), *v-Ros* (viral Ros), *v-Fps* (Fujinami avian sarcoma viral oncogene (*v-Fes*)), Etk/BMX (epithelial and endothelial tyrosine kinase), and Lck (lymphocyte-specific protein-tyrosine kinase), constitutively activate Stat3 in the context of oncogenesis (Bowman *et al*., 2000). Genetic evidence for the intrinsic oncogenic potential of Stat3 derives from an experimentally designed constitutively active mutant of Stat3. The “Stat3

c.a." protein is sufficient for mediating certain aspects of cellular transformation of rodent fibroblast cells when stably expressed, and cells transformed by "Stat3 c.a." have the ability to form tumors in nude mice (Bromberg *et al*., 1999). Blocking of Stat3 DNA binding with antisense oligonucleotides or a dominant-negative Stat3 protein, Stat3-β, further established the critical role of Stat3 in oncogenesis (Reviewed in Bowman *et al*., 2000; Song and Grandis, 2000). In all cases, inhibition of persistent Stat3 signalling suppressed the transformed phenotype. In several cases, Stat3 was not shown to be essential for viability of normal cells (Ebong *et al.,* 2004). In contrast, many cancer-derived cell lines that contain constitutively activated Stat3 are dependent on this. Introduction of dominant negative Stat3 or Stat3 antisense oligonucelotides leads to induction of apoptosis, decreased angiogenesis, or growth arrest of these cells (Akira, 2000; Garcia *et al.,* 2001; Bromberg, 2002).

Because many different cytokines are known to activate STATs (Ihle, 2001), it is not surprising that constitutive activation of Stat3 is observed downstream of aberrant cytokine signalling derived from either autocrine or paracrine sources. In the context of cytokines, IL-6 signaling through the gp130 receptor subunit is particularly relevant in multiple myeloma and prostate cancer because IL-6-mediated activation of Stat3 has a key role in preventing apoptosis and stimulating growth in cancer cells derived from these tumors. Stat3 activation is also linked to a number of receptors with intrinsic tyrosine kinase activities that are independent of the cytokine receptor gp130 subunit (Buettner *et al.*, 2002). With regard to growth factor receptor signalling, the role of Stat3 in oncogenesis is well investigated in squamous cell carcinoma of the head and neck (SCCHN) and breast cancer. Human SCCHN cells, but not normal mucosal epithelial cells, typically over-express both the TGF-α and its cognate receptor, EGF receptor (Song and Grandis, 2000). It has been demonstrated that TGF-α /EGF receptor-mediated growth of transformed epithelial cells is dependent on the activation of Stat3.

Cellular transformation by activated Stat3 and its relatives occurs through the transcriptional regulation of specific genes. Many Stat3 target genes are known, including antiapoptotic proteins, proliferation-associated proteins, and proangiogenic factors **(Tab. 3)**. Still other genes must be regulated indirectly by Stat3, as shown by cDNA array studies, many of which may contribute to oncogenesis or tumor progression, *e.g.*, flotillin, phospholipid scramblase 1, caveolin-2, growth arrest specific-6 (Gas 6), CD-44, osteopontin and PDGF (Brocke-Heidrich *et al*., 2004).

Tab. 3: *Stat3 target genes*

Stat3 target gene	Reference
fra-1	Zerbini *et al*., 2003
fra-2	Rydziel *et al*., 2000
c-fos	*e.g.*, Ruff-Jamison *et al.*, 1994; Servirei *et al*., 1998
c-jun	*e.g.,* Fujitani *et al.,* 1994; Zhang *et al.,* 1999
c-myc	*e.g.* ,Yamanaka *et al.*, 1996; Narimatsu *et al.*, 1997; Bowman *et al*., 2001
c-myb	*e.g*., Yamanaka *et al*., 1996; Minami *et al.*, 1996; Narimatsu *et al*., 1997
fos-B	Rydziel *et al*., 2000
fas (together with c-jun)	*e.g*., Catlett-Falcone *et al.*, 1999; Ivanov, 2001
bcl-2	*e.g.,* Fukada *et al*., 1996; Epling-Burnette *et al*., 2001 ; Brocce-Heidrich *et al.*, 2004
bcl xl	*e.g.,* Zushi *et al*., 1998; Alas and Bonavida, 2003
mcl-1	*e.g.,* Puthier *et al*., 1999; Epling-Burnette *et al*., 2001
E-cadherin	*e.g.,* Rivat *et al*., 2004
SOCS-3	*e.g.,* Starr *et al*., 1997
PIAS-3	Russell and Richards, 1999
VEGF	*e.g.,* Niu *et al*., 2002; Wei *et al*., 2003
pim-1	Demoulin *et al*., 1999; Narimatsu *et al*., 2001
pim-2	Shirogane *et al*., 1999
cyclin D1	*e.g.,* Cressman *et al*., 1996
ICAM-1	*e.g.,* Caldenhoven *et al*., 1996; Romano *et al*., 1997
CD-18	*e.g.,* Wooten *et al*., 2000;
jun B	*e.g.,* Yamanaka *et al*., 1996;Rydziel *et al*., 2000
jun D	*e.g.* Zerbini *et al*., 2003; Rydziel *et al.*, 2000
β-catenin	*e.g.* Yamashita *et al*., 2002; Uttamsingh *et al.*, 2003
p19/ink4d	*e.g.* Tanaka *et al.,* 2000; Demoulin *et al*., 2001
α (M) integrin	Panopoulos *et al*., 2002
β (2) integrin	Panopoulos *et al*., 2002
β-actin	Naruishi *et al*., 2001; Efferth *et al.*, 2000
VLA-5 (α5 β1 integrin)	Naruishi *et al*., 2001
p 27/kip1	*e.g*., Kortylewski *et al*., 1999; Wooten *et al*., 2000
α 1-antychymo-trypsin (pACT)	Terstegen *et al.*, 2001

3.2. Cell Invasiveness, a Major Parameter of Malignancy in CRC

3.2.1. Acquisition of Invasive Properties by Carcinoma Cells

Metastatic spread of tumor cells is one of the major risk factors affecting clinical prognosis. Cancer cells possess a broad spectrum of migration and invasion mechanisms. These include both individual and collective cell-migration strategies.

As tumors become increasingly malignant, cells within them develop the ability to invade into surrounding normal tissues and through tissue boundaries to form daughter neoplasias (metastases) at sites distinct from the primary tumor. There are several sequential steps in this process including cell migration into the surrounding host tissue, infiltration and penetration into vessels for dissemination, attachment to capillary beds of distant organs, cell extravasation and subsequent growth in tissues. Invasion and metastasis are not unique for cancer as they also occur during embryonic development and in many non-cancerous diseases, such as ectodermal cell invasion during mesoderm formation, migration of neural crest cells and leukocyte motility (Le Douarin, 1993; Springer, 1994; Thier, 2002). Thus, the so-called cell migration genes may be frequently utilized by cancer cells to allow for metastatic spread of disease. Highly invasive cancers are usually characterized by aberrant activity of specific intra- or extracellular molecules such as protein kinases, phosphatases, transcriptional factors, proteolytic enzymes, and others (Reviewed in Sliva, 2004).

Tumor invasion can be considered as a process in which cells are activated, then move away from their initial location, by modifying their adhesiveness to the extracellular matrix, expressing new adhesion molecules and degrading the extracellular matrix components by the active secretion of proteases. These events require complex cross-talking between endothelial and tumor cells, extracellular matrix components, and cellular elements of the microenvironment. Adhesion molecules, such as integrins and cadherins, play a major role in signalling from outside to inside a cell, thereby controlling a cell's ability to sense and interact with its local environment (Allgayer *et al.,* 1997; Perl *et al.,* 1998). In the case of gastrointestinal carcinoma, E-cadherin, CD-44 (Cluster Designation-44) and ICAM-1 (Intracellular Adhesion Molecule 1) have been shown to be important for invasiveness (de Both *et al*., 1999; Alexiou *et al*., 2001; Masaki *et al*., 2001).

Importantly, proteolytic enzymes and their inhibitors (*e.g.*, matrix metalloproteinases and TIMPs) not only have the ability to break down the components of the extracellular matrix,

but are also involved in the release of factors which can positively or negatively affect the growth of the tumor cells (*e.g.*, Schwartz *et al*., 1994; Heiss *et al*., 1995; Cairns *et al*., 2003**)**. Both processes are under the tight regulation of a balance between stimulating and inhibiting factors. The existence of common mechanisms of regulation and the presence of naturally-occurring factors that inhibit invasion make the inhibition of both processes possible. Tumor cells may develop adaptive mechanisms that enable the tumor to withstand the treatment, particularly when only one mechanism or one process is inhibited. In post-extravasational stages of metastasis, the roles of adhesion and proteolysis are accompanied by other processes such as apoptosis, dormancy, growth factor-receptor interactions and signal transduction. Recent work has also demonstrated that not only the *immediate* cellular microenvironment (*i.e.*, specific cell-cell and cell-matrix interactions), but also the *extended* cellular microenvironment (*i.e.*, vascular insufficiency and hypoxia in the primary tumor), can modify cellular gene expression and enhance metastasis (Reviewed in Cairns *et al*., 2003). Mechanisms of invasiveness and metastasis also involve a transcription deregulation of proto-oncogenes, such as c-Met. The c-Met protein contains a tyrosine kinase domain that initiates a range of signals to regulate various cellular functions (Bottaro *et al.,* 1991). Hepatocyte growth factor (HGF)/scatter factor (SF) produced by stromal fibroblasts acts in a paracrine manner on cancer cells *via* the c-Met receptor (Trusolino *et al*., 2002). The activation of this receptor can induce proliferation, motility, adhesion, and invasion in tumor cells (*e.g.*, Jiang *et al*., 1993; Uchiyama *et al*., 1996). Over-expression of c-Met protein has been correlated with tumor progression and prognosis in breast cancer, gastric cancer, hepatocellular, endometrial, and nasopharyngeal carcinomas (Kuniyasu *et al*. 1993; Ueki *et al*., 1997; Ghoussoub *et al*., 1998; Wagatsuma *et al*., 1998; Qian *et al*., 2002). c-Met has been suggested to be associated with colorectal cancer (CRC) progression (Trusolino *et al*., 2002). Bilchik *et al*. (2001) reported on the utility of c-Met as an RT-PCR marker for detecting micrometastasis of CRC in sentinel lymph nodes. Takeuchi *et al.* hypothesized that over-expression of c-Met mRNA in primary CRC can predict tumor invasion and regional metastasis. Interestingly, Stat3 is one of the mediators activated by c-Met and is involved in c-Met-induced cell motility (Boccaccio *et al.*, 1998).

3.2.2. Role of Tumor Associated Proteases und Protease Inhibitors in Invasiveness of Gastrointestinal Carcinoma Cells

Matrix degradation in the course of invasive growth is mediated by the concerted action of several proteinases, including members of the serine, cysteine, aspartate, and MMP families (Brinckerhoff *et al*., 2000). It is well established that various proteases are frequently over-expressed in tumors, and that tumors with strong protease expression are often more aggressive than those with lower expression levels.

This is evident for the urokinase type-plasminogen activator (uPA) system, which consists of the 55 kDa serine protease uPA, its membrane-bound receptor uPA-R, and the specific inhibitors plasminogen activator (PAI)-1 and -2. uPA activates plasminogen to form plasmin, resulting in the degradation of surrounding matrix elements and simultaneous activation of collagen, which in turn degrades basal membranes. The inhibitors and PAI-1 are essential for the transcellular recirculation of uPA-R, thereby guaranteeing a dynamic and flexible proteolytic system at the surface of tumor cells. Moreover, PAIs are speculated to protect inner tumor segments from self-destruction by excess proteolysis and to induce neo-angiogenesis of newly established metastases (Boyd, 1989; Schmitt *et al*., 1992; Nekarda *et al*., 1994; Schwartz *et al.*, 1994; Heiss *et al*., 1995). The urokinase-receptor (u-PAR) promotes plasminogen-dependent extracellular matrix degradation in gastric or colon cancer. Over-expression of u-PAR has been reported to occur mainly at the transcriptional level in malignant cells, and has been shown to indicate a poor clinical prognosis of cancer patients. (Reviewed in Allgayer, 2003).

The majority of connective tissue destruction is carried out by the Matrix-Metalloproteinases (MMPs), a family of zinc-dependent enzymes that degrades all components of connective tissues. Currently, 26 human MMPs have been identified, and these enzymes are classified according to their substrate specificity and structural similarities. There are four major subgroups: (*a*) interstitial collagenases; (*b*) gelatinases; (*c*) stromelysins; and (*d*) MT-MMPs. The interstitial collagenases degrade structural collagens types I, II, and III, which is followed by further breakdown by gelatinases and stromelysins.

The gelatinases are effective primarily against type IV collagen, although a limited ability to degrade stromal collagens has been noted. The stromelysins have broad substrate specificity, degrading non-collagen matrix molecules, such as proteoglycans, laminin, and fibronectin, but they indirectly mediate collagen degradation by contributing to the

activation of other latent MMP family members. The MT-MMPs represent membrane-bound forms of the enzymes, which activate latent MMP-2 and which can cleave collagen at the classic site (Parsons *et al*., 1997; Curran and Murray, 1999; Nagase and Woessner, 1999; Velesco *et al*., 2000).

MMP expression is low in most normal cells under physiological conditions. In normal tissues, they are thought to play a major role in tissue growth and remodelling (Behera *et al*., 2004); however, MMP expression is dramatically increased in a variety of cancer types, where it is indicative of invasive disease with a poor clinical prognosis (Airola *et al*., 1999; Walker *et al*., 1999; Hofmann *et al.*, 2000; Nikkola *et al*., 2002). Specific mechanisms exist, by which individual matrix metalloproteinases, especially membrane-type matrix metalloproteinases, interact with cell adhesion molecules and cytoskeletal proteins, thus dynamically contributing to colorectal tumor invasion (Leeman *et al*., 2003). For example, matrilysin (MMP-7) is activated at an early stage of colorectal tumourigenesis by the β-catenin signalling pathway. A particularly important member of the MMP family is the interstitial collagenase MMP-1, which degrades the most abundant proteins of the extracellular matrix (collagen types I and III). MMP-1 levels increase during colorectal carcinoma progression (Baker *et al*., 2000; Yamashita *et al*., 2001;) where they are associated with shorter disease-free survival (Murray *et al*., 1998; Shiozawa *et al*., 2000). In *in vitro* assays, this increase was necessary to induce colon carcinoma cell invasion through a synthetic extracellular matrix (ECM). Aberrant expression of MMPs has also been documented in head and neck carcinomas, astrogliomas, gastric cancer, and melanomas (Reviewed in Curran and Murray, 1999).

Several recent studies addressed signalling pathways which are regulated in colorectal cancer development. The expression of MMPs is greatly modulated by cytokines and growth factors, which induce cellular responses by activating intracellular signalling cascades including the mitogen-activated protein (MAP) kinase *via* specific cell surface receptors (*e.g.*, Korzus *et al*., 1997; Yu *et al*., 2002; Udayakumar *et al*., 2003). Among the nuclear substrates of MAP kinase, some were found that are directly involved in MMPs transcriptional regulation. These include the gene products of *fos* and *jun* oncogenes which compose the activator protein-1 (AP-1) transcription factor, as well as *ets* family members, for example extracellular signal-regulated kinases (Erk) 1/2 site in the MMP-1 promoter (*e.g.*, Curran *et al*., 1999; Brinckerhoff *et al*., 2000; Horiuchi *et al*., 2003; Tower *et al*., 2003; Huntington *et al*., 2004). Interstitial collagenase 1 (MMP-1) and tissue inhibitor of metalloproteinases 1 (TIMP-1) genes are co-ordinately up-regulated by growth factors as

well as by several nuclear and non-nuclear oncogenes. This can be explained by the combined actions of transcription factors operating through composite ets/Ap-1 motifs in some mammalian promoters, including MMP-1 and TIMP-1 (Korzus *et al*., 1997).

4. STUDY OBJECTIVE

A very interesting emerging target in cancer therapy is the signal transducer and activator of transcription - 3 (Stat3). It was recently shown that Stat3 is persistently active in the great majority of malignant colorectal tumors (colorectal carcinoma, CRC). However, its role in development and progression of this disease has not been investigated so far.

One major purpose of this work is an analysis of functions of Stat3-activation in colorectal cancer cells, by studying its influence on parameters such as cell proliferation, invasion properties, and anchorage-independent growth. Moreover, the project intends to correlate the expression of malignancy-associated proteinases with aberrant Stat3 activity, thereby possibly identifying novel target genes of Stat3 in the context of CRC.

Another goal of this work is to investigate potential origins of persistent Stat3 activation in colorectal cancer tissue. Methods of discriminating between paracrine and autocrine mechanisms of Stat3 are to be developed with the goal of obtaining information on the nature of the factor(s) that induce Stat3 activity in CRC cells.

5. MATERIALS AND METHODS

5.1. Materials

5.1.1. Biological materials

5.1.1.1. Tumor biopsies

Tumor biopsies were obtained from colorectal cancer patients after approval of the study by the local ethical committee. Patients were not treated with chemotherapy prior to resection. Biopsies were snap frozen in liquid nitrogen immediately after resection. Frozen blocks were disintegrated mechanically and total RNA was isolated by homogenization in TRIzol-Reagent (Gibco BRL), as described below.

5.1.1.2. Cell lines

Colon carcinoma cell lines HT-29 and SW-480 were purchased from the American Type Culture Collection (ATCC).

HT-29 - DSMZ Nr. ACC 313

SW-480 - DSMZ Nr. ACC 299

The establishment and characterization of the low passage number cell lines COGA-1 and COGA-3 has been described previously (Vécsey-Semjen *et al*., 2002). COGA-1 was derived from a high-grade mucinous carcinoma of the *coecum*; this tumor can be classified as hereditary non-polyposis colon cancer (Lynch II). COGA-1 cells did not show chromosomal instability.

All cell lines were cultured in RPMI 1640 medium (Life Technologies, Inc) containing 10% fetal calf serum (FCS; Greiner GmbH), 200 mM L-Glutamine (Life Technologies, Inc), 100 mM sodium pyruvate (Gibco BRL) and 100 µg/ml gentamycin (Gibco BRL). Cells were cultivated in either coated culture tissue plasticware (Greiner Labortechnik) or non-coated Petri dishes for microbiology (Greiner), as indicated in the Results section. All cultures were maintained by continuous passaging (on average two times a week) when subconfluent: plates were washed once with phosphate-buffered saline (PBS), then trypsinized once with 2.5 x trypsin- ethylenediaminetetraacetic acid (EDTA) (0.125% trypsin in 1.325 mM EDTA 4Na; Biochrom AG) in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4{*}7\ H_20$, 1.4 mM KH_2PO_4, pH 7.3). Trypsinization was stopped with medium containing 10% FCS; cells were centrifuged at 120 x *g* for five minutes and resuspended in fresh medium in appropriate dilutions.

All cell lines were grown in a humidified incubator at 37°C and 5% CO_2. For storage, cells were frozen in liquid nitrogen in RPMI 1640 medium containing 20% FCS and 10% DMSO (Sigma Chemicals).

Cells were checked for mycoplasma contamination by Stratagene Mycoplasma Plus™ PCR Kit according to the manufacturer's protocol.

5.1.2. Antibodies

Name	Company, Product	species	Epitope recognized	Dilution
phospho (p)-Tyr Stat3	Cell Signaling, 9131	rabbit	Y705 p-92 Stat3-α & p-83 Stat3-ß	1:1,000
Stat3 C-20	Santa Cruz, sc-482X, 91065	rabbit	p-92 Stat3 C-terminus	1:1,000
goat anti-rabbit IgG-POD	Sigma	rabbit		1:10,000
MMP-1	Calbiochem	mice		1:50

5.1.3. Other reagents

Cell biological solutions and reagents were obtained from Greiner, Biochrom, Sigma Aldrich, DIFCO; reagents used for biochemical assays (salts, acids, buffers, etc.) were commercially available analytical-grade reagents from Sigma Aldrich, Roth, Fluka, R&D Systems, Bio-Rad, Gibco BRL and Serva with qualities "pure for analysis" ≥99%. IL-6 was purchased from R&D Systems GmbH (Wiesbaden). Human MMP-1 promoter/luciferase reporter plasmid was obtained from Prof. C. E. Brinckerhoff (Dartmouth Medical School, Hanover, New Hampshire). Retroviral constructs were generated by Dr. E. Pfitzner (Georg-Speyer-Haus, Institute for Biomedical Research, Frankfurt am Main). cDNA macroarrays on nylon membranes substrates were generously provided by Prof. B Wiederanders (Friedrich-Schiller University Jena Medical School, Institute of Biochemistry, Jena).

5.2. Methods

5.2.1. Cell biological methods

5.2.1.1. DNA constructs

The humane MMP-1 promoter/luciferase reporter plasmid -4372 ("hMMP-1luci") (bacterial vector pGL3, ampicillin-resistant), used in this study contains the firefly luciferase gene under the transcriptional control of the MMP-1 promoter and was obtained from Prof. C. E. Brinckerhoff (Dartmouth Medical School, Hanover, New Hampshire). A variant of the MMP-1/luciferase construct contains a single nucleotide polymorphism (SNP) located at -1607 bp, where an insertion of a guanine base (G) creates the sequence, 5'-GGAT-3', the core binding site for members of the Ets family of transcription factors (Wasylyk *et al*., 1993). The 4.3 kb MMP-1 promoter DNA fragment containing 1 G or 2 G, respectively, at -1607 bp **(Fig. 3)** and the MMP-1 promoter/reporter plasmids have been described previously (Rutter *et al*., 1997; Rutter *et al*., 1998).

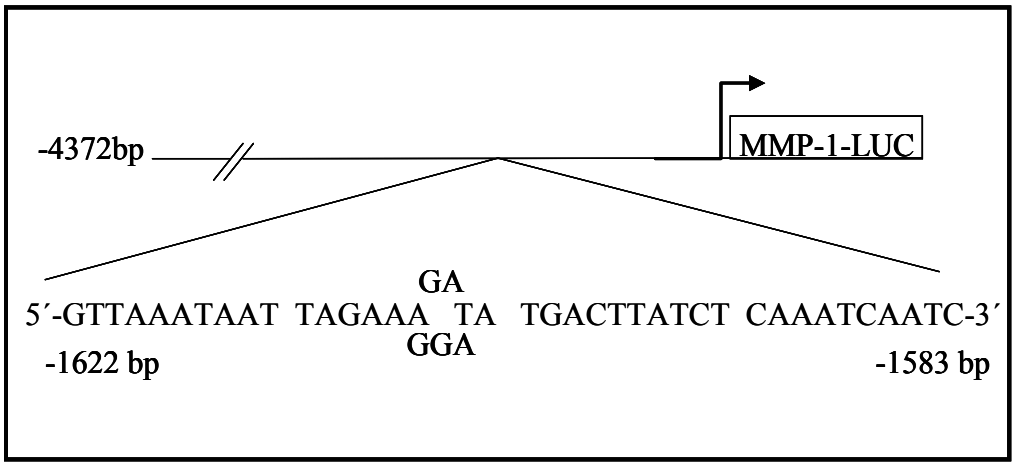

Fig. 3: *Segment of the MMP-1 promoter containing the SNP (for details see text).*

5.2.1.2. Cell Stimulation with Cytokines and Conditioned Media

For cytokine stimulation, HT-29 or COGA-1 cells were incubated with 20 ng/ml recombinant human IL-6 (R&D Systems GmbH, Wiesbaden) medium for 30 min (western blot), overnight (reporter gene assay) or 24 h (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 in 5.2.3.1..

A conditioned medium from cells cultivated on a non-coated plastic surface was obtained by seeding 1.8 x 10^6 cells from a confluent cell culture flask into 8 ml RPMI 1640 and incubating this cell suspension in a microbiological Petri dish for 4 days. After this period, medium was cleared from cells by centrifugation (10 min at 120 x *g*), and passed through a sterile filter with a pore size of 0.2 µm (Greiner). For stimulation experiments, cells in tissue culture flasks were incubated with undiluted conditioned medium for 30 min and further treated for cytokine stimulation assays.

5.2.1.3. Long-term Cell Proliferation Assay

Aliquots of 10^5 cells were suspended in 2 ml RPMI 1640 medium and allowed to adhere in individual wells of 6-well cell culture plates (Greiner bio-one). They were grown in the absence or presence of 20 ng/ml IL-6 for up to nine days. Medium with or without cytokines was changed every two days. At intervals, cells from individual wells were detached from the plastic surface in 0.5 ml 0.05% trypsin/0.02% EDTA (Biochrom, Berlin) for 2 min at 37° C before 1.5 ml medium was added to restore the original volume. Aliquots of cell suspensions were stained with 4% trypan blue (Sigma) in 0.9% NaCl and counted using a Neubauer chamber. The cell concentration was then extrapolated to the total volume of the culture.

5.2.1.4. Soft Agar Assay for Anchorage-Independent Growth

Stat3 induced cell transformation was analyzed using soft agar assay performed as described in Huang *et al.* (2001). Anchorage-independent growth has been shown to correlate with tumorigenicity (Huang *et al*., 2001). Briefly, cells were assayed by seeding 10,000 cells in 500 µl 0.6% agar (Agarose nobile, DIFCO) diluted in RPMI 1640 medium with 10% FCS into six-well plates lined previously with 500 µl 0.5% agar medium **(Fig. 4)**. The plates (in triplicate and repeated twice) were cultured at 37°C for 2-3 weeks for various experiments, and 250 µl fresh medium was added every three days. The colonies then were stained with 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide (MTT, 500 µg/ml, Sigma) at 37° C for 2 h before photographing and counting.

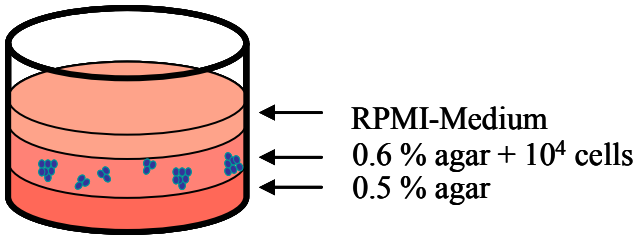

Fig. 4: *Schematic representation of the soft agar assay.*

5.2.1.5. In vitro Cell Invasion Assay

Cell invasiveness was determined by the modified Boyden Chamber method. Each plate contains 6 usable transwells (CORNING COSTAR Corporation, Cambridge, MA 02040 USA), consisting of a separate top well which contains the filter, and a corresponding lower well. The upper side of polycarbonate membranes (6.5 mm in diameter, 8 µm pores) was coated with 200 µl Matrigel (Becton Dickinson, Bedford, MA) diluted 1:50 with ice-cold serum-free RPMI 1640 (40 µg/filter), then dried overnight at 37° C.

In order to determine the invasive properties of each cell line, 2×10^5 cells were suspended in 1 ml RPMI 1640 supplemented with 0.1% FCS and plated on the Matrigel-coated filters **(Fig. 5)**. As chemoattractant, 2 ml medium with 10% FCS was used in the lower compartment of each transwell unit. IL-6 (20 ng/ml) was added to the cell medium 24 h before the assay, and was maintained throughout the entire cultivation period. After 24 h of incubation, cells that had invaded the lower compartment of the system were centrifuged (120 x *g* for 5 min at 20°C), resuspended in 1 ml of medium, stained with trypan blue and counted under a microscope in four predetermined fields at a magnification of 200 x. Results are expressed as percentages of invasive cells compared to the total number of cells initially placed in the upper compartment of the chamber. The average percentage from three wells was determined, and the entire invasion assay was performed at least four times.

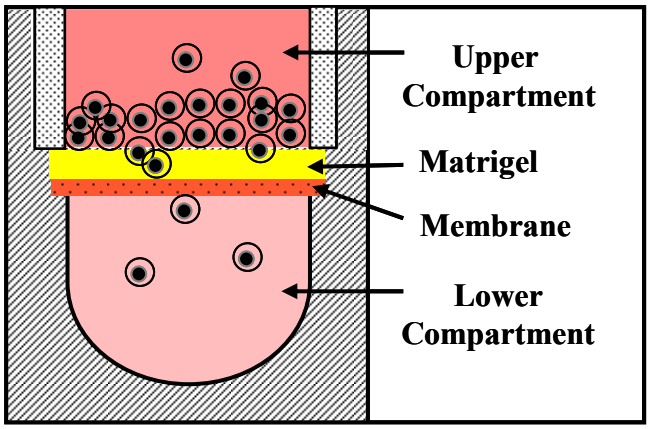

Fig. 5: Schematic representation of the Matrigel-based in vitro invasion assay in a modified Boyden chamber. *Active penetration of the Matrigel layer by colon carcinoma cells as a result of the release of matrix-degrading enzymes is indicated. Most invasive colon carcinoma cells that penetrate the Matrigel and cross the micropore filter (8 µm pore size) do not adhere to the lower surface of the filter and can be recovered from the lower compartment 24 hours after placing the total colon carcinoma cell population in the chamber.*

5.2.2. Immunological methods

5.2.2.1. Immunohistology

Experiment was performed by Dr. Schütz (University of Leipzig, Institute of Pathology). Briefly, tissue was embedded in paraffin and subsequently cut into 4 µm-thick sections. In order to reduce endogenous peroxidase activity, the deparaffinized sections were treated with 0.3% H_2O_2. After blocking unspecific binding sites with normal goat serum (Dako Diagnostik), the sections were incubated with the monoclonal antibodies (dilution 1:50, 4°C, overnight), followed by goat-anti-mouse biotin and streptavidin-horseradish peroxidase (BioGenex, San Ramon, Calif., USA) and developed with diaminobenzidine. Finally, all specimens were coverslipped using Aquatex (Merc, Darmstadt, Germany). Negative controls were performed using an irrelevant monoclonal antibody.

5.2.2.2. Cytokine Determination in Cell Supernatant

The "*RayBio™ Human Cytokine Array*" protein array system (Tebu) was used according to the manufacturer's instructions.

Two array membranes spotted with 79 capture antibodies, six positive controls and three negative controls **(Tab. 4)** were blocked with 2ml 1 x Blocking Buffer (Tebu-bio) at room temperature for 30 min. Membranes were then incubated with 1 ml of conditioned medium at 4°C overnight. After an extensive washing with Wash Buffer I and II (Tebu-bio) to remove unbound cytokines, membranes were then incubated with biotin-conjugated anticytokine antibodies, at a dilution 1:1,000 with Blocking Buffer for 1 h at room temperature. After washing, membranes were incubated with streptavidin-horseradish peroxidase conjugated antibodies at a dilution 1:1,000 with 1 x Blocking Buffer for 1 hr at room temperature. Unbound materials were washed off. Finally, the signals were detected using the "Chemiluminescence imaging system" (Tebu-bio). The relative expression levels in protein arrays were determined by densitometry as described in Section 4.2.4.5. Positive control was used to normalize the results from the 2 membranes being compared.

Tab. 4: *The pattern of cytokine capture antibodies on the "RayBio™ Human Cytokine Array"*

A	B	C	D	E	F	G	H	I	J	K
Pos	Pos	Pos	Pos	Neg	Neg	ENA-78	GCSF	GM-CSF	GRO	GRO-α
I-309	IL-1 α	IL-1ß	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10
IL-12 p40/p70	IL-13	IL-15	IFN-γ	MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	MIP-1ß
MIP-1δ	RANTES	SCF	SDF-1	TARC	TGF-ß1	TNF- α	TNF-ß	EGF	IGF-I	Angiogenin
Oncostatin M	Thrombopoietin	VEGF	PDGF-BB	Leptin	BDNF	BLC	Ckß 8-1	Eotaxin	Eotaxin-2	Eotaxin-3
FGF-4	FGF-6	FGF-7	FGF-9	Flt-3 Ligand	Fractal-kine	GCP-2	GDNF	HGF	IGFBP-1	IGFBP-2
IGFBP-3	IGFBP-4	IL-16	IL-10	LIF	LIGHT	MCP-4	MIF	MIP-3 α	NAP-2	NT-3
NT-4	Osteoprotegerin	PARC	PIGF	TGF-ß2	TGF-ß3	TIMP-1	TIMP-2	Neg	Pos	Pos

Abbreviations: *Pos,* positive control*; Neg,* negative control. All other abbreviations used are standard.

5.2.2.3. Analysis of MMP-1 Activity

The levels of active MMP-1 in the supernatants from the HT-29 colon carcinoma cells were determined, using the commercially available "Human active MMP-1 Fluorescent Assay Fluorokine E" (R&D Systems) **(Fig. 6)**.

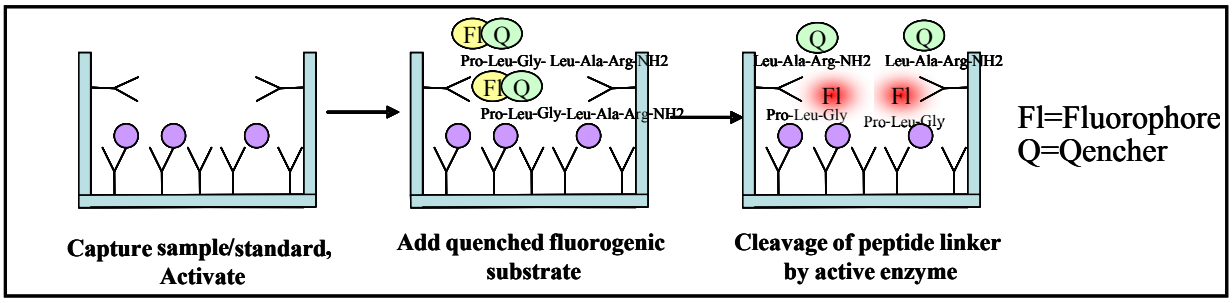

Fig. 6: *Principle of the Fluorokine E human active MMP-1 activity assay (schematic).*

An Immunoassay was performed according to the manufacturer's instructions (R&D Systems). The HT-29 cells were cultured for 4 days to confluency in RPMI 1640 with 10% FCS in the presence or absence of 20 ng/ml IL-6. After centrifugation (4°C; 120 x *g*; 5 min), 150 µl of supernatant from HT-29 cells and varying concentrations of MMP-1 standard were added to 96-well plates, containing 100 µl of Assay Diluent buffer, in duplicate. The plates were incubated for 3 h at room temperature on a horizontal orbital microplate shaker set. Unbound materials were washed out four times with 200 µl of Wash Buffer (R&D Systems). 200 µl of the activation reagent (0.5 M 4-aminophenylmercuric acetate (APMA) in dimethyl sulfoxide (DMSO) were added to each standard well for pro-MMP-1 activation. The plates were incubated for 2 h at 37°C in a humidified environment. After washing, 200 µl of 1 mM fluorogenic substrate in DMSO (R&D) were added for 20 h at 37°C. The relative fluorescence units (RFU) of each well were determined by using a fluorescence plate reader (SOFT max PRO 3.1.1) with the following parameters: excitation wavelength set to 320 nm and emission wavelength set to 405 nm. The duplicate readings for each standard, control, and sample were averaged. Standard curves **(Fig. 7)** were generated by plotting the mean RFU for each standard on the y-axis against the concentration on the x-axis. The concentrations of different samples were determined from the standard curves.

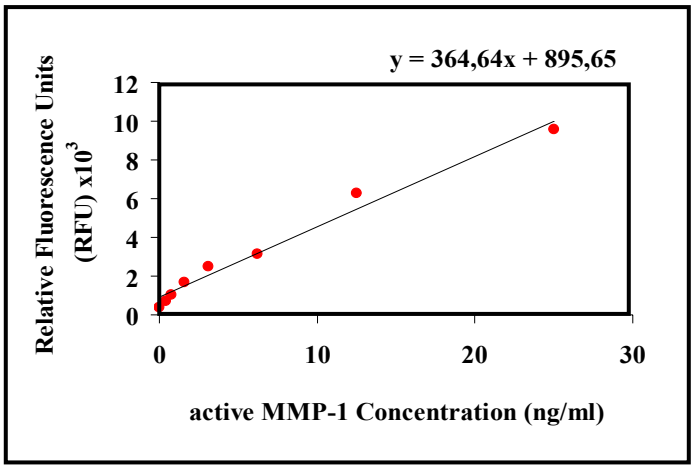

Fig. 7: A standard curve for the active MMP-1 in HT-29 colon carcinoma cells. *The addition of APMA to the standard (0 - 25 ng/ml of pro-MMP-1) caused activation of MMP-1, this was accompanied by a proportional increase of the fluorescence level.*

5.2.3. Western Blot

5.2.3.1. Preparation of Cell Extracts

Culture dishes of adherent cells were rinsed twice with ice-cold PBS, cells were scraped off and pelleted by centrifugation (5 min, 480 x *g*). Pellets were mixed with WCE-buffer (20 mM Hepes, pH 7.9, 400 mM NaCl, 1 mM EDTA, 20% glycerol, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 5 µg/ml leupeptin, and 100 µM sodium ortho-vanadate) and vortexed extensively, followed by 3-4 freeze-thaw cycles in liquid nitrogen. The extracts were cleared by centrifugation at 19,000 x *g* for 30 min at 4° C. Supernatants were subjected to protein determinations followed by Western blots or stored at –80°C.

5.2.3.2. Determination of Protein Concentration

Protein concentration was determined by a Bradford assay (BIO-RAD) compared with a standard curve of bovine serum albumine (BSA) (New England BioLabs), dilution series 0-20 mg/ml, according to the manufacturer's protocol and measured with Spekol 1200 (Analytik AG, Jena) in Roti^{R} Quant Cuvette (ROTH), λ=595 nm.

5.2.3.3. SDS-Polyacrylamide Gel Electrophoresis

Discontinuous SDS-PAGE gel electrophoresis under denaturing conditions was carried out according to standard procedures using the Mini Gel system (BIO-RAD).

For Western blots a 7.5 % separating gel was used (for higher densities the recipe was adjusted). For two gels: 3.3 ml distilled water

2 ml running gel buffer (1.5 M Tris pH 8.0)

2.5 ml acrylamide (AA:bis-AA = 39:1; BIO-RAD)

4 ml Glycerine (40%)

50 µl 20% sodiumdodecylsulfat (SDS)

50 µl 10% APS

10 µl N`N`N`N-tetramethyl-ethylendiamin (TEMED) (Electran; BDH)

Stacking gel: 2 ml distilled water

1.3 ml stacking gel buffer (0.5 M Tris pH 6.8)

0.7 ml acrylamide

1 ml Glycerine

25 µl 20% SDS

40 µl APS

10 µl TEMED

20 µg of whole cell extract were solubilized in gel loading buffer (62.5 mM Tris/ HCl pH 6.8; 2% SDS; 25% Glycerol; 1‰ phenolblue; 5% ß-mercaptoethanol), boiled for 10 min and separated on a 10% acrylamide SDS Gel. The gel run was performed (in 25 mM Tris Base, 250 mM glycine pH 8.3, 0.1% SDS) at 120 V until the front reached the bottom.

5.2.3.4. Semi-dry Electroblotting

Proteins were transferred from the gel onto a nitrocellulose membrane (Schleicher&Schüll GmbH) by electro-blotting in semi-dry blotting buffer (1 L: 3.23 g glycine, 5.8 g Tris pH 7.5, 1.7 ml 20 % SDS, 200 ml methanol) at 0.8 mAmp/cm^2 membrane for 60 min.

5.2.3.5. Immunoblotting

The membrane was stained with Ponceau-Red (0.1% Ponceau-Red in 5% acetic acid; Sigma) to check for successful transfer and equal loading, then nitrocellulose membranes were blocked in 1 x NET-G buffer (10 x :1.5 M NaCl, 50 mM EDTA, 500 mM Tris-HCl pH 7.5, 0.5% Triton X-100, 0.2% Gelatine, Serva-researchgrade № 22151) for 1 hour at room temperature. The first antibody Stat3 p-Tyr 705 (Cell Signaling Technology, Beverly MA) and Stat3-α (c-20, Santa Cruz) were diluted in blocking solution and incubated for 36 h in a 1:1,000 dilution at 4°C. Then the membrane was washed 5 times for 10 min each in

NET-G, incubated for 1 h at room temperature with a secondary antibody, peroxidase-conjugated anti rabbit IgG (Roth), at a dilution of 1:10,000, then washed again 5 times for 10 min each in NET-G. Detection was achieved by ECL™ (enhanced chemiluminiscence detection; Amersham Pharmacia Biotech) for 1 min. This method is based on an enzymatic reaction, performed by the secondary antibody-coupled horseradish peroxidase, which leads to emission of light that can be detected on an X-ray film - Hyperfilm™ECL™ (Amersham Pharmacia Biotech). Protein size was determined by comparing the migration distance in the gel with the position of the bands of a prestained molecular weight marker SDS-7B (SIGMA).

5.2.3.6. Stripping of Blots

Blots were stripped for 30 min at 55°C, while being shaken in 2% SDS, 50 mM Tris-HCl pH 6.7, 100 mM β-mercaptoethanol. The membrane was then carefully washed in large volumes of NET-G, blocked again, and the standard procedure was repeated with another antibody.

5.2.3.7. Coomassie Staining

Coomassie staining was used to compare the quality and concentration of the samples. Gels were stained for about 30 min in 40% methanol, 10% glacial acetic acid, 0.25% Coomassie R250 (Serva) and subsequently destained in 40% methanol, 10% glacial acetic acid until bands were clearly visible. Gels were then dried on filter paper.

5.2.4. RNA and DNA Analytical Methods

5.2.4.1. RNA Extraction

Frozen blocks of colon carcinoma tumor samples were disintegrated mechanically. HT-29 cells were placed in 100-mm plates at 1.8 x 10^6 cells/plate and grown to 80% confluency (48-72 h). After that, cells were washed with 5 ml ice–cold PBS. The total cellular RNA was isolated from confluent monolayers of cells or cryopreserved human colonic tumor resection specimens using TRIzol-Reagent (Gibco BRL) and the protocol provided by the manufacturer. One ml TRIzol was resuspended with 100 mg tumor sample or HT-29 cells, then 200 µl chloroform was added and samples were shaken gently by hand for 15-30 sec then incubated at room temperature for 2-3 min. Then they were centrifuged at 16,000 x *g* for 15 min at 4° C. The upper aqueous phase of each of the samples containing RNA was transferred to new 2 ml sterile tubes, and 200 µl Isopropyl alcohol was added. The samples were vortexed vigorously and incubated at RT for 10 min. Then they were

centrifuged at 16,000 x *g* for 10 min at 4° C and the supernatants were carefully removed so as not to disturb the RNA pellets. The pellets were washed using 75% Ethanol (1 ml) and centrifuged at 6,000 x *g* for 5 min at 4°C. Then the pellets were dried for 10-20 min at room temperature. After addition of 20-30 µl RNase-free H_20, the RNA was quantified by optical density in "HELLMA^R-Quarzglas-SUPRASILR-Cuvette (Type No.105.202.008-QS, "QS1000" light path 10 mm) using a spectrophotometer "Gene QuantII RNA/DNA Calculator" (Pharmacia Biotech) set at an absorbance level of 260 nm. The quality of RNA was checked by the electrophoresis of 1 µg samples in 1.5% agarose gel (Gibco BRL) in TRIS/ACETATE/EDTA buffer (TAE), previously stained with 2.5 µl ethidium bromide (10 mg/ml, BIO-RAD), and visualized under UV light. The size of RNA fragments was determined by a comparison with DNA ladders (1 kb or 100 bp ready load; Gibco BRL). No significant degradation was observed in any RNA sample. All the RNA samples were stored at -80°C prior to reverse transcription.

5.2.4.2. Reverse Transcription

First strand cDNA was synthesized from 5 µg total RNA using M-MLV RNase H(-) Point Mutant reverse transcriptase (Promega Corp., Madison, WI) in a final volume of 30 µl. Total RNA was first incubated with 750 ng Oligo-dT^{15}-Primer (Promega Corp., Madison, WI) or 200 ng of random hexameres (Promega Corp., Madison, WI) in a preheated PCR thermal cycler "TECHNE" (PROGENE) at 65°C for 15 min, then cooled on ice for 5 min. The following contents were combined in a labelled 0.25 mL PCR tube and mixing by pipetting: 1 x reverse transcriptase (RT) buffer (Promega Corp., Madison, WI), 1 mM dNTP mixture (Amersham Pharmacia), 10 mM dithiothreitol (DTT) (Invitrogen), 20 units of RNAsin recombinant ribonuclease inhibitor (Promega Corp., Madison, WI), 240 units of M-MLV reverse transcriptase and diethylpyrocarbonate treated water. To generate digoxigenin-labeled cDNA macroarray probes, 2 µl of one mM Digoxigenin-dUTP (Roche) was added to the reaction mix. The tubes were replaced in the PCR thermal cycler. After incubation at 42°C for 75 min, the RT reaction was terminated by heating at 72°C for 15 min. The reaction was stopped by addition of 1 µl EDTA (200 mM). Then 3 µl of 4 M LiCl and 90 µl of ice-cold 100% ethanol were added and samples were incubated at -80°C for 30 min. After centrifugation at 19,000 x *g* for 15 min at 4°C the supernatants were then carefully removed so as not to disturb the cDNA pellets, and the pellets were washed using 70% ethanol (500 µl). The samples were centrifuged at 6,000 x *g* for 5 min at 4°C; pellets were dried for 10-20 min at room temperature and 50 µl of TE-buffer was added. Five µl of the RT samples were electrophoretically separated on 1%

agarose gel (Gibco BRL) in TAE previously stained with ethidium bromide (BIO-RAD), and visualized under UV light. Size of cDNA was determined by comparison with DNA ladders (1 kb or 100 bp ready load; Gibco BRL). The newly synthesized cDNA was amplified by TaqMan Real-Time PCR or hybridized in human cDNA macroarray system **(Fig. 8)**.

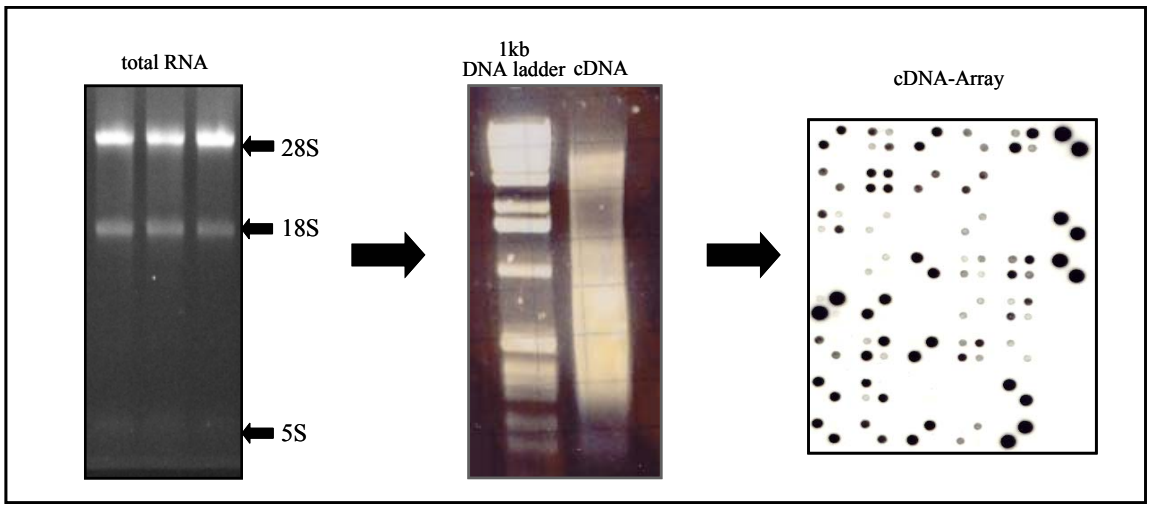

Fig. 8: *Quality control of total RNA and cDNA employed in cDNA macroarray experiments (for details see text).*

5.2.4.3. Protease Expression Analysis by cDNA Arrays

cDNA macroarrays on nylon membranes substrates were prepared as follows: PCR fragments of 200- to 500 bp length were derived from cDNAs representing the following collection of human intra- and extracellular proteases and protease inhibitors: matrix-metalloproteinases; membrane-type matrix metalloproteinases; a disintegrin and metalloproteases ADAM; cathepsins; tissue inhibitors of metalloproteinases; caspases; urokinase-type plasminogen activator uPA, urokinase-type plasminogen activator receptor (uPAR); integrins; plasminogen activator inhibitors PAI; cystatins; tumor necrosis factor α converting enzyme (TACE), emmprin, heparanase, tissue factor, granzyme H and bikunin **(Tab. 5)**.

Tab. 5: *A map for the location of cDNA spotted onto human cDNA macroarray*

	1	2	3	4	5	6	7	8	9	10	11	12
A	ADAM 8	ADAM 9	Cathep. B	Cathep. F	MMP1	TIMP1	MT2-MMP	Caspase 1	Integrin α_2	Integrin β_1	β-Aktin	
B	ADAM 9	ADAM 8	Cathep. F	Cathep. B	TIMP1	MMP1	Caspase 1	MT2-MP	Integrin β_1	Integrin α_2		β-Aktin
C	ADAM 10	ADAM 11	Cathep. D	Cathep. H	MMP2	TIMP2	MT3-MP	Caspase 2	Integrin α_4	Integrin β_2		
D	ADAM 11	ADAM 10	Cathep. H	Cathep. D	TIMP2	MMP2	Caspase 2	MT3-MP	Integrin β_2	Integrin α_4		
E	ADAM 15	TACE	Cathep. K	Cathep. L	MMP3	TIMP3	MT4-MP	Caspase 3	Integrin α_5	Integrin β_3	GAPDH	
F	TACE	ADAM 15	Cathep. L	Cathep. K	TIMP3	MMP3	Caspase 3	MT4-MP	Integrin β_3	Integrin α_5		GAPDH
G	ADAM 20	ADAM 21	Cathep. S	Cathep. V	MMP7	TIMP4	MT5-MP	Caspase 4	Integrin α_6	Integrin β_4	GAPDH	
H	ADAM 21	ADAM 20	Cathep. V	Cathep. S	TIMP4	MMP7	Caspase 4	MT5-MP	Integrin β_4	Integrin α_6		GAPDH
I	uPA	EF1α	Cathep. W	Cathep. Z	MMP9	GFP	MMP19	Caspase 6	Integrin α_v	Integrin β_5		
J	EF1α	uPA	Cathep. Z	Cathep. W	GFP	MMP9	Caspase 6	MMP19	Integrin β_5	Integrin α_v		
K	uPAR	PAI-1	Cystat. A	Cystat. B	MMP11	Ubiqitin	Caspase 8	Caspase 7		Integrin β_7		
L	PAI-1	uPAR	Cystat. B	Cystat. A	Ubiqitin	MMP11	Caspase 7	Caspase 8	Integrin β_7			
M	Emmpr	PAI-2	Cystat. C	Tissue factor	MMP13	Hepara nase	Caspase 9	Caspase 10	rS9			
N	PAI-2	Emmpr	Tissue factor	Cystat. C	Hepara nase	MMP13	Caspase 10	Caspase 9		rS9		
O	Emmpr II	c-ets-1	Pro 1073	Granzy. H	MT1-MMP	Bicunin	IAP3	CEA		R13a		
P	c-ets-1	Emmpr II	Granzy. H	Pro 1073	Bicunin	MT1-MMP	CEA	IAP3	R13a			

To represent "housekeeping genes", probes for the following cDNAs were also included: ß-actin, transcription factor c-ets-1, carcinoembryogenic antigen (CEA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ubiquitin, elongation factor EF1 α and ribosomal protein S9. PCR fragments were purified by preparative agarose gel electrophoresis and concentrations were adjusted to 20 ng/µl in H_2O. DNA solutions were heated to 95^oC for 5 min, chilled, then exposed to 100 mM NaOH for 20 min at room temperature. Subsequently, samples were adjusted to 2 x sodium chloride and sodium citrate solution (SSC)/125 mM Tris/HCl pH 7.5. Aliquots containing 5 ng were spotted in duplicate onto positively charged nylon membranes (1 417 240, Roche) 7 x 10 cm in size.

PCR primers used to generate the probes are shown in **Tab. 6.** Membranes were stored at -20^oC and used for comparative analysis of cDNA obtained from tumor samples or HT-29 cells transfected with Stat3 derivatives.

Tab. 6: *Primers for human cDNA macroarrays*

Primername	Dimension	Primersequenz sense	Primersequenz antisense
hADAM 81/ 2	523 bp	5´-cct gca tga caa cgt aca gc -3´	5´- gca ctc ctg gca gca ggt ac-3´
hADAM 91/ 2	694 bp	5´-act tta gca gtt gca gtg ca -3´	5´- gga aga tct gat cct agc tg-3´
hADAM 101/ 2	590 bp	5´-gat gaa tgc tgc ttc gat gc-3´	5´- tga gcc aca atc cat tca gc-3´
hADAM 111/ 2	524 bp	5´-cca ctg ttc gag cag atg c-3´	5´- ctg gtt gta ctc gtc gat gc-3´
hADAM 151/ 2	471 bp	5´-agg tgc aca gtg tgc atc tg-3´	5´- cag ctg cag ttc agc tca gt-3´
hTACE1/ 2	724 bp	5´- tgt gaa gat gtt gct aga gc –3´	5´- ctc act gct att acc tgt gc -3´
hADAM 201/ 3	702 bp	5´- gca cac cag atg gag ttg ca-3´	5´- cca cta gat tcc cac agt ac-3´
hADAM 211/ 2	585 bp	5´-ctt ggc cta gcc tat gtt gc-3´	5´- agt agg cac tgt cac tac ag-3´
Integrine			
halpha21/ 2	722 bp	5´-gac aag tgg ttc aac tct ggt ca-3´	5´-agc agg gta gcc tac atc gc-3´
halpha41/ 2	674 bp	5´-gct gat tta cag gtt tct gca-3´	5´-tgg cat tct cca gta gta gtc-3´
halpha51/ 2	493 bp	5´- cca cca tgt cta tga gct ca -3´	5´- ctt ggt cca ttg cac agc tg -3´
halpha61/ 2	500 bp	5´-gta cag ttg ttg gcg agc aag-3´	5´-gaa ggc tcg cat gag aat gtc-3´
halphaV1/ 2	735 bp	5´-ctt agc aag act ttc ctg tgc-3´	5´-gtc tca gtc cac agt aat gac-3´
hbeta11/ 2	514 bp	5´- gtt ggt aga cat tgt gaa tgc -3´	5´- agg aac att cct gtg tgc atg –3´
hbeta21/ 2	562 bp	5´-tgc acg aag ttc aag gtc ag-3´	5´-tgg tca gct tca gca cgt gc-3´
hbeta31/ 2	667 bp	5´- atg gac ctg tct tac tcc atg -3´	5´- cat tgc tgg aat cca tgg ac -3´
hbeta41/ 2	620 bp	5´-acc tgt aca tcc tca tgg ac-3´	5´-gtg aag ctt ctc gta gta gc-3´
hbeta51/ 2	632 bp	5´- agt tgt cag tct ggg atc ag –3´	5´- tgt gct gat gtc tgt cga gc –3´
hbeta71/ 2	675 bp	5´-cta cct tat gga cct gag ct-3´	5´-tgt acc acg ttg ctg gag tc-3´
hBikunin1/ 2	606 bp	5´-tac aat gtc act gac gga tc-3´	5´- cat atg tgt tct tca cca gct-3´
hbAktin1/ 2	635 bp	5´- acc acg gcc gag cgg gaa atc -3´	5´- gag ccg ccg atc cac acg gag ta -3´
hCaspase 1 1/ 2	701 bp	5´-aga ccc gag ctt tga ttg act-3´	5´- acg gca ggc ctg gat gat ga-3´
hCaspase 2 3/ 4	897 bp	5´- gga cgc agg ata ttg gga gtg tg- 3´	5´- acg gca ggc ctg gat gaa gaa-3´
hCaspase 3 1/2 (CPP 2/ CPP 3)	1037 bp	5´- agt tag cga gcc ctg ctc aca ctc-3´	5´- tac cct ctg cag cag gagagt agg tc-3´
hCaspase 4 1/ 2	1137 bp	5´- tgt tcc cta tgg cag aag g-3´	5´- tgc cag gaa aga ggt aga aat-3´
hCaspase 6 1/ 4	624 bp	5´-cgc gtt tgg ctg caa tga gc-3´	5´- ctc cag cag gca gcg tgt aa(a) c-3´
hCaspase 7 1/ 2	912 bp	5´- atg gca gat gat cag ggc tgt att-3´	5´- cta ttg act gaa gta gag ttc ctt ggt ga-3´
hCaspase 8 4/ 5	670 bp	5´- caa acc tcg ggg ata ctg tct gat-3´	5´- ctg ttt ccc cat gtt ttt ctt gtc-3´
hCaspase 9 3/ 4	1016 bp	5´- agg aca tgc tgg ctt cgt ttc t-3´	5´- gcc ctg gcc tta tga tgt ttt a-3´
hCaspase 10 3/ 4	813 bp	5´- aag ccg agt cgt atc aag gag agg-3´	5´- cca ggg gca cag gga ata cta-3´
hCathepsin B 1/ 2	539 bp	5´- gca gcc tca gcc acc cag at-3´	5´- cca cca tta cag ccg tcc cca cac-3´
hCathepsin D 1/ 2	895 bp	5´- cac ggg ctc ctc caa cct gt-3´	5´- agt gta gta gcg gcc gat gaa gac-3´
hCathepsin F 1/ 2	582 bp	5´- cgg gcc aag ggt cgc tgt a-3´	5´- act tgg ctt gct tca tct tgt tgc-3´
hCathepsin H 1/ 2	646 bp	5´- cgt gga ctg gcg gaa aaa-3´	5´- cag agg gat ggg gta gga g-3´
hCathepsin K 3/ 4	514 bp	5´- gtg tgg ttc ctg ttg ggc ttt tag-3´	5´- tcc ttt gtt tcc cca gtt ttc tcc-3´
hCathepsin L 1/ 2	612 bp	5´- ccg ggg agg gca gtt gag-3´	5´- cct tga ggc cca gag cag tc-3´
hCathepsin S 1/ 2	664 bp	5´- atg gca aac aat aca agg aa-3´	5´- tga cgc gca tct aca cc-3´
hCathepsin V 1/ 2	742 bp	5´- ata tgg cgc gaa tga aga agg atg-3´	5´- aaa gcc gta gcc aac cac cag aac- 3´
hCathepsin W 1/ 2	750 bp	5´- tgc tca ccg cct gga cat ctt t-3´	5´- gcc cat atc ccc tcc tct gat ttg-3´
hCathepsin Z 1/ 2	521 bp	5´- ggc cct cca ccc tcc tgt c-3´	5´- ggc gcc ctt ccc atc ctt at-3´
hCEA 1/ 2	378 bp	5´-tga ata caa gtt tct gat acc ac-3´	5´- gaa ctt gtg caa taa cta tat tac-3´
hc-ets-1 1/ 2	636 bp	5´-gac atc tta tgg gaa cat cta g-3´	5´- tgg tcc act gcc tgt gta gc-3´
hCystA 1/ 2	388 bp	5´-atc ctg tcc agc aaa gaa gc-3´	5´- cag caa gga tca tga ctc ag-3´

Primername	Dimension	Primersequenz sense	Primersequenz antisense
hCystB 1/ 2	370 bp	5´- ggt gag gtc cca gct tga ag -3´	5´- gca aaa gca gtc gca gaa tc -3´
hCystC 1/ 2	377 bp	5´-agc aac gac atg tac cac ag-3´	5´-tct cct ggt gca ggc aca tg-3´
hEFa 1/ 2	289 bp	5´-tgc ccc agg aca cag aga ct-3´	5´-ctg tgt cgg ggt tgt agc ca-3´
hEmmpI1/ 2	570 bp	5´-cat gct ggt ctg caa gtc a-3´	5´-gtg tat gat ggg aat cta cg-3´
hEmmpII1/ 2	578 bp	5´- agt gct tgc aag att cca ag-3´	5´-gct tct gcg gtt ctg gag tc-3´
hGAPDH 3/ 4	269 bp	5´- ggc tct cca gaa cat cat cc-3´	5´- ggg tgt ggc tgt tga agt ca-3´
hGrzH1/ 2	473 bp	5´-tgc tga cag ctg ctc act g-3´	5´-gag aat acc ttg ggc tac gt-3´
hHepa1/ 2	450 bp	5´-cta cca gga gca att gct ac-3´	5´-cca ttc aaa tag tag tga tgc-3´
hIAP3 1/ 2	512 bp	5´- tgg cac gag cag ggt ttc-3´	5´- cca ggc acg atc aca agg tt-3´
hMMP - 1 1/ 2	677 bp	5´- agg gtc aag cag aca tca tg-3´	5´- agc atc gat atg ctt cac agt -3´
hMMP - 2 1/ 2	650 bp	5´- act cag cca gca cct tgg ag-3´	5´- agc cca aag cag ggc tgc gt-3´
hMMP - 3 1/ 2	630 bp	5´-ttg cag tta g(a)g aac atg ga-3´	5´-atc cag ctc gta cct ca-3´
hMMP - 7 1/ 2	519 bp	5´-ggc atg agt gag cta cag tg- 3´	5´-cca gcg ttc atc ctc atc ga-3´
hMMP - 9 1/ 2	610 bp	5´-ctt cta cgg cca cta ctg tg-3´	5´-cac tgc agg atg tca tag gt-3´
hMMP - 11 1/ 2	589 bp	5´-ctc agt act ggg tgt acg acg g-3´	5´-aca gtc gct ggc agg tga c-3´
hMMP - 13 1/ 2	821 bp	5´-gtc tgg aga tat gat gat ac-3´	5´-cat cta cta ttc tta cca ctg-3´
hMT1-MMP 1/ 2	450 bp	5´-gca gaa gtt tta cgg ctt gca-3´	5´-gca gag tca aag tgg gtg tc-3´
hMT2-MMP 1/ 2	469 bp	5´-agt cac cgg tgt gct cga c-3´	5´-atg cag gtc agt gct gga ga-3´
hMT3-MMP 1/ 2	399 bp	5´-agc tcg tcc atc cat tga ag-3´	5´-tca atg cat atc gct ttc gac-3´
hMT4-MMP 1/ 2	568 bp	5´-agc tgt cta agg cca tca c-3´	5´-cgc atg atg gag tgt gca g-3´
hMT5-MMP 1/ 2	658 bp	5´-tga aga ggt gcc ata cca tg-3´	5´-cca gaa ctg ctc gat ctg ca-3´
hMMP - 19 1/2	729 bp	5´-gac cgg gcc cct tgt tcc-3´	5´-tct acg gtc ttg cgc ctg cta c-3´
hPAI-1 1/ 2	687 bp	5´- gat cga ggt gaa cga gag t-3´	5´- cac agt gga ctc tga gat g-3´
hPAI-2 1/2	712 bp	5´- atc ctg atg cga ttt tgc ag-3´	5´- atg ctt ctc aga atg gat ct-3´
hPro1073 (1323)	681 bp	5´- taa gct tat tat cta tgg ctg aag aca aac ac-3´	5´- ttc agt tgc cag gaa aga ggt aga aat a-3´
hR13a 1/2	631 bp	5´- agc ggc tgc c(g)a aga tgg-3´	5´- tgg gct cag acc agg agt c-3´
hrS9 1/ 2	663 bp	5´- cgc agg cgc aga cgg tgg aag c-3´	5´- tgg cag gaa aac gag aca atc-3´
hTIMP11/ 2	501 bp	5´-aga cca cct tat acc agc g-3´	5´-gac act gtg cag gct tca gt-3´
hTIMP21/ 2	532 bp	5´-gat cca gta tga gat caa gc-3´	5´-tgt cag agc tgg acc agt c-3´
hTIMP31/ 2	445 bp	5´- ctt ctg caa ctc cga cat cg -3´	5´- gta gtg ttt gga ctg gta gc-3´
hTIMP41/ 2	534 bp	5´-agt ctt cat cca tct gtg ca-3´	5´-cag taa cag cta caa ggc tag-3´
hTF1/ 2	399 bp	5´- ctt cag gca cta caa ata ctg -3´	5´-aag tcc gtt cat ctt cta cg -3´
huPA 1/ 2	645 bp	5´-cat caa gtt cca tcg aac tg-3´	5´-cca ggt aga cga tgt agt c-3´
huPAR 1/ 2	907 bp	5´-cat gca gtg taa gac caa cg-3´	5´-aca gtc tgg cag tca tta gc-3´
hUbiquitin 1/ 2	234 bp	5´- atg cag atc ttc gtg aaa acc-3´	5´- tta aca gcc acc cct cag-3´

Hybridization of labelled cDNAs to protease cDNA macroarrays on nylon membranes was done using Dig Easy Hyb (Roche). The membrane was prehybridized overnight at 42°C in 6 ml of DIG Easy Hyb hybridization solution, supplemented with 150 µg/ml denatured herring sperm carrier DNA (Invitrogen). The filter was then hybridized with 50 µl oligo-dT^{15}-primed Digoxigenin–labeled cDNA probe. After 36 h hybridization at 42°C in 3 ml DIG easy hyb hybridization solution and 150 µg/ml denatured herring sperm DNA, the membrane was washed once in 2 x SSC with 0.1% SDS, once in 1 x SSC with 0.1% SDS at room temperature and then washed three times at 68°C using prewarmed 0.5 x SSC and 0.1% SDS and twice at 68° C using prewarmed 0.1 x SSC and 0.1% SDS. After that nylon membrane (Roche) was blocked in 1 x blocking buffer (Roche) for 1 h and incubated with digoxigenin antibody (Roche) for 30 min at room temperature in a 1:10,000 dilution. The filters were then washed three times for 10 min in 150 mM NaCl, 100 mM maleic acid (pH 7.5) and 0.3% Tween-20. Finally, the signals were detected using the Chemiluminescence substrate CSPD (Roche). CSPD was diluted 1:100 in detection solution (0.1 M Tris-HCl, pH 9.5; 0.1 M NaCl) and membranes were incubated in this solution for 5 min at room temperature. The cDNA array membranes were exposed to Hyperfilm^{TM}ECL^{TM} (Amersham Pharmacia Biotech) for 40 min at room temperature and scanned using HPScanjet 4470pro scanner (Hewlett Packard).

5.2.4.4. Densitometric Analysis

For the quantitative analysis of cDNA arrays, the "AIDA" software (version 3.11; Raytest, Straubenhardt) was used. First, the contrast of the scanned X-ray film images was optimized. Then a grid template (schematic representation of an array) was created to associate the correct gene information with the corresponding gene spots. Using orientation spots (spots printed along the borders of arrays to help users to orient arrays properly) the arrays were matched to the corresponding spots on the grid template. Then the alignment was fine-tuned and the grid template lined up closely with the spots. The background value was determined by setting the median intensity to match the "blank space" between the different panels of the array. The background value is used to calculate the adjusted intensity for a gene. After generating a report the following characteristics were shown:

Area calculated as a dimension of the signals or background in mm^2

Integral calculated as a total signal from the gene spots in pixels (double-spotted array)

The correlation factor and correlation integral were calculated as follows:

cF= A(Signal)/mqA (Background)

cF - correlation factor

A - Area

mq - average from the 3 "blank spaces"

cI= I(Signal)-(cF x mqI (Background))

cI - correlation Integral (adjusted intensity for a gene)

I – Integral

The signal intensity between the two arrays being compared was normalised. Normalisation allows for adjustments to the hybridisation signals if different amounts of messenger RNA (mRNA) were used for generating cDNA probes or to correct for different exposures. Several studies have demonstrated that the most thorough way to normalise mRNA expression levels is to use a set of "housekeeping genes" as hybridisation standards (Adams *et al.*, 1995). "Housekeeping genes" produce the mRNAs necessary for a cell's basal metabolism. In contrast to the cDNA included on macroarrays, which are under tight transcriptional control, the levels of housekeeping gene transcripts remain relatively constant in different tissues, cells, developmental stages, and diseases (Adams *et al*., 1995). The mean signals derived from four "housekeeping" mRNAs (GAPDH, ß-actin, ubiquitin, R13α), spotted on the same membrane, were set as 100% for normalisation. Relative signal intensity was expressed as percentage of this maximal intensity.

5.2.4.5. Real-Time Quantitative RT-PCR (qRT-PCR)

Quantitation of cDNA from specific mRNA transcripts was accomplished by qRT-PCR (ABI/Prism® 7000 Sequence Detection System; Applied Biosystems) using TaqMan technology (TaqMan Master Mix; Applied Biosystems).

TaqMan utilizes a gene-specific probe containing both fluorescent and quencher tags, which hybridise to the cDNA between the two PCR primers. As PCR proceeds, the 5' nuclease activity of the *Taq* polymerase cleaves the probe, releasing the tag such that the fluorescence is proportional to the amount of specific amplified product present.

This procedure is based on the time point during cycling when amplification of the PCR product is first detected, rather than by the amount of PCR product accumulated after a

fixed number of cycles. The parameter threshold cycle (Ct) is defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe passes a fixed threshold above the baseline. The MMP-1 gene copy number in unknown samples is quantified by measuring Ct and by using a standard curve to determine the starting copy number, as described by the manufacturer (1997 User Bulletin no.2; Applied Biosystems).

A standard curve was constructed for both MMP-1 and 18S rRNA gene, as an endogenous control **(Fig. 9)**. Serial dilutions (in duplicate) of cDNA (equivalent to a cDNA amount from 100 ng to 100 pg of initial total RNA) from HT-29 cells were made in sterile water. The series of diluted human cDNAs were aliquoted and stored at -80° C until use.

The target amounts of the unknown samples were divided by the endogenous reference amount to obtain a normalized target value. Final results, expressed as n-fold differences in MMP-1 gene expression relative to 18S rRNA gene and calibrator were calculated. Direct comparisons between levels cannot be made between different primer/probe sets.

Pre-designed fluorogenic Assays-on-Demand™ TaqMan™ probes and primer pairs for MMP-1 (Hs00233958_m1) and 18S rRNA (Hs99999901_s1) were obtained from Applied Biosystems Incorporated (Foster City, CA; http://www.appliedbiosystems.com). The MMP-1 and 18S rRNA 18uM gene specific primers predicted to amplify 150- and 120 bp DNA fragments, respectively.

The following FAM (6-carboxy-fluorescein)-labeled probes were used for PCR amplification:

MMP-1 gene 5’ AAAGACAGATTCTACATGCGCACAA 3’

18S rRNA gene 5’ TGGAGGGCAAGTCTGGTGCCAGCAG 3’

qRT-PCR was performed using a 96-well optic tray on the ABI Prism 7000 sequence detection system (Applied Biosystems). A total reaction volume of 25 µl contained the 12.5 µl of 2 x TaqMan Universal Master Mix, No AmpErase UNG (Applied Biosystems, part number 4324018), 3 µl cDNA (described above), 1.25 µl of 20 x Assays-on-Demand™Gene Expression products (Applied Biosystems, part number 4331182) for MMP-1, consisting of

20 x mix of unlabeled PCR primers and 5 uM TaqMan MGB probe (FAM dye-labeled MMP-1), and sterile water. The PCR thermal cycle condition was set up at 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min.

For each reaction well, the fluorescence signal of the reporter dye (FAM) is divided by the fluorescence signal of the passive reaction dye (ROX) to obtain a ratio defined as the normalized reporter signal. Experiments were performed in triplicate for each sample data

point. Each PCR run included the four points of the standard curve, a no-template control, and the unknown cDNAs. Data collection and analysis was performed with SDS version 1.0.1. Software (Applied Biosystems). Data were then exported and further analyzed in Excel (Microsoft, Redmond, WA).

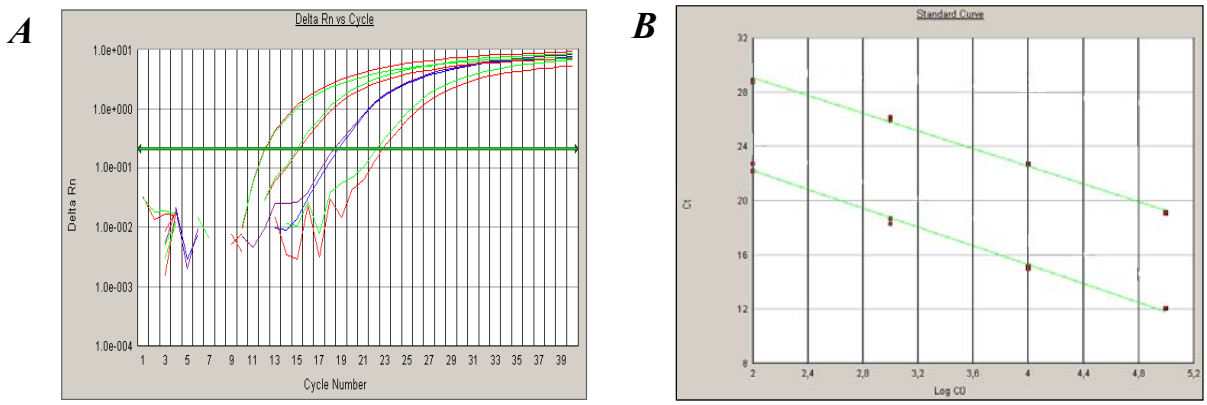

Fig. 9: Representative quantitative real-time RT-PCR analysis for MMP-1 mRNA and 18S rRNA copy levels. ***A:*** *Amplification plot of serially diluted cDNA (100 ng to 100 pg) from HT-29 cells, tested for 18S rRNA expression as endogenous control (in duplicate). Delta Rn on the Y axis indicates the fluorescent signal of the cleaved probe in the amplification reaction, measured real-time during cycling. The PCR cycles are indicated on the X axis. After a phase with exponential amplification (steep part of the curve), the amount of PCR product stabilizes by increasing shortage of reagents. The cycle at which the amplification plot crosses the ΔRn value of ~0,1 was defined as* C_T. ***B:*** *standard curves for 18S rRNA (below) and MMP-1 m RNA (above) in serial dilution ranging from 100,000 to 100 pg of HT-29 cDNA. Both standard curves were made for each biopsy sample or cell line. The* C_T *value was indicated on the Y axis and the amount of cDNA (in pg) on the X axis (logarithmic expression). Using the calibration curves of the standard cell line HT-29, the amount of MMP-1 mRNA or 18S rRNA in a sample can be quantified accurately.*

5.2.4.6. DNA Transient Transfection and Luciferase Assay

HT-29 colon carcinoma cells as well as stable HT-29/Stat3 transfected cells were grown in RPMI 1640 medium containing 10% fetal calf serum (FCS). Transfections were performed using the PolyFect reagent (QIAGEN) following the manufacturer's instructions. Briefly, exponentially growing HT-29 were plated at a density of 7 x 10^5 cells/well in six-well cluster plates (Greiner) in 2 ml RPMI 1640, 10% FCS and grown until 80-90% confluent (24-36 h). The cells were then washed, placed in 1 ml/well fresh full RPMI 1640, and cotransfected (using Polyfect reagent) with 1 µg of chimeric humane MMP-1 promoter/luciferase construct and 0.1 µg of the pRL-TK *Renilla* luciferase vector (Promega) as an internal control for transfection efficiency. Both types of DNA were diluted with growth medium containing no serum, antibiotics or proteins, to a final volume of 100 µl; the solution was mixed and 9 µl of PolyFect Transfection Reagent/well added.

The mixture was incubated at room temperature for 5-10 min to allow complex formation. 1ml of full medium was added to the complexes and the transfection mixture immediatly transferred to the dish. PolyFect is non-toxic, and cells can be incubated until harvest without medium change. After 14 h, the cells were gently washed with RPMI 1640 and incubated with fresh RPMI containing IL-6 (20 ng/ml). The cells were harvested 8 h later using reporter lysis buffer (Promega, Medison, WI). The luciferase and renilla activities were determined using the Dual-Luciferase Reporter Assay System kits from Promega according to manufacturer's instructions. Luciferase activity 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.

5.2.5. Xenograft Experiments

Experiments were performed by Florian Corvinus and Richard Moriggl. Briefly, a total of 10^5 cells of the HT-29 cell line was suspended in PBS and injected subcutanously in a volume of 100 µl into the neck of 5-8 week male athymic Hsd:NMRI-nu/nu mice (Harlan). Mice were dissected after the tumors had reached a size of approximately 2,000 mm². Tumor specimens were treated further in the same manner as the human biopsies (see above).

5.2.6. Statistical Analysis

All numerical values reported represent mean values +/- standard deviation (±S.E.) determined by use of Microsoft Excel Software.

6. RESULTS

6.1. Stat3 activity is associated with enhanced proliferation, oncogenic transformation and invasiveness in colon carcinoma cell lines

6.1.1. IL-6 activates Stat3 in colorectal tumor cell lines

Constitutive Stat3 activity was observed in more than 90% of surgical tumor specimens from colorectal carcinoma patients by Western blot and Electrophoretic Mobility Shift Assay (Corvinus *et al*., 2004). As high Stat3 activation was also evident in malignantly transformed colon epithelium cells, established cell lines (HT-29 and SW-480) and low passage cell lines (COGA-1, COGA-3) were employed as model systems to study aspects of both origin and consequences of Stat3 activity in colon cancer. These selected cell lines have been shown to be very similar and even identical to original tumors concerning many properties, *e.g.,* phenotype, markers, genetic changes (Vécsey-Semjén *et al*., 2002). Each human colon carcinoma cell line was analyzed for Stat3 expression and activity, and compared to clinical tumor specimens by Western blot using Stat3 or phospho-Stat3 (Tyr 705) antibodies. All investigated cell lines showed Stat3 expression. However, in contrast to surgical tumor specimens, neither HT-29 nor SW-480 cells displayed any constitutive activity of Stat3. To answer the question whether cytokines are involved in the activation of Stat3, cells were stimulated with various cytokines known to trigger Stat3 *via* the gp130 cytokine receptor chain shared by their receptors. Oncostatin M (OSM), Leukemia Inhibitory Factor (LIF), and Interleukin-11 did not evoke any detectable Stat3 activity (data not shown). In contrast, IL-6 induced profound Stat3 activity in both established colon carcinoma cell lines tested as indicated by Western blot with the phospho-Stat3-specific antibody. Both investigated COGA low passage cell lines also did not exhibit constitutive but only IL-6-inducible Stat3 activity **(Fig. 10)**. These results demonstrate that constitutive Stat3 activity inherent to tumors is lost during cultivation of cancerous colon epithelial cell lines and is regained by cytokine stimulation.

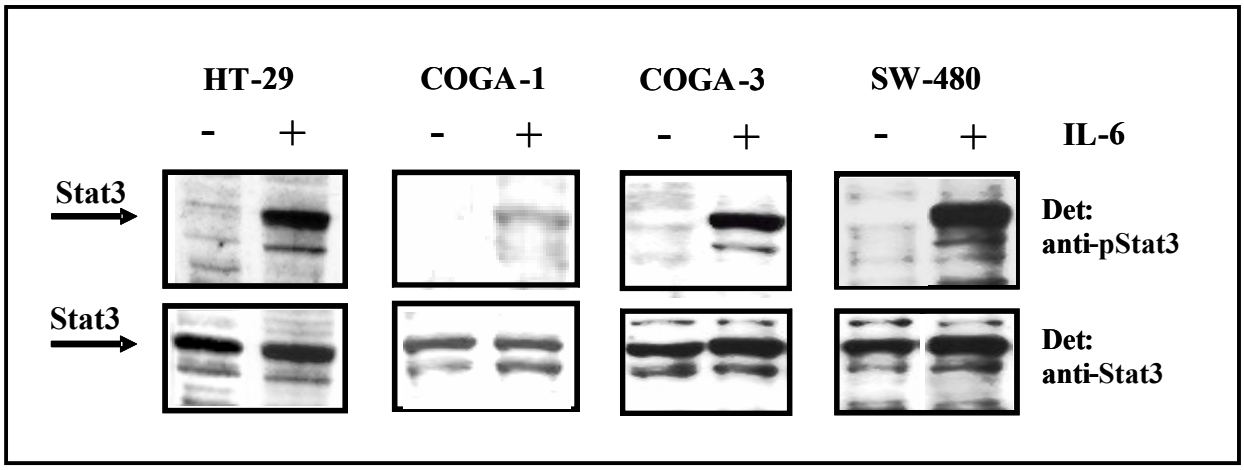

Fig. 10: Analysis of Stat3 activity in colon carcinoma cell lines following IL-6 treatment.
HT-29, COGA-1, COGA-3 and SW-480 cells were incubated (+) with 20 ng/ml of IL-6 for 30 min or left untreated (-). 20 µg of protein obtained from whole cell extracts were suspended in SDS loading buffer and subjected to SDS-PAGE analysis (10% polyacrylamide gel). Phosphorylation of Stat3 was determined by immunoblotting using the specific phospho-Stat3 (Tyr 705) antibody at a dilution of 1:1,000 (top). Membranes were stripped and reprobed with an antibody to Stat3 to assure equal loading (bottom).

6.1.2. Expression of Stat3 mutants in colorectal carcinoma (CRC) cells as a means to study the role of Stat3 in malignant cell transformation

In order to address the possible functional involvement of Stat3 to the malignant transformation of colon epithelium cells, various Stat3 derivatives were expressed in the colon carcinoma cell line HT-29. The following Stat3 variants were stably introduced into HT-29 cells by retroviral infection (cell lines were generated by Frauke Döll and Edith Pfitzner, Georg-Speyer-Haus, Frankfurt/Main): Wild type Stat3 (resulting in cell line HT-29/Stat3), constitutively active Stat3 that had been generated by introduction of a disulfide bridge which forces the formation of stable dimers (Bromberg *et al.*, 1999) (resulting in cell line HT-29/Stat3 c.a.), and a dominant negative mutant of Stat3 in which the tyrosine residue at the amino acid position 705 is replaced with a phenylalanine (resulting in cell line HT-29/Stat3 d.n.). This mutation prevents the Tyr 705 phosphorylation of the Stat3 which is required for its dimerization and nuclear translocation. As the retroviral vector encoded GFP, the fraction of cells expressing heterologous Stat3 could be checked by FACS analysis for fluorescence and was routinely above 70% (**Fig. 11** and E. Pfitzner personal communication).

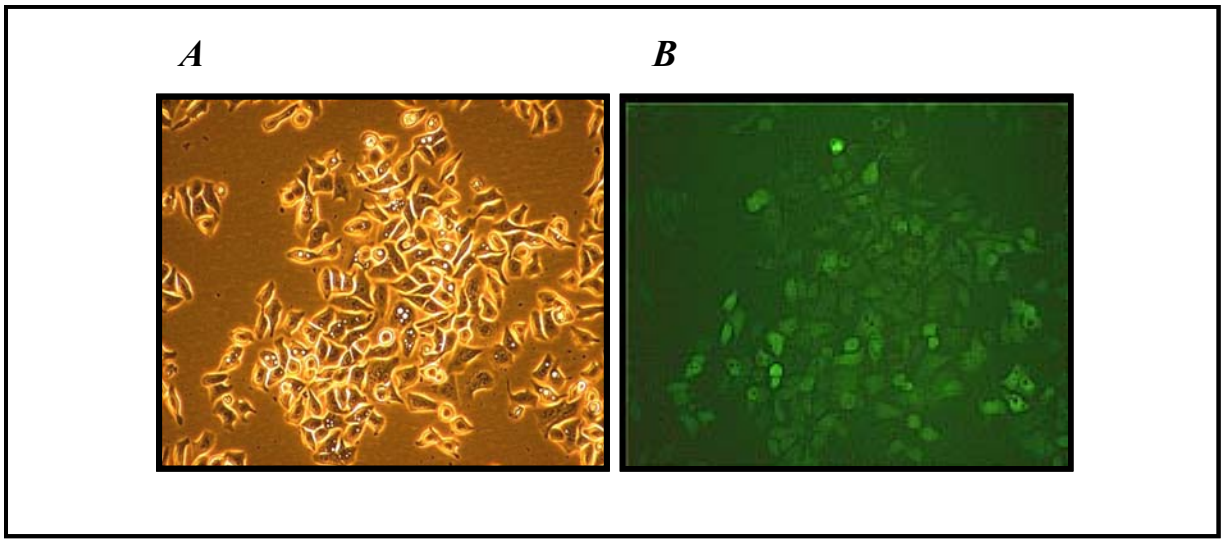

Fig. 11: Analysis of Stat3/GFP expression in HT-29/Stat3 cells by fluorescence microscopy. *Fluorescence of HT-29 cells over-expressing Stat3 was documented using an Axiovert 135M (Zeiss) fluorescence microscope at dark field representation (**A**) or employing a fluorescens-filter (BP 546; FT 580; LP 590) (**B**). Original magnification: 10 x.*

In this study, expression of Stat3 variants as well as tyrosine phosphorylation in response to IL-6 stimulation was analyzed by Western blot using phospho-specific antibodies for Stat3 **(Fig. 12)**. The introduction of a wild-type Stat3 cDNA into HT-29 cells led to a robust over-expression of the protein in comparison to parental cells. The abundance of tyrosine phosphorylated Stat3 in response to incubation with IL-6 was only slightly enhanced, probably due to a saturation of the IL-6 receptor system.

“Stat3 c.a.” and “Stat3 d.n.” were also expressed to higher levels compered to the endogenous Stat3 upon retroviral gene transfer. “Stat3 c.a.” was phosphorylated only to a limited extent. This last effect may be due to a poor association of “Stat3 c.a.” with the phosphorylated gp130 receptor chain and/or to a poor accessibility of the phosphorylation site for the Jak kinase. “Stat3 d.n.” completely blocked IL-6-dependent phosphorylation of endogenous Stat3.

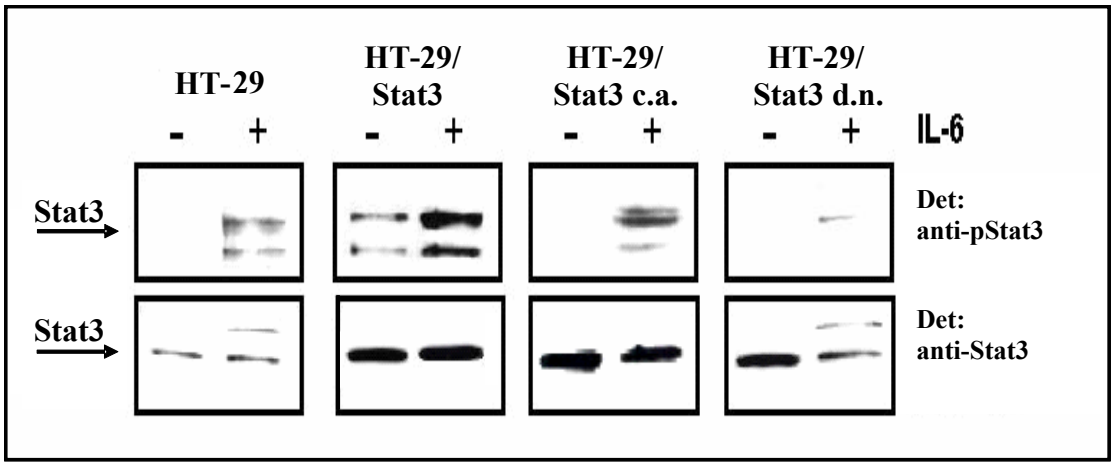

Fig. 12: Expression and activation of heterologous Stat3 variants in HT-29 cells. *Western blot analysis of tyrosine phosphorylation (top) and expression (bottom) of retrovirally introduced Stat3 variants in HT-29 cells. Cells were left untreated (-) or incubated with 20 ng/ml of IL-6 for 30 min (+). 20 µg of protein from whole cell extracts was resuspened in SDS loading buffer and subjected to SDS-PAGE analysis (10% PAA gel). Phosphorylation of Stat3 was determined by immunoblotting using the specific phospho-Stat3 (Tyr 705) antibody at a dilution of 1:1,000 (top). Membranes were stripped and reprobed with an antibody to Stat3 to assure equal loading (bottom).*

6.1.3. Stat3 accelerates the proliferation of colon carcinoma cell line HT-29

Constitutive activation of the Stat3 in a wide variety of cancers raises the question of the contribution of this activity to cell proliferation control. To test this possibility, the effect of the heterologous Stat3 constructs on the proliferative behavior of HT-29 cells was analyzed. Proliferation of HT-29 cells was monitored over a cultivation period of nine days **(Fig. 13)**. The presence of IL-6 in the medium resulted in a faster cell proliferation, suggesting an involvement of gp130-triggered Jak/Stat3 signaling in growth control **(Fig. 13A)**. Confirming this notion, (over-) expression of "Stat3 c.a." and Stat3 led to a profound acceleration of cell proliferation, whereas expression of "Stat3 d.n." resulted in a significant decrease in the proliferation rate **(Fig. 13B)**. Infection of HT-29 cells with the empty retroviral GFP-vector had no significant effect on cell proliferation (data not shown). These results show that Stat3 promotes cell division and proliferation of the colon carcinoma cell line HT-29.

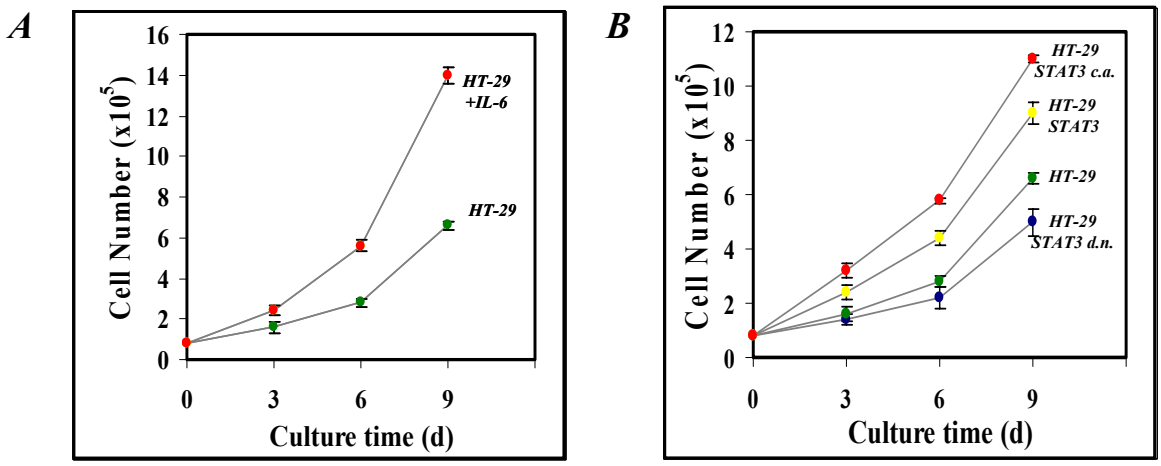

Fig. 13: Effects of Stat3 activity on HT-29 cell proliferation. ***A:*** *Proliferation of HT-29 cells in response to IL-6 stimulation. Samples of 5x10^4 cells of each cell line were seeded into individual wells of 6-well cell culture plates in a volume of 2 ml. After 3, 6, and 9 days, aliquots were counted using a Neubauer chamber, and cell numbers were extrapolated to the total culture volume. When present, the IL-6 was in the medium at a concentration of 20 ng/ml throughout the entire cultivation period. The results shown represent the mean of four independent experiments.* ***B:*** *Proliferation of HT-29 derivatives expressing constitutively active, wild type or dominant negative Stat3 variants in comparison to non-transfected cells. The results shown represent four independent experiments.*

6.1.4. Stat-3 promotes soft agar colony formation of CRC cell lines

The formation of colonies in soft agar indicates the potential of cells to grow independentely of cell anchorage and is a measure of oncogenic cell transformation. To examine the effect of Stat3 on transformed growth, HT-29 cells and derivatives (over-expressing Stat3 and "Stat3 c.a.") were compared with regard to anchorage-independent growth employing a colony formation assay.

Cells were seeded into 0.6% agar, overlaid with medium and incubated for 2 weeks before colonies were counted. **Fig. 14** demonstrates that over-expression of Stat3 in HT-29 cells significantly increased the number of colonies. A much more pronounced effect was induced by "Stat3 c.a.". These results indicate an influence of Stat3 activity on the oncogenically transformed cellular phenotype.

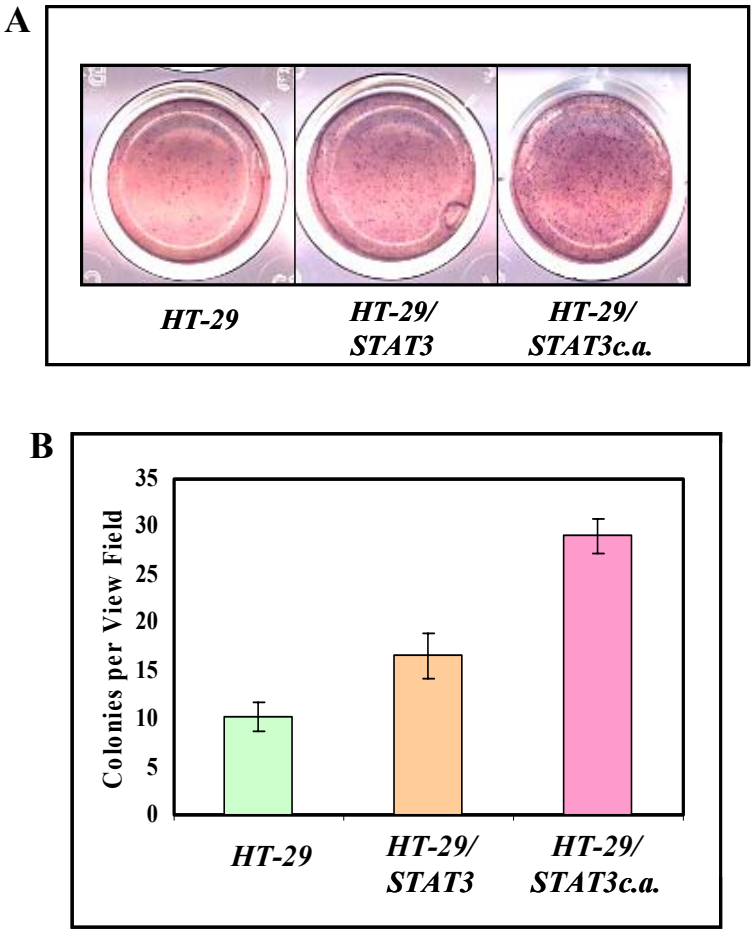

Fig. 14: Influence of Stat3 activity on colony formation of HT-29 cells in soft agar. *Stat3 derivatives over-expressing Stat3 or "Stat3 c.a." as well as non-transfected HT-29 cells were grown in soft agar for two weeks. Colonies formed were visualized by staining with MTT (500 µg/ml)* ***(A).*** *In each well, colonies in seven view field were counted. The values result from triplicate experiments* ***(B).***

6.1.5. Stat-3 activity enhances invasiveness of CRC cell lines

The results concerning the influence of Stat3 on proliferation and colony formation of HT-29 cells pointed towards enhanced migratory properties of this cell line in dependence of Stat3 activity. This was addressed by seeding the cells onto Matrigel® coated filters of chemotaxis Boyden chambers and measuring their ability to cross these filters as a measure of their invasive behavior *in vitro*. The basement membrane matrigel is an extract of the Englebreth-Holm-Swarm mouse tumor. In most cases, cells capable to penetrate the Matrigel layer *in vitro* are also highly metastatic *in vivo* (Janiak *et al*., 1994). Aliquots of $2x10^5$ cells of HT-29 cells and derivatives over-expressing Stat3 or "Stat3 d.n.",

respectively, were seeded onto a layer of Matrigel. After 24 h, the fraction of cells that had migrated through the gel and accumulated in a lower compartment was determined. **Fig. 15A** shows the invasion profile of tumor cells, expressed as the percentage of invasive cells compared to the total number of cells placed in the upper compartment of the chamber. With 25% of cells migrated through the Matrigel coated filters, "HT-29/Stat3 c.a." cells had the strongest invasive character, followed by Stat3 over-expressing cells (15%) and non-transfected HT-29 (7%). Moreover, to test possible stimulatory effects of IL-6, an activator of Stat3, on transmigration, cells were suspended in the appropriate medium supplemented with 20 ng/ml of IL-6. Presence of IL-6 in the medium (24 h before the assay as well as during the assay) resulted in enhanced cell invasiveness of non-transfected HT-29 cells (2.6-fold) and HT-29/Stat3 (1.4 -fold) **(Fig. 15B)**. The expression of "Stat3 d.n." completely blocked this effect.

Stat3 activity was found clearly correlated with the cells' ability to grow in an invasive manner.

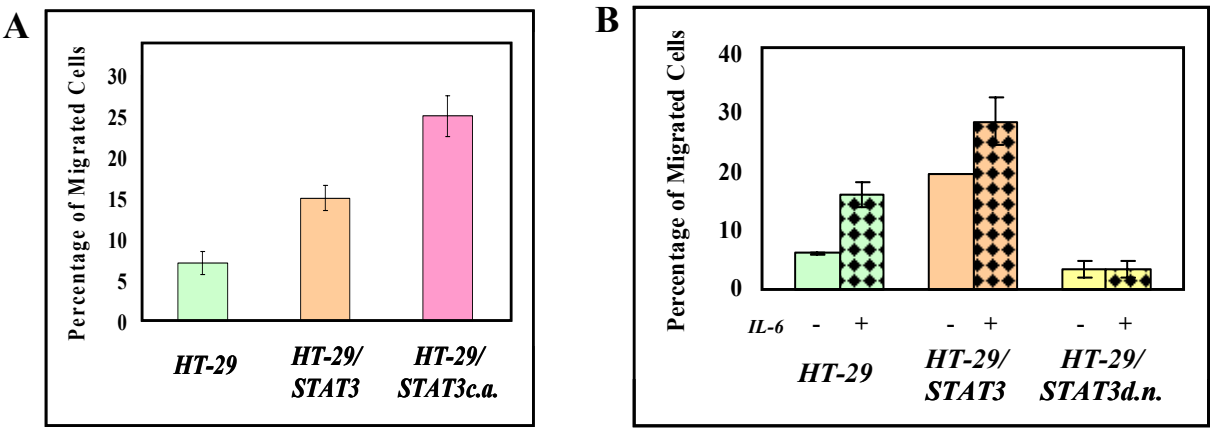

Fig. 15: Cell invasiveness in dependence of Stat3 activation in colon carcinoma cells.
A: *Quantitative evaluation of invasive growth of HT-29 cells and derivates expressing Stat3 variants. HT-29, HT-29/Stat3 and HT-29/Stat3 c.a. cells were seeded onto MATRIGEL™ coated membranes. After 24 h, cells which had migrated to the lower compartment of the system were counted. Cell invasion was determined as mean number of cells invaded through the MATRIGEL™ membrane (the rate of the invasion was expressed as the fraction of migrated cells compared to the total number of cells placed in the chamber). This assay was repeated four times in triplicates.* ***B:*** *Effect of IL-6 on the invasiveness of HT-29 cells and derivatives expressing Stat3 variants. HT-29, HT-29/Stat3 and HT-29/Stat3 d.n. were grown on MATRIGEL™ in the absence (-) or presence (+) of IL-6 (20 ng/ml) 24 h before and during the experiment. The experiment was repeated three times and each treatment was performed in triplicate.*

6.1.6. Stat3 has transforming effects on the COGA-1 low passage number CRC cell line

To exclude the possibility that the findings described so far are the consequence of specific properties of the HT-29 cell line, we studied the influence of Stat3 on the malignant properties of another colon carcinoma cell line (COGA-1). This low passage cell line is devoid of chromosomal instability (Vécsey-Semjen *et al*., 2002) and is, thus, a homogenous cell culture model which mirrors the tumor situation *in vivo* particularly well. Therefore, effects of Stat3 on proliferation, colony formation in soft-agar as well as invasiveness of COGA-1 cells were analyzed. As for HT-29 cells (see above), derivatives expressing heterologous wild type Stat3 and constitutively active Stat3 ("Stat3 c.a.") had been generated by retroviral infection (cell lines were obtained from Frauke Döll and Edith Pfitzner, Frankfurt/Main).

As shown in **Fig. 16**, Stat3 and, to a stronger extent, "Stat3 c.a." promote proliferation of the colon carcinoma cell line COGA-1. A profound stimulating effect of IL-6 on cell proliferation of COGA-1 cells was observed. COGA-1 cells over-expressing Stat3 and "Stat3 c.a." formed five to six times more colonies in soft-agar as non-transfected cells. Invasiveness of COGA-1 cells over-expressing Stat3 or "Stat3 c.a." was significantly higher compared to control cells.

These findings suggest an important general role of Stat3 in cell transformation and progression in the context of colon cancer.

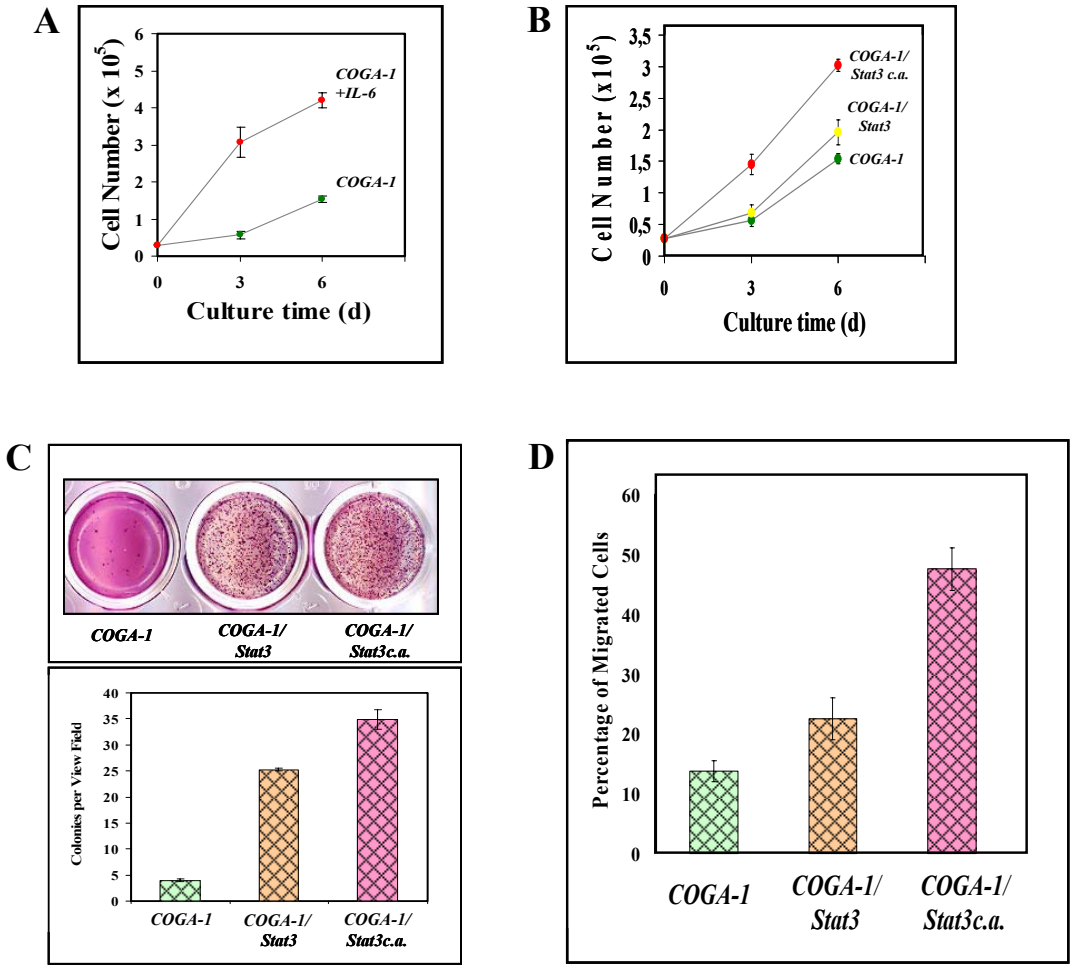

Fig. 16: Effects of Stat3 activity on cell transformation parameters in the low passage COGA-1 cell line. ***A:*** *Proliferation of COGA-1 cells upon IL-6 stimulation. The experiment was performed as in Fig. 13 with the following variations: individual cultures were started at a cell number of $2x10^4$; cell numbers were determined after a cultivation of 3 and 6 days. Four experiments were done independently. IL-6 was present in the medium at a concentration of 20 ng/ml throughout the entire cultivation period.* ***B:*** *Proliferation of COGA-1 derivatives expressing constitutively active or over-expressing Stat3 variants.* ***C:*** *Colony formation of COGA-1 and derivatives in soft agar. 10^4 cells were grown in soft agar for three weeks. Formed colonies were stained with MTT and counted.* ***D:*** *in vitro invasion through MATRIGEL™ of COGA-1 lines expressing Stat3 derivatives compared to non-transfected cells. After 24 h, cells which migrated to the lower well were counted. This assay was repeated four times in triplicates (the results shown represent mean ±SE).*

6.2. Elevated expression of a subset of proteinases in colorectal tumor biopsies coincides with constitutive activity of Stat3

6.2.1. Stat3 activity in colorectal tumors correlates with expression of tumor- associated metalloproteinases MMP-1, -3, -7 and -9.

The results obtained so far raised the question of how Stat3 enhances the invasiveness of colon carcinoma cells. It is logical that the mechanism(s) through which this persistently active transcription factor mediates cellular transformation and invasiveness implicate activation of specific genes. It is known that proteinases, in particular collagenases, are required as key lytic enzymes for the invasion process. Indeed, their inhibition by metalloproteinase inhibitors totally prevented the amnion- or matrigel invasion (Reviewed in Chambers and Matrisian, 1997). To understand the molecular mechanisms responsible for Stat3-mediated tumor invasion we analyzed mRNA from 15 cancer samples using human cDNA macroarrays covering 50 different protease probes (Tab. 5; compare Material and Methods). Samples of 5 µg total RNA isolated from tumor tissues were used to generate Digoxigenin-labelled cDNA, which was subsequently hybridized to the membranes.

All primary CRC samples have previously been tested for Stat3 activity (experiments were performed by Florian Corvinus). According to these results, the biopsies were divided in groups of high and low Stat3 activity and then tested for expression of tumor-associated proteinases by cDNA arrays (examples of raw images in **Fig. 17A**). In most tumor tissues of "Type I"(high Stat3 activity, left-hand side), high expression of invasion-associated matrix metalloproteinases MMP-1, MMP-3, MMP-7 and MMP-9 was found. In contrast, for "Type II" specimens (low Stat3 activity, right-hand side), no apparent expression of this set of MMPs was observed. The expression of MMPs -1, -3, -7 and -9 in a total of 15 tumor samples was quantified by densitometric determination of signal intensities on cDNA array membranes using the computer program "Aida" (version 3.11, Raytest, Straubenhart). To normalize the different exposures, mean signals derived from four "housekeeping"mRNAs (GAPDH, ß-actin, ubiquitin, R13α), spotted on the same membrane, were set 100% **(Fig. 17B).**

These data clearly indicate that tumor-specific expression of four MMPs occurs in conjunction with Stat3 activity.

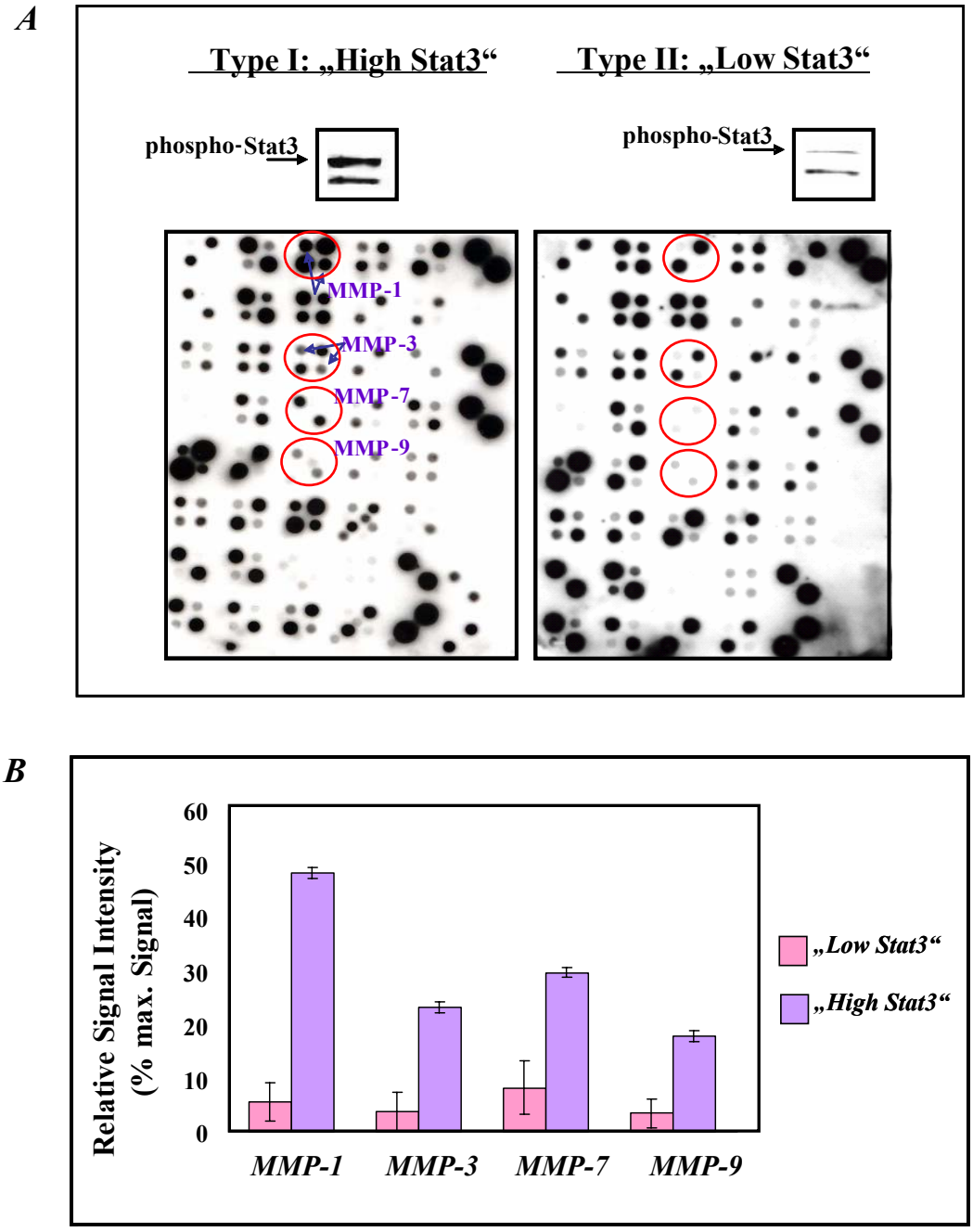

Fig. 17: Comparison of protease gene expression in colon carcinoma tumor samples with high versus low Stat3 activity using cDNA macroarrays. ***A:*** *Total RNA was isolated from tumor samples with high Stat3 activity (Type I) and with low Stat3 activity (Type II). Digoxigenin-labeled cDNA samples were generated from each RNA sample and hybridized to two identical human cDNA arrays on nylon membranes. Membranes were exposed to X-ray film at room temperature for 40 min.* ***B:*** *The relative expression levels of MMP-1, -3, -7 and -9 in 15 tumor samples were quantified by densitometry and normalized to the respective mean of four "housekeeping" control mRNAs (GAPDH, β-actin, ubiquitin and R-13α).*

6.2.2. Elevated expression of other proteinases and inhibitors of proteinases in Stat3-positive tumors.

Apart from the MMPs discussed in the previous chapter, less pronounced Stat3-related differences in expression were also found for other proteinases. mRNAs for Cathepsin B, tissue inhibitor of MMP (TIMP)-1, urokinase-type plasminogen activator (uPA), uPA receptor (uPAR), plasminogen activator inhibitor (PAI)-1, MMP-11, membrane-type MMP-1 (MT1-MMP), a disintegrin-like and metalloproteinase (ADAM)-8 were more abundant in "Type I" tumors **(Fig. 18)**. However, expression of most of these proteases was so diverse within the respective groups of tumor samples that the correlation with Stat3 activity remains disputable.

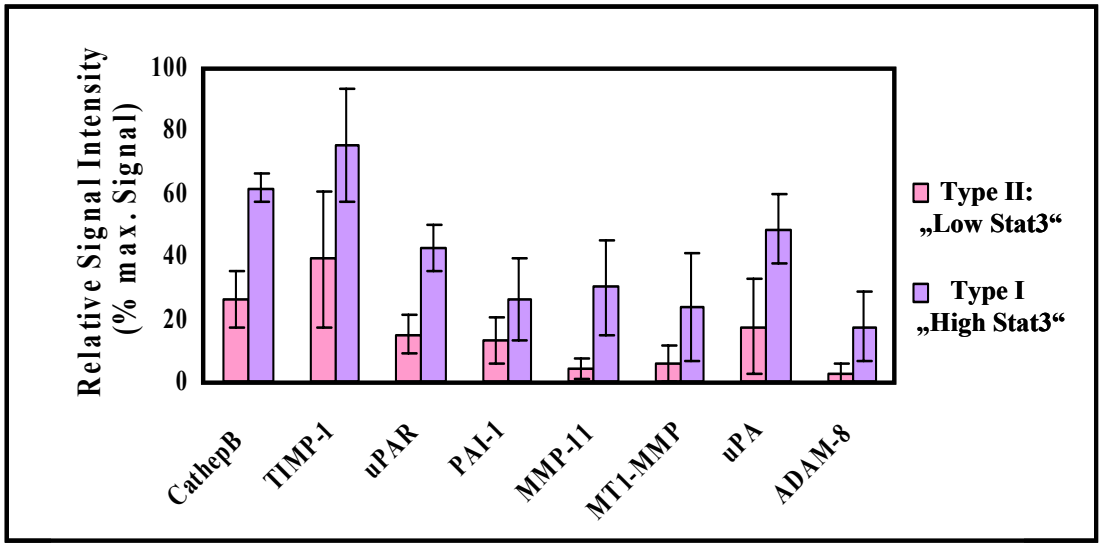

Fig. 18: Comparative expression analysis of proteases in CRC tumor samples associated with Stat3 activity to a moderate extent using cDNA macroarrays. *The relative expression levels of proteinases in 15 tumor samples (seven Stat3 positive "Type I " tumors and eight Stat3 negative "Type II" tumors) was quantified by densitometry and normalised as in Fig. 17.*

6.3. Stat3 activity influences expression and enzymatic activity of the matrix metalloproteinase -1 (MMP-1) in the HT-29 colon carcinoma cell line

6.3.1. Stat-3 activity associated with MMP-1 expression in CRC tumors

In the following, this study focuses on MMP-1 as a possible target gene relevant for the influence of Stat3 on cell transformation for several reasons:

-1) out of all proteases tested, MMP-1 showed the highest expression;

-2) MMP-1 is the most abundantly expressed MMP degrading type I and III collagen, major constituents of the extracellular matrix.

To confirm the results obtained by cDNA arrays, expression of the endogenous MMP-1 gene was measured by quantitative real-time PCR. The 18S ribosomal RNA gene was used as an endogenous control to normalise for differences in the amount of cDNA employed. Data were initially expressed as a threshold cycle (CT), defined as the point at which an increase in fluorescence above a baseline signal can first be detected. In contrast to the samples with low Stat3 activity, far more MMP-1 mRNA was measured in CRC specimens with high Stat3 activity **(Fig. 19)**.

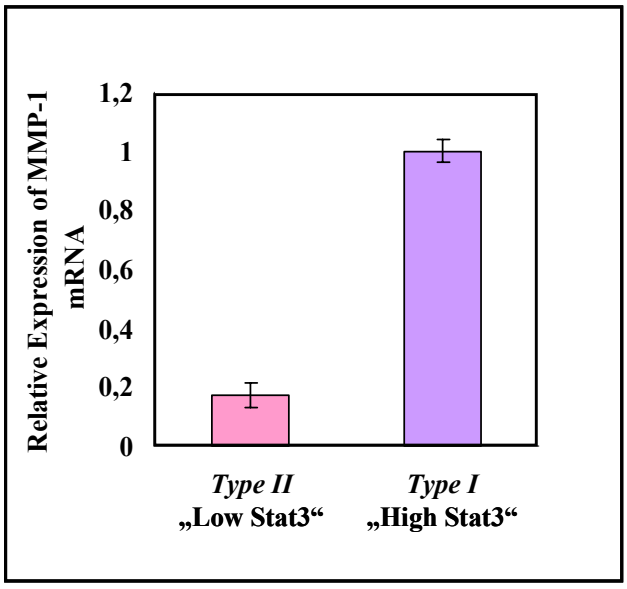

Fig. 19: Real-time PCR detection of MMP-1 mRNA levels in CRC biopsies of with low and high Stat3 activity. *Total RNA was isolated from tumor samples with high Stat3 activity (Type I) and with low Stat3 activity (Type II). Quantification of MMP-1 mRNA levels was measured by real-time RT-PCR. Three independent experiments were performed (each run in triplicate). Results were normalized and expressed as the means ±S.E. Relative MMP-1 expression was calculated by arbitrarily setting the expressions from samples "Type I" as 1 (100%).*

6.3.2. MMP-1 expression is co-localised to Stat-3 activity in colorectal tumors

Immunohistochemistry was performed on CRC biopsies to deteremine which cell types within the tumors are responsible for the biochemically detected MMP-1 signals, and to correlate the respective localisation of Stat3 activity and MMP-1 expression.

Fig. 20 shows a representative example of intense immunohistochemical MMP-1 staining in a colorectal carcinoma of high Stat3 activity (as previously shown by Western blot analysis, Florian Corvinus, pers. communication) in comparison to a tumor specimen with low Stat3 activity. Strong immunoreactivity of MMP-1 in the carcinoma cells themselves was found in all cases of colon carcinoma with high Stat3 activity. MMP-1 was not expressed neither in epithelial cells of normal intestinal crypts nor in stromal cells (*i.e.*, fibroblasts, inflammatory cells) around the tumor **(Fig. 20A)**. In contrast, expression of MMP-1 in tumor cells with low Stat3 activity was not significantly elevated compared to non-cancerous epithelial cells **(Fig. 20B)**, in which immunostaining of MMP-1 protein was only marginal as evident in mucosal sections of healthy colon tissue **(Fig. 20C).**

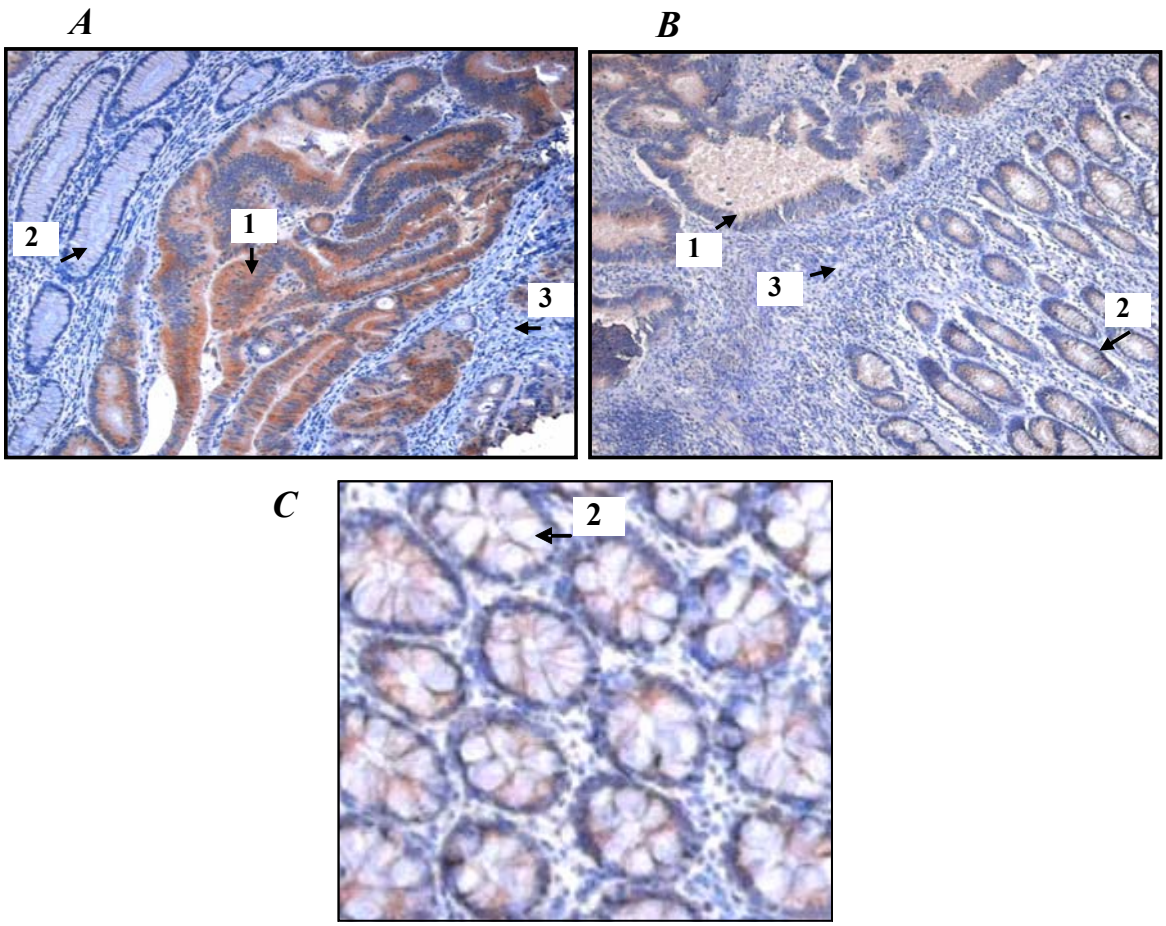

Fig. 20: Immunohistochemical staining for MMP-1 in representative, paraffin-embedded specimens of primary CRCs and control tissue. *Sections of a CRC biopsy with strong Stat3 activity* ***(A)****, a specimen with low Stat3 activity* ***(B)*** *and healthy control tissue* ***(C)*** *were treated with a specific antibody to MMP-1. Arrows indicate: 1 – de-differentiated tumor cells, 2 – normal epithelial cells, 3 – stromal cells. Original magnification in* ***A*** *and* ***B****: 20x, in* ***C****: 40x.*

6.3.3. Stat-3 activity in CRC cell lines correlates with the level of MMP-1 mRNA

To address a possible causal connection of Stat3 activity and MMP-1 expression, MMP-1 gene transcription was examined in the presence or absence of IL-6 in HT-29 cells transfected with Stat3 derivatives. The cells were treated with 20 ng of IL-6 for 24 h before mRNA levels were measured by real-time quantitative PCR **(Fig. 21)**. Expression of heterologous Stat3, and even more pronounced, of "Stat3 c.a.", increases the level of MMP-1 mRNA in HT-29 cells, indicating that Stat3 is involved in the induction of MMP-1 transcription. This notion is supported by the finding that stimulation by Interleukin-6, a Stat3 activator, influences MMP-1 expression in a positive way.

These results suggest that Stat3 may directly act on the transcription of the MMP-1 gene in the colon carcinoma cell line HT-29.

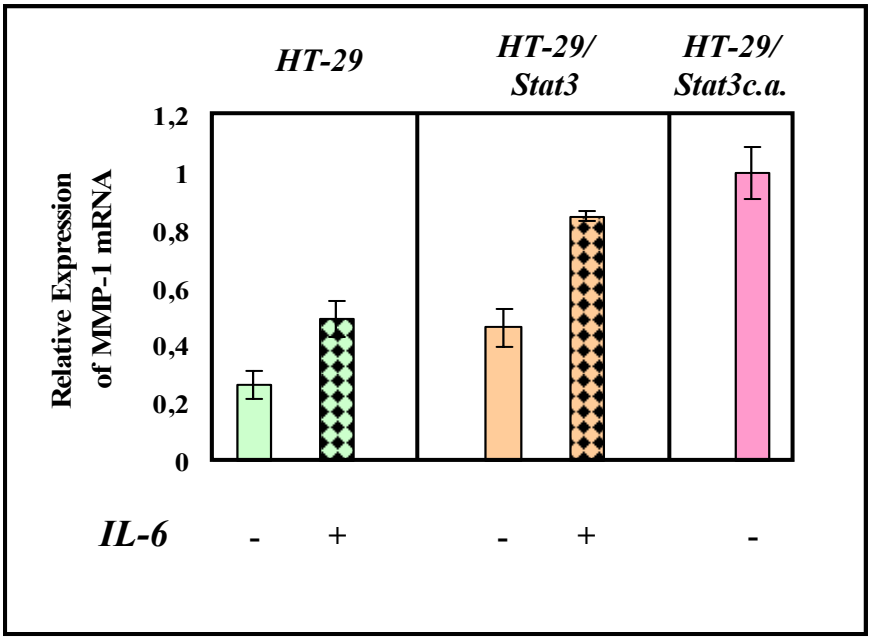

Fig. 21: Influence of Stat3 expression and IL-6-induced activation on the abundance of MMP-1 mRNA in HT-29 cells. *Confluent HT-29 cells cultured in RPMI 1640 with 10% FCS were treated with IL-6 (20 ng/ml) for 24 h. Total cell RNA was isolated, reversely transcribed, and the resulting cDNA was used for real-time quantitative RT-PCR with specific primers and probes for MMP-1 as described in Material and Methods. Three independent experiments were performed (each run in triplicate). The results were normalized and expressed as the means ±S.E. Relative MMP-1 expression was calculated by arbitrarily setting the expressions from samples with "Stat3 c.a." as 1 (100%).*

6.3.4. Stat3 activation in CRC cells correlates MMP-1 enzyme activity

To address in how far real-time PCR data reflected the actual protein expression and activity, an immunoassay for activated MMP-1 was employed. Supernatants from HT-29 cells and Stat3-expressing derivatives were subjected to an ELISA measurement for a fluorescing product of catalytically active MMP-1. Non-stimulated HT-29 cells secreted only a small amount of active MMP-1. In transfected cells, secreted MMP-1 activity was enhanced by heterologously expressed Stat3 (7.6-fold) and "Stat3 c.a." (8.5-fold) **(Fig. 22)**. The activation of Stat3 by IL-6 significantly increased levels of active MMP-1 secreted by both non-transfected (5-fold) and Stat3 over-expressing HT-29 cells (2-fold) compared to the respective non-stimulated cells. "Stat3 d.n." totally supressed the increase of fluorescence (>99%), and no MMP-1 activation was found even upon IL-6 stimulation.

These results indicate that Stat3 contributes to the induction of matrix metalloproteinase-1 activity secreted by HT-29 colon carcinoma cell lines, thereby possibly promoting the

degradation of collagen during invasive growth. Moreover, the data issued from the ELISA experiment are consistent to the results obtained by real-time PCR (see above).

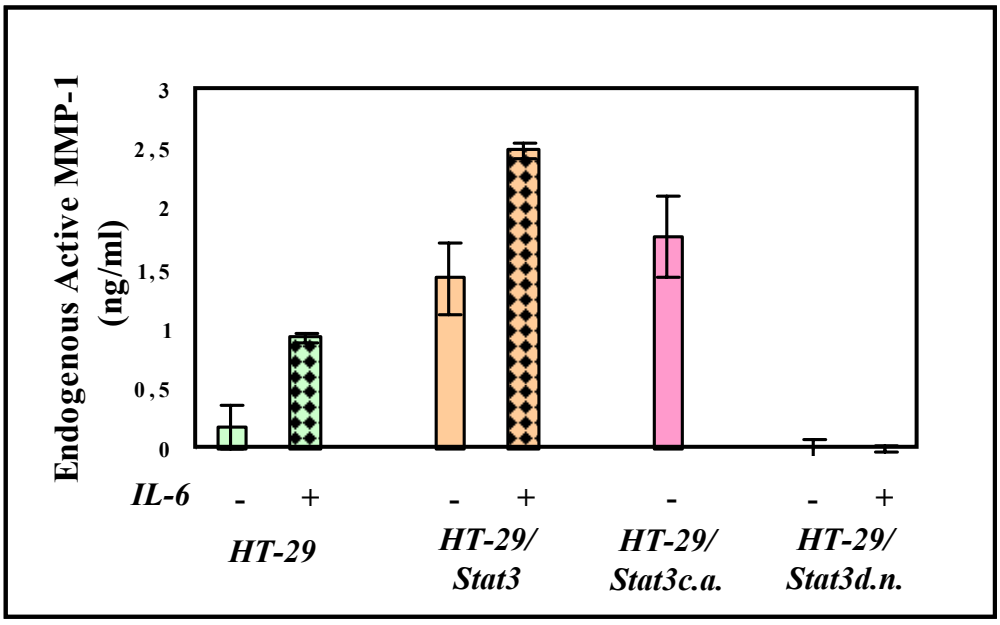

Fig. 22: Influence of Stat3 expression and activation by IL-6 on MMP-1 activity secreted by HT-29 cells. *HT-29/Stat3, "Stat3 c.a." and "Stat3 d.n." cells as well as non-transfected cells were cultured in RPMI 1640 with 10 % FCS. Cells were cultured to confluency for 4 d in the presence (+) or absence (-) of 20 ng/ml IL-6. Aliquots of the supernatants were used for the determination of MMP-1 activity as detailed in Material and Methods. This assay was repeated three times in duplicate (shown results represent mean ±SE).*

6.3.5. The MMP-1 promoter is regulated by Stat3 in HT-29 CRC cells

To obtain information about the possible connection between Stat3 and MMP-1 transcription, the human MMP-1 promoter (Rutter et al., 1997) was analysed for the presence of putative Stat recognition sequences and found to contains 14 Stat3 specific binding sites of the type **GAA**NNN**TTC** **(Fig. 23)**.

Fig. 23: Putative Stat3 binding sites in the human MMP1-Promoter. *The nucleotide sequence for the MMP-1 promoter clone is was obtained from the GenBank database (GenBank Accession Number AF023338, Rutter et al., 1997). Stat3 recognition elements are boxed, the two half sites of the Stat3 binding element are highlighted in red.*

```
   1 cctcacatat ttcaaatcca tctcaaattc acattcacag atgtaagagc tgggaaagga
  61 cggttttgac agggctgaac tgagctatgg tatgagtagc actcatcccc agaaagtctc
 121 ttggtttgaa ttttccggga aaggagctat agctgcaaaa atctgtttca caaatgtgct
 181 aactataagc attttccaca gtgtttaata aaccatgcag ataagaaaat attattgaca
 241 aacaaattaa taaaatgctc aaaataatct gatactaaat gcttgtagca tggcatgcaa
 301 atcaccaaaa ataaatgtgc tatgcttcat ataaaatctc cagtaaggct gggtgtggtg
 361 gctcacacct ataatcccaa cactctggga ggccgaggtg agagaactgc ttgaggccag
 421 gagtttgaga ctagcctggc caacatagtg agacctcatc tctacaaaaa atcttaaaaa
 481 tcagtgggac atggtggtgc acatctgtag ttctagctac ttgggagtct gaggcaggaa
 541 gattgcttaa gcccaagagt ttgaggtccc tacactccag cctaagcgac agagggagac
 601 cttgtctcta aataaataaa ttagttaatt gaatgtccag tcagttgata tatccaaatt
 661 cttcccatgg taattttaaa aactttagtc ttaggagagt aaaagtcatg gacataagac
 721 ttcttataaa caactcagcc taatgagaaa tagaccctgt atttaagtgt catttaagta
 781 tctatttctt cattgatcta ttcatttatt aactcctgta acaatcattt gcagacacct
 841 actatgttga ggtagtataa actataaatt caacaagttt gataagggaa ataagagaga
 901 ttgagtgaca gcttgaaggg gaggattctt tcaggcctgt gggaccgggt ggtggcatgg
 961 agacattatt gtggacttga gggagttaat gtgacagtcc tcgtgtctcc agacactttc
1021 tctctgttag ggaagcaaga tttctatccc cagagtatgt atgtgttatg tctggactgc
1081 agtggcacag aactgtgttc aacgagtgac taccgctctg ctgtgtgccc tgggacttgg
1141 ggttaattga tcaatcattt ctatccagaa ggtaaccatg aggactgacg gaaccagtgt
1201 gtaccaagtg tctgttaagt gtctggtcaa tggttatcca taaagctact gcatggccat
1261 atgtaggaag aatacacacc gtgagcaaat ttttcccacg tgtaactctc acaacaaaat
1321 agcattaaat acttaatgtt tctggctaaa gaccatttca agacttgcag gacaaaaaaa
1381 tagaaaaaat atctgacact caaatggagt tacaaaatta aaacggctga attccccagc
1441 ataaaaaaat atgaagcaag attgaaattt caagactaag tttaatatgg aaaaatacaa
1501 atatgtttga ggcctttcac agagcagcca gcatgaagca accaagaaaa ccacggaaat
1561 aatctggctg cctggaaata gtccggagtc agctgacaca gccacacgag agccctctta
1621 tgcttgtcat aaggggtaaa ggaataattt cagaaaatta catttaaaag agaattatgg
1681 gggaagaaga tgctcccaga ggaaacaaat agtatggatg tgaagagcaa atacaacttt
1741 aacatgtttt gaacttcttg gaaactatgc taagtttagg cattgctagg atttggtatg
1801 atttaatccc cagctttctg ttctaaattt ttgttttctt ttttactctc aaataaatca
1861 tatgctagca ccagctgcaa agttacatat gttgtattag acgatcttcc atgaatacct
1921 aactggaaat tccaagattc agggccatgt gaatctaggc tggctgctta accaaaactt
1981 aatttaattt ttttcgttta ttttaggaaa aaaaattaac gaaaagatgt ttcaagcaac
2041 cagtttccaa tccacgtcag caactatgac atttaatgaa acactgtgag catttagcat
2101 gagagctctg gactcagatg cagggagctt tgctagagaa gggaggaaaa agcaggcatg
2161 atgtggcggg ttgtggggga ctccaaggct ctatttccaa cttccatcag agaacttctg
2221 ttttcacctg gttttcaaat ttgctttcca aaagggattt tgtttaagta aaggatacag
2281 aggtttataa aagtttgaaa acttctacat tgcaggatgt gcaggctctt gccagatggg
2341 acagtgtatg agactcttcc agggtgacgt cttaggcaat ttcctgtcca atcacagatg
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2521 actccttgac ttttaaaaca tagtctatgt tcaccctcta atatgaagag ccccttttcac
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2701 tcttaccctc ttgaactcac atgttatgcc acttagatga ggaaattgta gttaaataat
2761 tagaaagata tgacttatct caaatcaatc caagatatac tgaagtattg tttatgagta
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2941 ggccagtgtt tctccactgt atgttttcaa taaatgcttc caaggaagga gagtggggca
3001 tgagtagggg agctacagag ataaaccaac ttttcttacc aggaatgcta cagatagcac
3061 tggtgacacc ggtcaccagt acccaagaca atttaatgtg gaacataagt acaggaatac
3121 acatcttttca ttacagagcc atgtatttat tttaatgggc aggagatgct aaataagatc
3181 ttttgaatgg aggaatgcat aaatatatga atgaatgcat acatgaaaga ataaataaat
3241 gctgcctagc accaaggagc gaagatagac tcatatcaag ggaaacaagt atgattaaaa
3301 ataagacccc agagtcacgc tcagtctctt tccagccttt tcatcatccg gtacattcag
3361 acaagttttca ggggaaggatc ctatttgtcc catgataatg atgggcaagg ggtggggagt
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3421 tatctcatac tccgcctgtg gatgaggggt cttctcaggt aaggctctta aatcctaggc
3481 ctgagtaaat tttttcaaat tttatttag acagggtccc tctctgttgc ctaggctgga
3541 gtgcagcggc acaatcacag ctcaatgcag cctcaacctc ccaggcccaa gtgatcctcc
3601 cacctcagcc tcttcagtga ctaggactac aggtgcatga ctccatgctt ggctaacttt
3661 aaaaaatgtt tgtttgtttg tttgtttttt acagagatgg ggtctcacca tgttgcccag
3721 gctgatcttg aactcctggg ctcaagtgat tcccctgcct cggcctcctg aaattctggg
3781 attataggct tgagccacca tgcctggctc tgagtaaaga ttaagggaag ccatggtgct
3841 atcgcaatag ggtaccaggc agcttaacaa aggcagaagg gaacctcaga gaaccccgaa
3901 gagccaccgt aaagtgagtg ctgggggagc tgaacttcag tcagtacagg agccgaacag
3961 ccatcaggtg cgcagtgtta gtaattccac cctctgccct gggagcaagg tgtgtggaga
4021 aacctgtagc actttatgac catcagaacc agccttttc aaaaagacca tggagtactc
4081 tttgacctgt gtatataaca agaacctttc tcaaatagga aagaaatgaa ttggagaaaa
4141 ccactgttta catggcagag tgtgtctcct tcgcacacat cttgtttgaa gttaatcatg
4201 acattgcaac accaagtgat tccaaataat ctgctaggag tcaccatttc taatgattgc
4261 ctagtctatt catagctaat caagaggatg ttataaagca tgagtcagac acctctggct
4321 ttctggaagg gcaaggactc tatatataca gagggagctt cctagctggg atattggagc
4381 agcaagaggc tgggaagcca tcacttacct tgcactgaga aagaagacaa aggccagt
```

To further analyze the ability of Stat3 to directly influence the induction of the promoter activity of hMMP-1 a luciferase reporter construct driven by 4.3 kb of the human MMP-1 promoter (the sequence shown in Fig. 23) was employed. Parental HT-29 cells as well as derivatives over-expressing Stat3 and "Stat3 c.a." were transiently transfected with this construct and subsequently treated with IL-6. Luciferase activity measured 22 h after transfection was normalized by cotransfecting of HT-29 cells with a pRL-TK *Renilla* luciferase vector as described in Material and Methods.

Rutter et al. (1998) described that the incidence of the *2G* allele at the position -1607 bp (see Materials and Methods, Fig. 3) in the human (h) MMP-1 promoter possibly is correlated with aggressive tumors.

Therefore, both the *1G* and *2G* variant of the hMMP-1 promoter were included in the tests for Stat3 influence on promoter activity. HT-29 parental cells as well as HT-29 cells over-expressing Stat3 were transiently transfected in parallel with luciferase reporter constructs driven by MMP-1 promoter with either one or two G nucleotides at position -1607 and optionally stimulated with IL-6. **Fig. 24A** shows that a significant increase in transcription (ranging from 2-3-fold) was observed with the 2 G promoter construct compared to the 1 G promoter construct. IL-6 induced a similar relative effect (2.5-fold induction) on transcription with both 1 G and 2 G constructs in HT-29/Stat3 cells.

These data suggested that the presence of 2 G nucleotides at position -1607 in the MMP-1 promoter is important for transcriptional regulation of the hMMP-1 promoter by Stat3. For this reason, in the next experiments, the 2 G version of the hMMP-1 luciferase reporter gene construct was used.

To further investigate the role of Stat3 for MMP-1 transcription, HT-29 derivatives over-expressing Stat3 and "Stat3 c.a." were included in the study **(Fig. 24B)**. In both cell lines, a

clear increase in transcription of the hMMP-1 luciferase reporter construct was observed compared to non-transfected cells. IL-6 treatment strongly induced the promoter activity of the hMMP-1 promoter in HT-29 cells as well as in cells over-expressing Stat3 **(Fig. 24C)**. The results demonstrate that Stat3 has an immediate effect on transcriptional activity of the MMP-1 promoter in HT-29 cells. They underscore a potential functional relation of Stat3 activity and expression of proteases relevant for tumor cell malignancy.

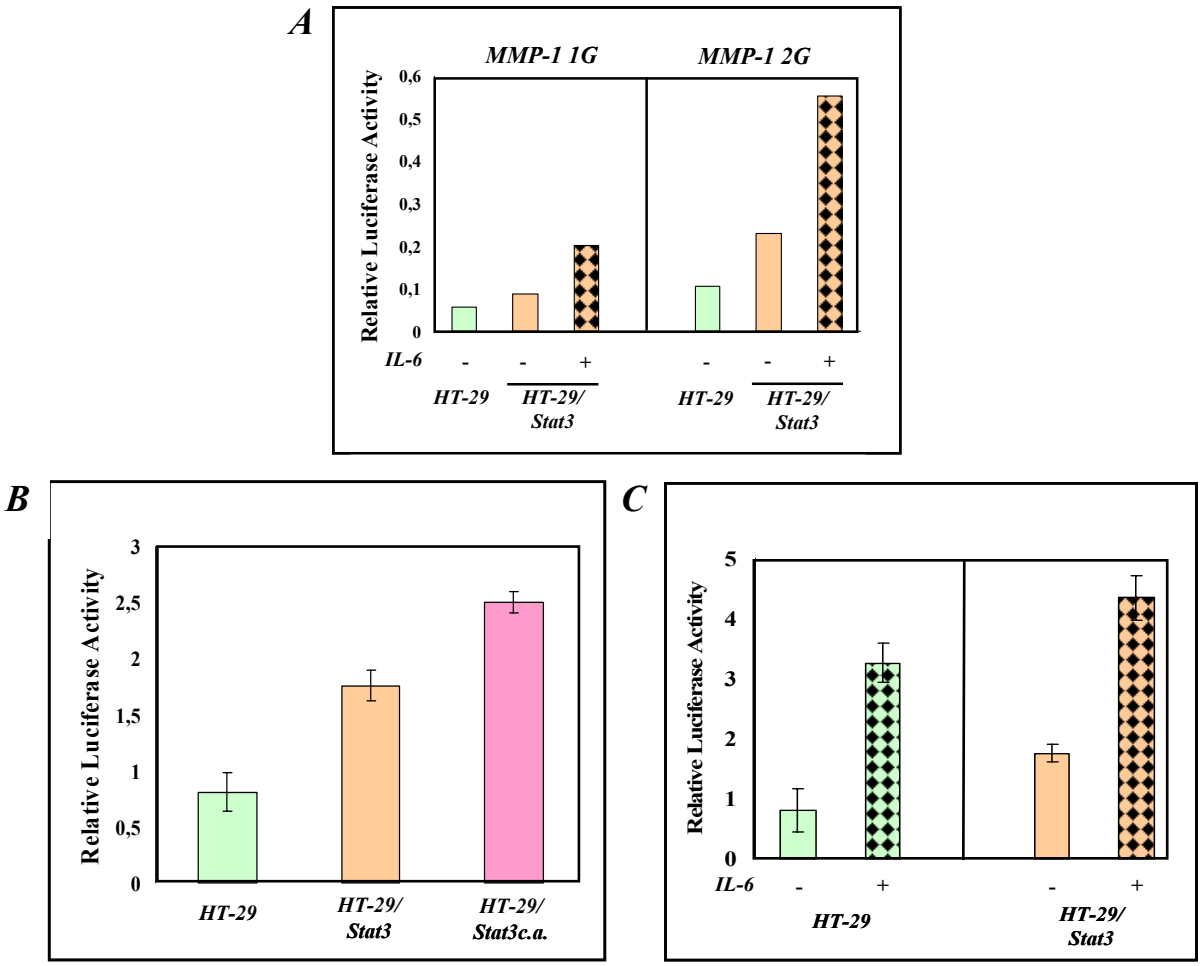

Fig. 24: Effects of Stat3 activity on transcriptional activitation of the human MMP-1 promoter.

A: *Effects of the single nucleotide polymorphism (SNP) at -1607 bp of the hMMP-1 promoter on Stat3-mediated promoter activity in HT-29 cells. HT-29 cells or HT-29 cells stably over-expressing Stat3 were transiently transfected with 1 µg of the 4,3 kb human MMP-1 1G- (left) or 2Gs (right) luciferase reporter plasmid together with 0.1 µg of the pRL-TK Renilla luciferase vector as an internal control. Cells were optionally treated (overnight) with 20 ng/ml IL-6 before luciferase activity was measured. The results were normalized to renilla activity.* ***B:*** *Induction of human MMP-1 promoter activity by means of stable transfection of HT-29 cells with Stat3 or the "Stat3 c.a." mutant. The experiment was performed as described under A. Three independent transfections, each run in triplicate, were performed and the results were normalized to renilla activity and expressed as the means ±S.E.* ***C:*** *Transcriptional activation of the hMMP-1luci reporter gene in HT-29 cells following stimulation with IL-6. Non-transfected HT-29 and HT-29/Stat3 cells were transiently transfected with hMMP-1luci 2G reporter plasmid and stimulated with IL-6 as described above.*

6.3.6. Constitutive Stat3 activity and MMP-1 expression are correlated in tumors derived from a colon carcinoma cell line

It was previously shown that the constitutively active state of Stat3 in colon carcinoma cells is not lost inevitably by cultivation, and can be restored by injecting tumor cells into mice (Corvinus et al., 2004). To analyze the differences between colon carcinoma cells in culture and in the tumor context with regard to Stat3 activity and MMP-1 abundance, the xenograft tumors derived from HT-29 cells (Corvinus et al., 2004) were subjected to the analysis of MMP-1 expression by immunohistochemistry. A specific antibody for MMP-1 revealed high expression of the protease in the cytoplasm of tumor cells **(Fig. 25A)**. To approach the mechanisms underlying MMP-1 expression in colon carcinoma cells, MMP-1 mRNA production in HT-29 cells negative for Stat3 activity and Stat3-positive xenograft tumor tissue was compared by real-time RT-PCR. HT-29 colon carcinoma cells showed low expression of MMP-1 mRNA. In contrast, expression of MMP-1 mRNA in HT-29 xenograft tumor was much higher **(Fig. 25B)**. Taken together, MMP-1 expression parallels the activity of Stat3.

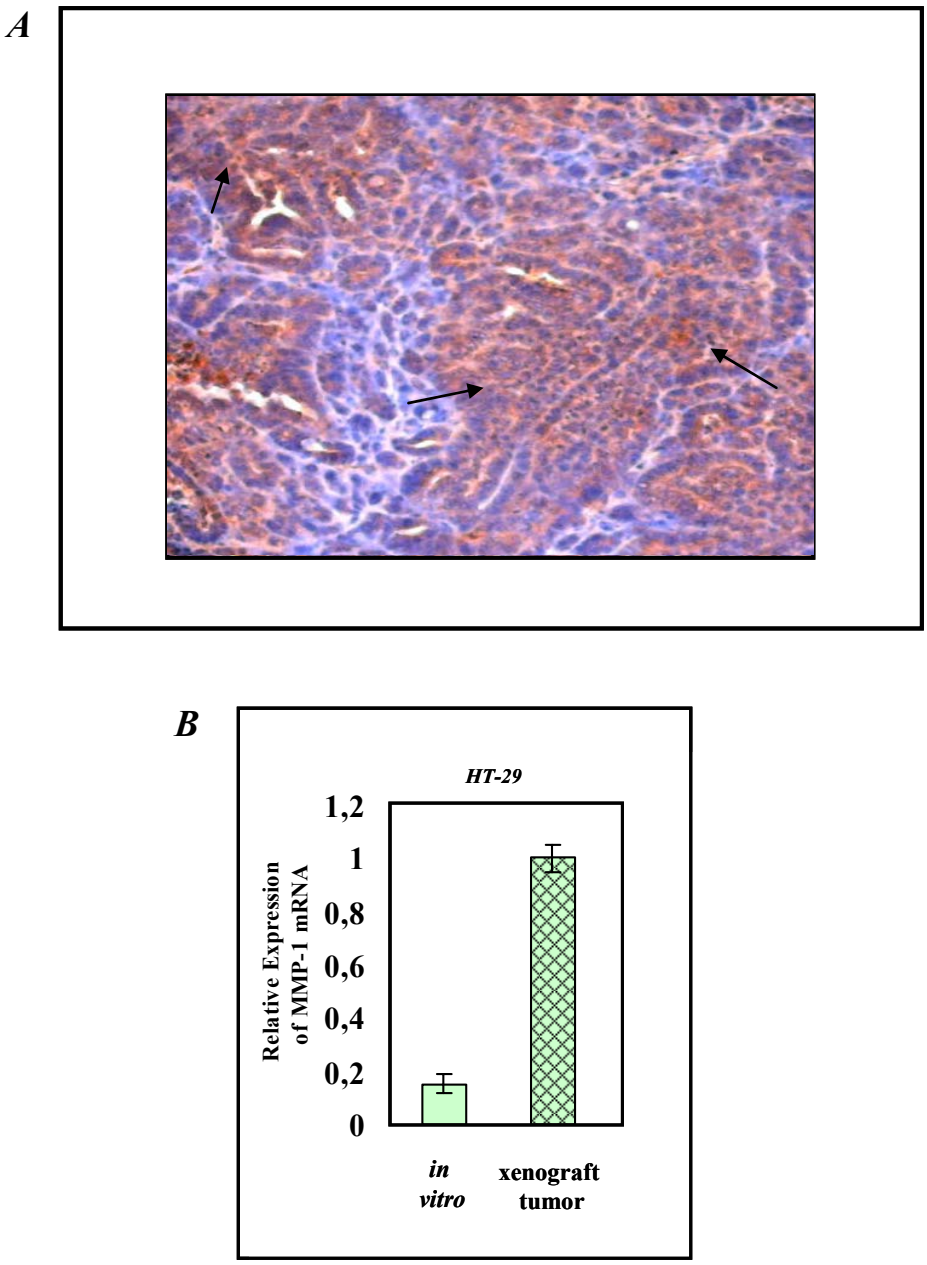

Fig. 25: Comparison of MMP-1 expression in HT-29 xenograft tumors and HT-29 cells grown in culture. ***A:*** *Histological examination of a HT-29-derived xenograft tumor for MMP-1 expression. Sections from a representative tumor induced by injection of HT-29 cells into nude mice were reacted with an antibody specific for MMP-1. Arrows indicate region of intense MMP-1 staining.* ***B:*** *Expression of MMP-1 mRNA in HT-29 cells and xenograft tumors. Total RNA was harvested from confluent HT-29 cells or xenograft tumors and MMP-1 mRNA levels were measured by quantitative real-time PCR. Three independent experiments, each run in triplicate, were performed, and the results were normalized and expressed as means ±S.E. Relative MMP-1 expression was calculated by arbitrarily setting the expression in samples from xenograft tumors as 1 (100%).*

6.4. Evidence for an autocrine activation loop driving Stat3 activity and concomitant MMP-1 expression in colon carcinoma cells

6.4.1. Non-adhesive growth of colon carcinoma cells induces Stat3 activity via an autocrine loop

The results described so far show that Stat3 activity directly influences malignant cell behavior and expression and enzymatic activity of MMP-1 in the colon carcinoma cell line HT-29. Because there is no significant Stat3 activation in HT-29 cells in culture, but arises upon implantation into mice, experiments presented in the following address potential mechanisms of Stat3 activation in xenograft tumors. To this aim, growth conditions were provided that would more closely resemble to the tissue environment than standard cell culture. It was observed that HT-29 cells cultivated on the non-coated plastic surface of Petri dishes displayed a more de-differentiated appearance compared to cells grown in regular cell culture flasks, like rounding-up, detachment and formation of clusters in suspension **(Fig. 26A)**. It was tested if these morphological changes coincided with altered Stat3 activation. Notably, cells growing on non-coated surfaces showed high Stat3 activity in non-stimulated HT-29 cells and in HT-29 cells over-expressing Stat3 with regard to tyrosine phoshorylation **(Fig. 26B)**. The effect was not evident in cells transfected with dominant negative Stat3. This finding was in opposition with the situation observed with cells grown in regular culture flasks (compare Fig. 10 and 12).

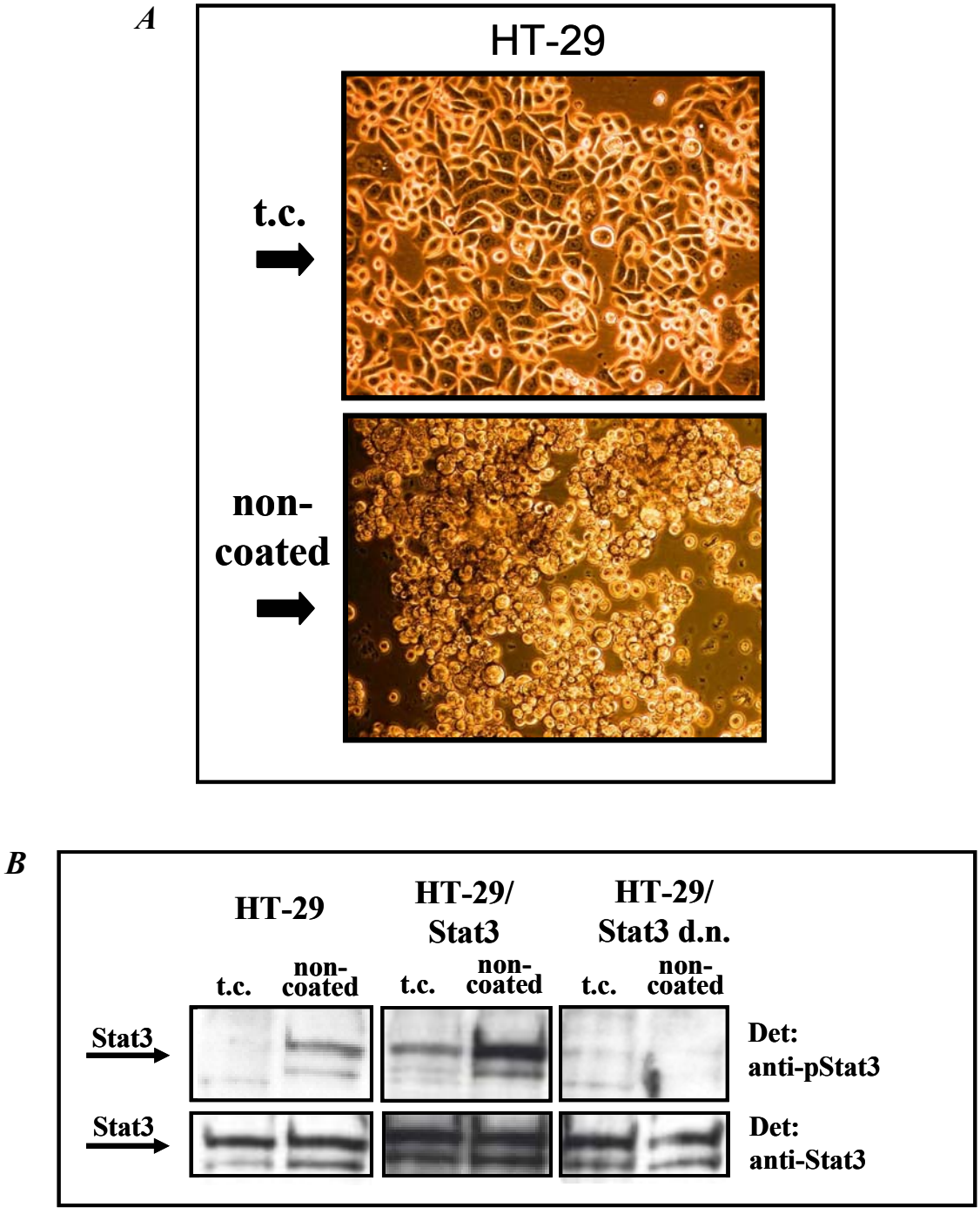

Fig. 26: Comparison of growth behavior and Stat3 activity in HT-29 cells cultivated on coated and non-coated surfaces. ***A:*** *Growth characteristics of HT-29 cells cultivated on regular tissue culture plasticware ("t.c.") and on non-coated surfaces ("non-coated").* ***B:*** *Western blot analysis of tyrosine phosphorylation (top) and expression (bottom) of endogenous, constitutively active ("c.a.") and dominant negative ("d.n.") Stat3 variants in HT-29-derived cell lines. Cells were grown for 4 days. They were then lysed and probed with specific antibody for phosphorylated Stat3 (top) and Stat3 (bottom), respectively.*

Next, it was addressed whether the Stat3 activation in cells grown in contact with non-coated surfaces may derive from the release of a Stat3 activating factor acting *via* an autocrine/paracrine mechanism. To test this possibility, conditioned medium from HT-29 cells as well as from HT-29 cells over-expressing Stat3, grown on non-coated plastic material for 3 days, was used to cultivate a new batch of cells in regular culture flasks. The Stat3 activity status was determined after 30 min **(Fig. 27)**. Interestingly, exposure of cells to conditioned medium induced high Stat3 tyrosine phosphorylation in parental cells and in the derivative over-expressing Stat3. Conditioned medium from HT-29/Stat3 cells had a stronger effect than medium from parental cells, indicating that Stat3 activity is directly related to the production of the activating factor. This notion is supported by the observation that expression of "Stat3 d.n." blocked the secretion of stimulating activity from cells grown on non-coated surface. These findings strongly indicate the existence of a secretory factor from HT-29 cells, which is capable of inducing Stat3 tyrosine phosphorylation through an autocrine pathway under conditions promoting cellular de-differentiation.

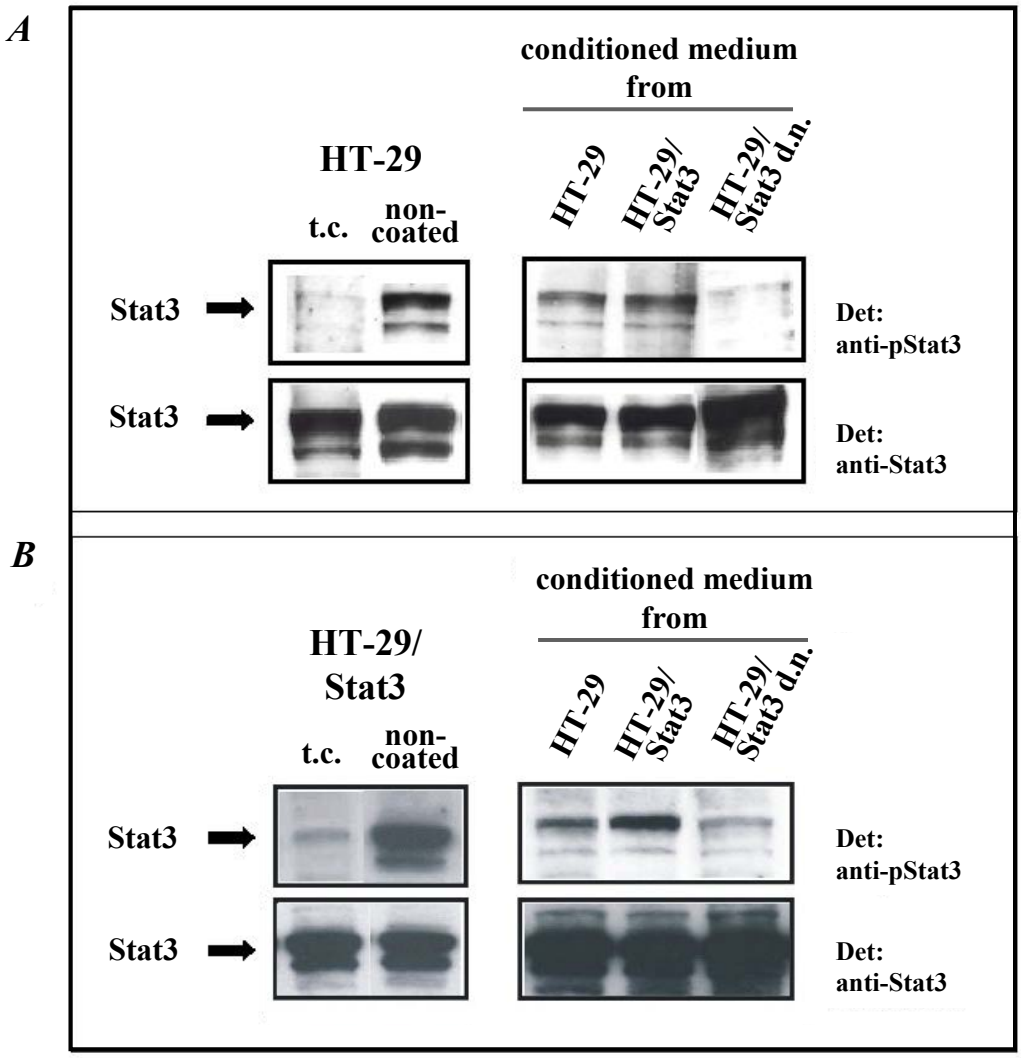

Fig. 27: Analysis of Stat3 activating properties of culture medium conditioned by HT-29 cells grown on non-coated surfaces. ***A:*** *Left-hand side: HT-29 cells were grown for 4 days on tissue culture (t.c.) or non-coated plastic material. Right-hand side: cells were grown for 30 min on tissue culture plastic surface in medium conditioned by the indicated cell lines grown on non-coated surface. Conditioned medium was obtained by centrifugation of cells and subsequent sterile filtration of the supernatant. Cells were harvested after treatment, lysed and subjected to Western blot analysis with a specific antibody for phosphorylated Stat3. Blots were reprobed with anti-Stat3.* ***B:*** *Same analysis for HT-29 cells over-expressing Stat3.*

6.4.2. Non-adhesive growth of HT-29 colon carcinoma cells enhances expression of the MMP-1 gene

In order to examine the effect of the non-adhesive growth of HT-29 cells on the expression pattern of MMP-1, the level of MMP-1 mRNA was measured by real-time quantitative PCR. HT-29 cells were incubated on tissue cell culture flasks or on microbiological Petri dishes for 4 days in RPMI 1640 before isolation of total RNA. It was found **(Fig. 28)** that the MMP-1 mRNA copy number in HT-29 and HT-29/Stat3 cells from the Petri dishes was significantly higher than the one from cells growing in regular flasks (4-fold and 3-fold, respectively). These results corroborate previous findings, *i.e.* MMP-1 expression is in correlation with the Stat3 activity in HT-29 cells.

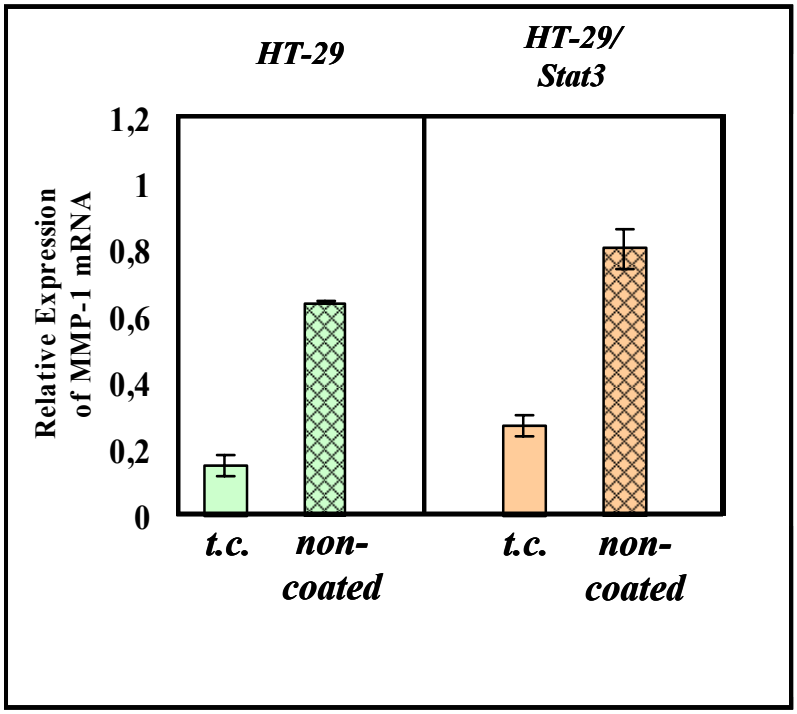

Fig. 28: Influence of growth conditions of HT-29 derivatives on MMP-1 expression. *HT-29- and HT-29/Stat3 cells were grown for 4 days on regular tissue culture plasticware ("t.c.") or on non-coated surfaces. The total RNA was isolated and MMP-1 mRNA levels were measured by quantitative real-time RT-PCR. Three independent experiments, each run in triplicate, were performed. Results were normalized and expressed as the means ±S.E.*

6.4.3. Non-adhesive cultivation of HT-29 cells induces the secretion of chemokines into the growth medium

To identify potential autocrine/paracrine factor(s) responsible for Stat3 stimulation in HT-29 cells, cytokines and growth factors were screened for specific appearance in the Stat3-activating conditioned medium. Abundance of 79 different factors were compared in conditioned media from HT-29 cells grown in regular cell culture flasks and on the non-coated plastic surface of Petri dishes using a commercial human cytokine array (Tebu-Bio). Membranes immobilized with 79 capture antibodies specific for cytokine and growth factors were exposed in parallel to the two media. Binding of specific antigens was subsequently detected by a mixture of detection antibodies.

In the growth medium of the colon carcinoma cells cultivated on non-coated plastic surface, elevated abundance of six proteins was identified in comparison to control medium: monocyte chemotactic protein (MCP)-1, angiogenin (Ang), interferon gamma inducible protein (IP)–10, IL-1α, macrophage inflammatory proteins (MIP)-1δ and -3α **(Fig. 29A and B)**. Surprisingly, all these proteins belong to groups of chemokins taking part in inflammatory processes. Two of them were described as important factors in tumor formation (angiogenin and MCP-1), for one of these proteins (MCP-1), an inducibility by Stat3 has been reported (Kim et al., 2002, also see Discussion). The cytokine arrays showed also that the abundance of IL-10, oncostatin M (OSM), growth regulated oncogene (GRO), macrophage colony stimulating factor (M-CSF) and MIP-1ß was reduced in the medium from the HT-29 cells grown on non-coated surface **(Fig. 29C)**. Further studies are required to evaluate the significance of these findings.

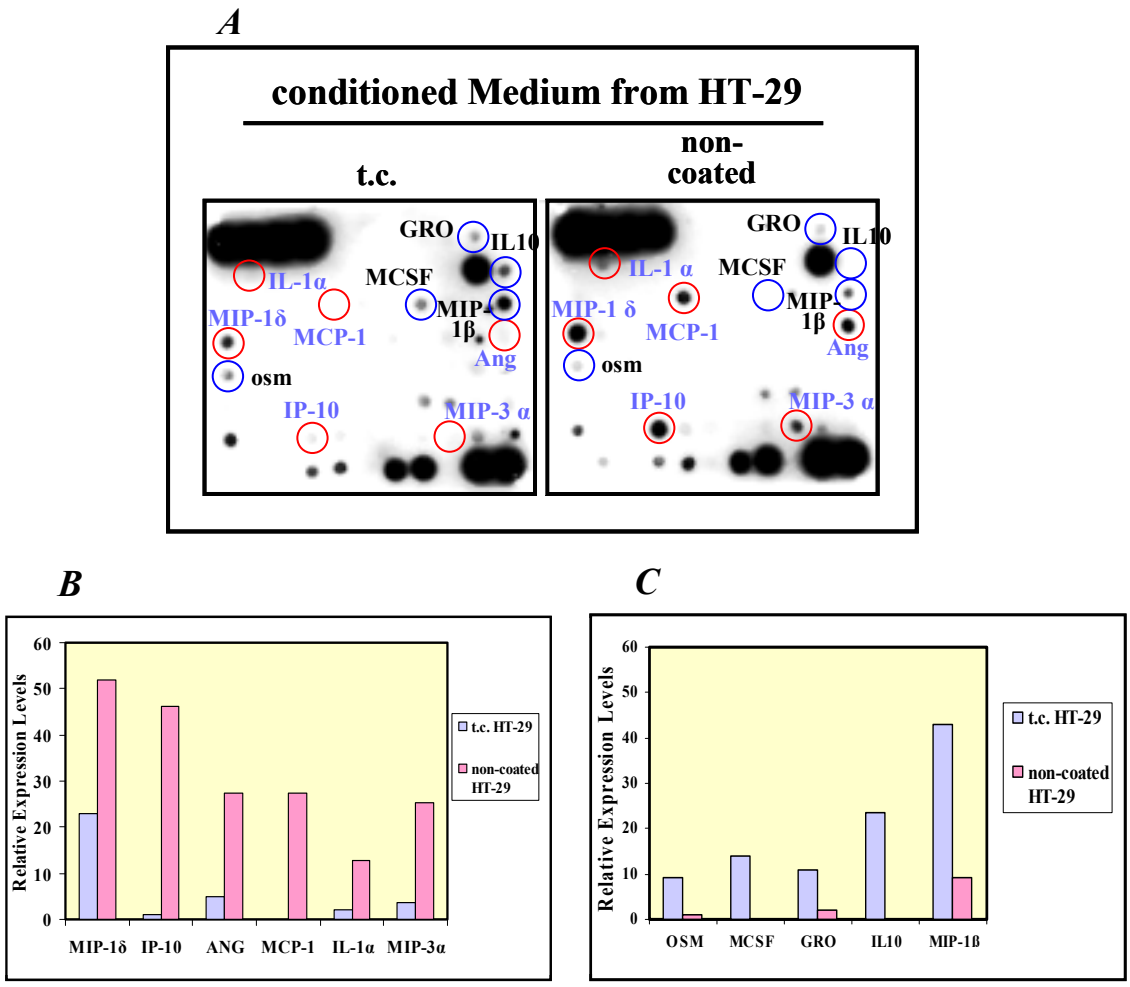

Fig. 29: Comparative analysis of the abundance of cytokines and growth factors in conditioned medium from cells grown on coated and non-coated surfaces. ***A:*** *Simultaneous detection of multiple cytokines and growth factors using a protein array. Conditioned media issued from HT-29 cells growth in regular cell culture flasks (left) and on non-coated plastic surface of Petri dishes (right) were incubated with cytokine array membranes. The membranes were then incubated with a combination of multiple biotin-conjugated antibodies to cytokines. Signals were visualized by enhanced chemiluminescence (compare Materials and Methods).* ***B:*** *Relative expression levels of factors up-regulated in conditioned medium issued from HT-29 cells growth on Petri dishes in comparison to control medium. The intensities of signals were quantified by densitometric determination. In order to normalize the different exposures, the mean signals derived from six positive controls were set 100 % percent.* ***C:*** *Relative expression levels of factors down-regulated in conditioned medium issued from HT-29 cells growth on Petri dishes in comparison to control medium. The levels were normalized to the mean of six positive controls which was set 100%.*

7. DISCUSSION

Carcinogenesis is a process dependent upon the decrease of tumor suppressor and increase of oncoprotein activity. During the multistep process of tumorigenesis, cells lose their normal ability to sense and repair DNA damage and to regulate cell cycle progression and apoptosis (Brinckerhoff *et al*., 2000). Simultaneously, they acquire abnormal patterns of growth factor signalling, angiogenesis, and invasive growth.

Tumor invasion and metastatic processes entail the loss of cell adhesion, degradation of the extracellular matrix as well as the basement membrane, and concomitant induction of cell movement (Liotta and Kohn, 1990). Various molecules promoting motility, such as growth factors and proteases, have been implicated in these processes. Tumor cells may display high constitutive expression of the matrix metalloproteinases (MMPs), even in the apparent absence of external stimuli. What mechanisms drive this expression? One possibility is that it results from errors in the regulation of signal transduction pathways targeted to the collagenase/MMP promoters (Mengshol *et al*., 2000; Dechow *et al*., 2004). This work provides evidence for a novel connection between erroneous signal transduction and protease expression in colorectal tumor cells *via* the signal transducer and activator of transcription Stat3.

While the STATs are not known to contribute directly to cell cycle checkpoint regulation or DNA repair, they contribute to tumorigenesis through their intimate connection to growth factor signalling, apoptosis, and angiogenesis. In addition, because these molecules play key roles in immune responses, defective STAT signalling can favor tumor development by compromising immune surveillance (Bromberg, 2002).

Stat3 is persistently activated in a large fraction of primary colon cancers as shown both by biochemical and immunohistochemical analyses (Corvinus *et al*., 2004). The over-expression of p-Stat3 is correlated with clinical stage and nodal metastasis in colorectal cancer (Ma *et al*., 2003). However, mutations in the Stat3 gene have not been identified in these cancers; hence, it remained to be determined how endogenous Stat3 is constitutively activated. The research presented here suggests that Stat3 activity in colorectal carcinoma (CRC) can be the result of not only paracrine, but also autocrine stimulation mechanisms.

This study elucidates the role of Stat3 activity in the progression of colorectal cancer. It was demonstrated in two different cell models that Stat3 promotes proliferative processes, and cellular transformation which can be tested by anchorage-independent growth in soft agar. The results showed that expression of dominant negative mutant Stat3 blocked IL-6-induced Stat3 activity and abolished the transformation response in colorectal carcinoma cells. Thus, the activated Stat3 molecule can independently mediate cellular transformation of these cells. The effect has also been observed in other transformed cell types, *e.g.*, glioblastoma cells (Rahaman *et al*., 2002) and squamous epithelial cells (Grandis *et al*., 1998). Fittingly, inhibition of Stat3 activation by the januskinase (Jak) pathway (AG 490) leads to decreased proliferation and survival potential of cancer cells (*e.g.*, Sriuranpong *et al*., 2003). Activation of Stat3 has been shown to target Cyclin D1, c-Myc, and Bcl-x, all of which are mediators of proliferation and antiapoptosis. Blocking constitutive Stat3 expression results in growth inhibition and apoptosis of Stat3-positive tumor cells *in vitro* and *in vivo* (Turkson and Jove, 2000). For the first time, Bromberg *et al*. (1999) have demonstrated anchorage - independent growth of fibroblasts transfected with constitutively active Stat3 in soft agar, and subsequent forming of tumors from these cells in nude mice. Similar effects have also been demonstrated by Uttamsingh *et al*. (2003) in embryo fibroblasts. The results of this work indicate that Stat3 can mediate oncogenic transformation not only of fibroblasts, but also of many others types of cells, including colorectal epithelial cells.

Moreover, not only does constitutive activation of Stat3 induce cellular transformation of colon cancer cells, but it can also enhance tumor cell motility and invasiveness. These results confirm those reported in another study in which the invasion of diverse human cancer cell lines and human dermal microvascular endothelial cells through Matrigel was found to be increased with Stat3 activation and suppressed by Stat3 dominant negative ("Stat3 d.n.") mutants (Niu *et al*., 2002; Yahata *et al*., 2003). Collectively, these results underscore the importance of this Stat3 pathway in the development of an invasive phenotype.

The mechanism of transformation by Stat3 is thought to be through the genes it transcriptionally regulates. Transcriptional regulation of genes by activated Stat3 is likely to be dependent upon the cellular context and thus the mechanism of transformation. Although there is some information about mechamisms of Stat3-driven proliferation, much less is known about transcriptional regulation of genes responsible for other aspects of malignancy. For example, it has been shown that HGF, a Stat3 activator, induced

invasiveness of the CaCo-2 human colon cancer cell line, and HGF stimulation is associated with increased production of MMP-1, -2 and -9. In contrast, anti-HGF antibodies inhibited protease production as well as cell invasion (Kermorgant *et al*., 2001). In line with this, the correlation between Stat3 activity and expression of tumor-associated proteinases was studied in this work. This study focused on MMP-1 as possibly relevant to Stat3-mediated transformation because its expression was the highest out of all tested proteases. The prognostic significance of MMP-1 expression in colorectal cancer has been reported. High levels of MMP-1 expression have been correlated with metastatic spread of tumors and poor prognosis of colorectal cancer (Curran and Murray, 1999; Shiozawa *et al*., 2000; Horiuchi *et al*., 2003). This study shows that up-regulation of MMP-1 mRNA is dependent upon Stat3 acitivity, as determined by real-time quantitative PCR. The results suggest that MMP-1 is expressed neither in colorectal tumors with low Stat3 activity nor in HT-29 cells. In contrast, expression of MMP-1 mRNA in corresponding tumors with a high Stat3 activity and in Stat3 transfected cells was clearly elevated. One of the major questions that comes up in assessing mRNA copy numbers of a gene is the relative association with respective protein expression in a specimen. The mRNA copy number of a gene does not always correlate with the expression of protein product because of the variant post-transcription events, such as mRNA modification, translation efficacy, half-life of the mRNA, translation modification, and protein turnover in the cell (Takeuchi *et al*., 2003). Results presented here demonstrate that MMP-1 mRNA copy number detected by real-time quantitative RT-PCR correlated well with MMP-1 protein expression. It is likely that expression as shown by immunohistological staining of tumor samples reflects MMP-1 activity, although the actual enzymatic activity could only be tested in colorectal carcinoma cell lines. Immunohistological methods showed that primary colorectal carcinoma (CRC) tumors, exhibiting a more intense Stat3 activity, demonstrated a remarkable increase in MMP-1 staining intensity, relative to primary CRC without Stat3 activity. These results were confirmed by ELISA for MMP-1 activity in supernatants of Stat3 transfected cells. Moreover, IL-6 stimulation of Stat3 in HT-29 cells was shown to enhance MMP-1 mRNA expression as well as MMP-1 endogenous activity. Future work will include tissue co-localisation of Stat3 and MMP-1 in tumor samples, directly tested by simultaneous immunohistological staining of both proteins. It would be very interesting to investigate whether elevated Stat3 activity and MMP-1 expression also coincides in other malignant tumors.

One of the intriguing questions concerning MMP-1 expression is that of the cell type producing this enzyme within tumor tissue. This study showed that MMP-1 is localized in colon carcinoma cells themselves, but is not found in stromal cells of tumors or in normal mucosal tissue specimens. These results corroborate previous findings (Nomura *et al*., 1996; Yamashita *et al*., 2001) and suggest that MMP-1 expression by the carcinoma cells themselves is one of the important factors affecting local tumor invasion and metastasis of colorectal cancer.

The enzymatic activity of MMPs was formerly considered to be specifically inhibited by tissue inhibitor of metalloproteinases (TIMPs). However, a recent study has shown that high levels of TIMP-1 and TIMP-2 are associated with aggressive cancers (Curran and Murray, 1999; Baker *et al*., 2000). Interestingly, in this experiment cDNA arrays revealed up-regulation of TIMP-1 mRNA in Stat3-positive samples from colorectal carcinoma, as compared to Stat3-negative tumors.

These results demonstrate that the Stat3 pathway not only plays an important role in colorectal carcinoma proliferation and transformation, but is also a driving force behind over-expression and elevated activity of MMP-1 in HT-29 cells. Thus, constitutive activation of this pathway may allow colon carcinoma cells (at least in certain situations) to mutate into an invasive phenotype, thereby facilitating metastasis. Involvement of Stat3 in invasiveness has also been described for other types of a cancer. As discussed by Yu *et al*. (2002), Stat3 activation is required for the progression of multistage carcinogenesis of mouse skin epithelial cells. Both basal and IL-6 induced expression of MMP-1 and MMP-3 were inhibited by a dominant-negative mutant of Stat3. In another study, blocking of endogenous gp130 signalling towards Stat3 in breast cancer cell lines by a dominant-negative gp130 protein resulted in inhibition of invasion and metastasis of breast cancer cells in a nude mouse model (Selander *et al*., 2004). In renal cell carcinoma a high frequency of Stat3 activation was associated with metastatic disease. A high level of p-Stat3 was a significant indicator of a poor prognosis (Horiguchi *et al*., 2002). Several studies indicate that Stat3 promotes angiogenesis, growth, and metastasis of human pancreatic cancer by directly regulating Vascular Endothelial Growth Factor (VEGF) expression (Wei *et al*., 2003).

One purpose of this work was to reveal a direct connection between Stat3 and MMP-1. The promoter region of the MMP-1 gene contains various transcription factor binding sites including several proximal activator protein–1 (AP-1) and PEA-3 sites (which bind

members of the Fos and Jun family of transcription factors), STAT binding sites, cognate element for GATA binding factors, and heat shock factors. AP-1 is regulated by mitogen-activated protein kinase (MAPKs) and is a pivotal transcription factor that regulates cytokine production and expression of MMPs (Whitmarsh and Davis, 1996; Brauchle *et al*., 2000; Cho *et al*., 2000), whereas STAT proteins are frequently involved in mediating cytokine-induced intracellular signals leading to expression of inflammatory molecules, including MMPs (Kisseleva *et al*., 2002). The relative effect of AP-1 and STAT proteins in MMP expression varies depending upon cell type, stimulus and the MMP being studied. For example, a specific inhibitor of Jak3, which impairs Stat3 activation, showed almost 100% inhibition of MMP-1 and MMP-3 mRNA induction and protein expression levels in human chondrocytes. However, inhibition of extracellular signal-regulated kinases (Erk) 1/2 in human chondrocytes had little effect on the expression of MMP-1 (Behera *et al*., 2004). Li *et al*. (2001) demonstrated that Jak3-specific inhibitor in primary and human chondrocytes blocked oncostatin M (OSM) - stimulated Stat1 tyrosine phosphorylation, DNA-binding activity of Stat1, as well as MMP-1, -3 and -13 expression. In contrast, a Jak2-specific inhibitor, AG 490, had no impact on these events.

It has been demonstrated that Stat3 plays an important role in FGF-1–induced matrilysin (MMP-7) expression. Transient transfection with dominant negative Stat3 inhibited FGF-1–induced transactivation of the matrilysin promoter. The 1.2-kb region of the human matrilysin promoter contains a number of cytokine inducible SH2-containing protein (CIS) elements that are known to bind Stat3 (Udayakumar *et al*., 2002). It was recently shown that tyrosine-phosphorylated Stat3 is causally related to MMP-9 promoter activity (Dechow *et al*., 2004). Moreover, a high-affinity Stat3-binding element was identified in the MMP-2 promoter. Transfection of constitutively activated Stat3 into poorly metastatic C23 melanoma tumor cells directly activated the MMP-2 promoter, whereas the expression of a dominant-negative Stat3 in highly metastatic C4 tumor cells inhibited the MMP-2 promoter (Xie *et al*., 2004).

This work shows that Stat3 directly up-regulates the expression of MMP-1 in HT-29 colorectal cancer cells. The MMP-1 promoter contains fourteen putative Stat3-binding sites (for the DNA binding specificity for Stat3 see Ehret *et al*., 2001). An MMP-1 promoter luciferase construct was directly induced at least 3-fold by “Stat3 c.a.” when transfected into HT-29 cells.

The level of MMP-1 expression, and consequentially its potential to mediate connective tissue degradation and tumor progression, can be influenced by a genetic variation in the

MMP-1 promoter. This variation is a single nucleotide polymorphism (SNP) located at-1607 bp, where an insertion of a guanine base (G) creates the sequence, 5 '-GGAT-3 ', the core binding site for members of the Ets family of transcription factors. Genotyping of 100 normal individuals indicated that the distribution of this SNP in the normal population is approximately: 30% - 1G homozygous; 30% - 2G homozygous; and 40% - 1G/2G heterozygous. However, in tumor cells cultured *in vitro*, the incidence of the 2G allel rises to 62%, supporting the hypothesis that there is a correlation with aggressive tumors. This *in vitro* correlation has been upheld *in vivo* in studies of ovarian and endometrial carcinomas. In both studies, the patients had a significantly higher incidence of the 2G allel compared with non-cancer controls. Furthermore, patients with the 2G allel expressed higher levels of MMP-1 protein (Reviewed in Brinckerhoff *et al*., 2000). These data have formed the basis for experiment with 1G- and 2G- MMP-1 promoters in this work. The results showing that an intact Ets binding site enhances Stat3-driven promoter activity suggest an additive contribution of Ets and Stat3 to MMP-1 transcription.

It will be interesting to gain more precise understanding of the contribution of the different transcription factors involved in the regulation of the MMP-1 promoter, by applying methods, like Electrophoretic Mobility Shift Assays (EMSA) and mutational analysis. Considering the substrate specificity of MMPs for extracellular matrix components, the involvement of Stat3 in the up-regulation of MMP-1 expression is of importance. Type I and type III collagens are the main structural components of the stroma in the gastrointestinal tract. Degradation of these structural components is required for further invasion, and such degradation is due mainly to the action of MMP-1. Indeed, increased collagenolytic activity against type I and type III collagens in cancer tissue extracts is reportedly associated with deeper invasion of colorectal cancer (Horiuchi *et al*., 2003). It is important to mention that in the context of this work the promoters of other MMP family members, over-expressed in p-Stat3-positive tumors, have been inspected for potential Stat3 cognate elements, namely MMP-3, -7 and -9. All three promoters containe Stat3-binding sites (not shown).

In spite of extensive research on Stat3, the mechanisms which trigger excessive Stat3 activity in cancer are poorly understood. Unlike other signalling molecules, no naturally occurring genetic mutations or amplifications of Stat3 associated with oncogenesis have been identified so far (Burke *et al*., 2001). This indicates that persistent Stat3 activity is most probably due to dysregulated events upstream of Stat3 within the signalling

pathways, *e.g.*, activation or mutation of physiological STAT activators such as the Janus kinases. Alternatively, Stat3 activation in cancer could result from over-expression of growth factors and their receptors or inhibition of negative regulators (Bromberg, 2002). Various recent findings, including those shown in this work, point to an important role of cytokines and growth factors secreted by tumor cells.

In contrast to tumor cell lines originating from other tumors, all colon carcinoma cell lines employed in this study were negative for Stat3 activity in the absence of cytokine stimulation. Interestingly, they showed profound activity of Stat3 upon implantation into immunodeficient mice and xenograft tumor formation (Corvinus *et al*., 2004). Attempts to promote cancerous cell behavior by providing culture conditions favoring non-adhesive growth for the colon carcinoma model cell line HT-29 (thus, mimicking aspects of the tumor tissue situation) yielded evidence that Stat3 activation may be induced *via* autocrine stimulation in the tumor. These results are in line with those by Vécsey-Semjén *et al*. (2002) who demonstrated that low passage COGA-1 cells were non-invasive on Matrigel-coated membranes, but were clearly tumorigenic in nude mice. Moreover, HT-29 and colon-38 colon carcinoma cells were clearly invasive and metastatic *in vivo*, but not *in vitro* (de Both *et al*., 1999). In both systems investigated (xenograft tumors and cells cultivated on the non-coated plastic surfaces), induction of Stat3 activity was paralleled by enhanced expression and activity of MMP-1. These findings are consistent with the results of Aparicio *et al*. (1999), who showed *in vitro* that rat colonic cancer cells did not express MMP-1; however, its expression was very high in ascitic fluids and solid tumors formed by these cells. *In vitro*, MMP inhibitor batimastat did not affect rat colon cancer cell proliferation, and slightly reduced cell invasion. However, *in vivo*, batimastat treatment totally prevented peritoneal carcinomatosis and liver metastasis development, and significantly prolonged survival. All these results support the possibility of an autocrine activation loop of Stat3 in CRC cells that is connected to the control of MMP-1 activity.

Autocrine loops involving factors that trigger Jak/STAT pathways have been identified as crucial for malignant cell behavior in several tumor models. In breast carcinoma cells, a mechanism of autocrine-mediated Stat3 activation has been identified which correlates with cell proliferation (Li and Shaw, 2002). In brain tumors, Stat3 is considered to play a central role in autocrine activation of the Vascular Endothelial Growth Factor (VEGF) system (Schaefer *et al*., 2002). Various examples of IL-6-triggered autocrine stimulation of tumor cells have been published recently, such as acute myeloid leukemia cells (Schuringa *et al*., 2000), melanomas cells (Moretti *et al*., 1999), breast cancer (Knupfer *et al*., 2004),

esophageal carcinoma (Leu *et al*., 2003), head and neck squamous cell carcinoma (Sriuranpong *et al*., 2003), prostate cancer (Royuela *et al*., 2004) renal carcinoma (Angelo *et al*., 2002; Horiguchi et al., 2002), and also human cervical cancer (Wei *et al*., 2001).

Surprisingly, this work revealed that none of the cytokines of the IL-6 family were over-expressed in conditioned growth media with high Stat3 activating potency. Instead, up-regulation of six chemokines (MCP-1, Ang, IP-10, IL-1α, MIP-1δ and MIP-3α) was found in supernatants of HT-29 cells grown in non-adhesive conditions.

Considering the possible role of MCP-1 in autocrine Stat3 activation, a wealth of evidence suggests that MCP-1 may play an important role in tumor formation. In contrast to the majority of normal cells, many human and murine tumor cells are known to constitutively produce high levels of MCP-1, *e.g.*, human glioblastoma (Huang *et al*., 2002), melanoma (Nesbit *et al*., 2001), ovarian cancer (Hefler *et al*., 1999), breast carcinoma and lung cancer (Wong *et al*., 1998). Additionally, clinical studies suggest that high expression of MCP-1 is a significant indicator of early relapse of human breast cancer (Ueno *et al*., 2000). Moreover, MCP-1 expression may contribute to the highly malignant phenotype of murine mammary adenocarcinoma cells (Neumark *et al.,* 1999) and is capable of inducing angiogenesis, a very critical aspect of tumor growth (Goede *et al*., 1999). Expression of MCP-1 is strongly associated with chronic inflammation, which may promote tumor development (de Boer *et al*., 2000). cDNA microarray technology revealed the association between the accumulation of MCP-1 and the development of drug resistance in ovarian cancer cells (Duan *et al*., 1999). Furthermore, Huang *et al*. (2002) showed that inhibition of MCP-1 in glioblastoma cells with anti-MCP-1 antibody reduced cell proliferation rates. Not surprisingly, MCP-1 promotes glioblastoma cell colony formation in soft agar. The authors also discuss an autocrine mechanism of MCP-1 activation in these cells. Very interestingly, the MCP-1 gene is reported to contain Stat-binding elements in promoter regions. AG 490, a Jak2 inhibitor, reduced induction of this gene, as well as nuclear factor binding activity and activation of Stat1 and -3 in gangliosides-treated microglia (Kim *et al*., 2002). MCP-1-induced tyrosine phosphorylation of cellular proteins involved the phosphorylation of non-receptor tyrosine kinases Lyn, Jak2, cytoskeletal binding protein paxillin and downstream transcription factors Stat-3 and Stat-5 (Mellado *et al*., 1998; Biswas and Sodhi, 2002).

Human angiogenin is an excellent substrate for the adhesion of HT-29 human colon adenocarcinoma cells. These cells adhere more quickly to human angiogenin than to fibronectin, laminin, collagen I, or collagen IV (Soncin *et al*., 1994). Etoh *et al*. (2000)

examined the relationship between the mRNA expression of angiogenin and clinicopathological features in 65 patients with colorectal cancer. They found a significant difference in the vascular involvement, lymph node metastasis, liver metastasis, and advanced stage in patients with high expression of angiogenin compared to those with low expression. Immunohistochemical analysis revealed that angiogenin was predominantly expressed in colon cancer cells (Kwon *et al*., 2002). Interestingly, a non-cytotoxic neutralizing monoclonal antibody prevents the establishment of HT-29 human tumor xenografts in up to 65% of treated athymic mice, and also prevents the growth of androgen-independent human prostate cancer tumors in athymic mice in a statistically significant, dose-dependent manner (Olson *et al*., 1994; Vallee and Riordan, 1997; Olson *et al*., 2002).

Furthermore, several studies have also shown that levels of MIP-3α mRNA and protein were rapidly up-regulated in TNF α- and IL-1-stimulated HT-29 and Caco-2 colorectal carcinoma cells, with kinetics similar to those seen for MCP-1 and IL-8 (*e.g.,* Kwon *et al*., 2002).

It is highly important to identify the nature of the autocrine Stat3 activator in colon carcinoma cells. Ways of achieving this aim include treatment of cells with recombinant chemokines to confirm the positive effects, and the use of specific antagonists and inhibitory antibodies to block the Stat3 activating effect of cell culture medium from HT-29 cells grown on the non-coated plastic surface of Petri dishes.

Prognosis in patients with colorectal carcinoma has conventionally been determined using a staging system based on the extent of primary tumor and the presence or absence of metastasis, as in Dukes' classification (Shiozawa *et al*., 2003). In the absence of curative surgical options for colonic cancers, chemotherapy is the primary method of treatment, albeit only a palliative one. This has prompted numerous investigations into better therapeutic strategies.

Increased knowledge of the molecular mechanisms underlying cancer initiation and progression has led to the identification of signalling pathways that have gone awry in tumor cells, thus providing new molecular targets for therapeutic intervention in cancer. Fundamental basic research has contributed not only to the identification of particular oncogenes involved in cancer development but also to the delineation of whole signalling pathways. This knowledge of signalling pathways has proven to be of even broader benefit for the development of cancer drugs. Targeting of molecules embedded in such pathways,

but not identical with the causal oncogene, is especially applicable when the causal trigger is difficult to inhibit (Gibbs, 2000).

In this context, this work contributes to promising developments put forward by others toward establishing the Stat3 signalling pathway as a valid molecular target for therapeutic intervention in a variety of human cancers (Bowman *et al*., 2000; Bromberg and Darnell, 2000; Turkson and Jove, 2000; Song and Grandis, 2000). Inhibition of STAT signalling has repeatedly been demonstrated to result in growth inhibition and induction of apoptosis in tumor cells harboring constitutive activation of Stat3 ((Burke *et al*., 2001; Niu *et al*., 2002; Real *et al*., 2002). Studies using normal mouse fibroblasts demonstrate that disrupting Stat3 signalling causes growth arrest but not apoptosis (Bowman *et al*., 2001), suggesting that blocking Stat3 signalling may not be excessively toxic. One possible explanation for the increased sensitivity of transformed cells to apoptosis compared to normal cells is that tumor cells may have become irreversibly dependent on STAT signalling for survival. The observed dependence of certain tumors but not normal cells on constitutive STAT activation for survival has wide implications for cancer therapy, offering the potential for preferential tumor cell killing. Since inhibition of STAT proteins is not necessarily toxic to normal cells, and even partial inhibition might be sufficient for successful treatment, STATs have become an attractive target for pharmaceutical research. It is also important to note that inactivation of STAT proteins might resensitize resistant cancers to apoptosis-inducing chemotherapeutic agents (Bowman *et al*., 2000).

The role of MMPs in tumor invasion and angiogenesis has been well established. Based on rational design techniques, several MMP inhibitors have been synthesized, and their therapeutic effect examined. Unfortunately, several large-scale clinical trials of broad-spectrum MMP inhibitors on malignant cancers failed or were suspended because of intolerable side effects, most likely due to blockage of MMP functions that are critical for normal physiological processes (Fletcher, 2000). Further understanding of the mechanism by which Stat3 activates MMP-1 gene transcription may help to identify new specific therapy strategies that are designed to block specific pathways, with subsequent inhibition of MMP synthesis.

Currently, several means of blocking STAT activation are in development, such as receptor antagonists, tyrosine kinase inhibitors, STAT DNA binding blockers, STAT SH2 domain inhibitors, and naturally occurring inhibitors such as protein inhibitors of activated STATs (PIAS), decoy oligonucleotides, and STAT depletion (Reviewed in Ravandi *et al*., 2002).

Some “signal transduction inhibitors” that, among other effects, impair STAT signalling, have already entered the clinical phase. For example, STI571 ("Gleevec"; by Novartis) (Druker *et al*., 1996; Druker *et al.,* 2001) blocks bcr-abl, v-abl, PDGFR and c-kit tyrosine kinase activities (Attoub *et al*., 2002; Scandura *et al*., 2002), all likely to affect STAT function. Inhibitors of Jak family kinases such as AG 490 block Stat3 activation and survival of human myeloma cells, breast, renal and prostate cancer cells (Meydan *et al.,* 1996; Horiguchi *et al.,* 2002). Furthermore, Src family kinase inhibitors have been shown to block Stat3 activation and induce apoptosis of breast cancer cells (Garcia *et al*., 2001), also epidermal growth factor receptor (EGFR) inhibitors block Stat3 activation and survival of human renal carcinoma cells (Kedar *et al*., 2002).

The development of more specific inhibitors of STAT signalling is being pursued extensively. This includes Stat3 anti-sense oligonucleotides, which block the growth and survival of squamous carcinoma cells of the head and neck (SCCHN) (Grandis *et al*., 2000; Leong *et al*., 2003), non-small lung cancer cells (Song *et al*., 2003), and prostate cancer cells (Barton *et al*., 2004). Protein inhibitors of constitutive Stat3 signalling have proven to be of great value in suppressing cancer cell growth *in vitro* and *in vivo* (Buettner *et al*., 2002). Gene therapy of a mouse model of melanoma using the dominant-negative Stat3 variant, Stat3-β, yielded inhibition of tumor growth and even tumor regression. Although only about 10–15% of the tumor cells were transfected *in vivo*, the Stat3-β induced anti-tumor effect was associated with massive apoptosis of melanoma cells, indicative of a potent bystander effect. This bystander effect is mediated in part by the release of soluble factors that are capable of inducing apoptosis and cell cycle arrest of non-transfected tumor cells (Niu *et al*., 2001). In addition, recent studies indicate that constitutive Stat3 signalling induces VEGF expression and tumor angiogenesis, suggesting that suppression of angiogenesis may be part of the therapeutic efficacy of Stat3 inhibitors (*e.g.*, Niu *et al*., 2002; Wei *et al*., 2003).

Taken together, the following strategies for targeting constitutive STAT signalling are under investigation: (*a*) development of receptor-ligand interaction antagonists, such as cytokine antagonists and receptor-neutralizing antibodies; (*b*) alteration of STAT interacting proteins, such as the PIAS and suppressors of cytokine signaling (SOCS) family members; (*c*) inhibition of STAT-activating serine kinases; (*d*) activation of STAT-specific phosphatases; (*e*) targeting of STAT-regulated genes involved in malignant progression; and (*f*) development of small molecule inhibitors that interfere with STAT dimerization and/or DNA binding (Reviewed by Buettner *et al*., 2002). A wealth of

findings in recent years, some of which are mentioned here, make the therapeutic potential of STAT factors in cancer appear very promising.

It is important to further investigate the possible prognostic significance of aberrant STAT activity in cancer. It has been shown in some studies that Stat3 activation is significantly associated with poor prognosis. For example, increased levels of phosphorylated Stat3 in HNSCC cells (Masuda *et al*., 2002) and in acute myeloid leukemia blasts (Benekli *et al*., 2002) are associated with lower survival rates. In addition, a significant association of high levels of p-Stat3 with metastasis was observed in renal carcinoma cells (Horiguchi *et al*., 2002). The number of CRC patients included in this work is unfortunately not sufficient for statistically significant correlation of Stat3 activation with clinical data und requires extension in the future.

Interestingly, activation of Stat1 in breast cancer is a favorable prognostic marker (Widschwendter *et al*., 2002). It has also been reported that following radiotherapy, patients with nasopharyngeal carcinoma with constitutive Stat5 activation, or activation of both Stat3 and Stat5, had better disease-free survival and overall survival than those without activated Stat5 (Hsiao *et al*., 2003).

8. CONCLUSION

The results of this work suggest that aberrantly activated Stat3 contributes to malignancy in colorectal cancer. It enhances parameters of cell transformation such as proliferation, anchorahe-independent growth and invasiveness. The expression of MMP-1, a proteolytic enzyme with importance for the invasive properties of colorectal tumors, relies upon Stat3 activation. The mechanism of Stat3 activation within colorectal tumors probably includes an autocrine stimulation loop. Stat3 is a potentially interesting both as a marker and a therapeutical target in colorectal carcinoma.

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10. SUPPLEMENT

10.1. Lebenslauf

Persönliche Daten

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09/1993-06/1994	Gymnasium № 22, Novosibirsk, Russland
06/1994	Abitur mit ausgezeichnetem Erfolg

Studium:

09/1994 - 06/2000	**Studium an der Novosibirsker Staatlichen Medizinischen Akademie, Fachrichtung „Heilwesen“ („Humanmedizin“).** Förderung durch ein Stipendium des Präsidenten der Russische Föderation (1998-99) Delegation zum allrussischen Pirogow-Forum für Ärzte in Moskau (1999)
06/2000	**Diplom mit Auszeichnung. Approbation.** seit September 2000: Mitglied der Allrussischen Immunologengesellschaft seit Oktober 2000: Mitglied der Russischen Medizinischen Gesellschaft
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Berufstätigkeit

04/1997-06/2000	Arbeit in der Intensivtherapie (Kreismilitärkrankenhaus 333) als Krankenschwester (während des Studiums), Novosibirsk
07/2000-07/2001	Assistenzärztin am Klinikum für Immunologie und Allergologie, Novosibirsk
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Stipendium

seit 06/2004	Landesgraduiertenstipendium zur experimentellen Bearbeitung der Promotion an der FSU Jena

Liste der Veröffentlichungen

Publikationen:

1. Corvinus F, Orth C, Moriggl R, Tsareva SA, Pfitzner E, Döll F, Kaufmann R, Huber L A, Zatloukal K, Beug H, Halbhuber K-J, and Friedrich KH (2004) Persistent STAT3 Activation in Colon Cancer is Associated with Loss of Cell Attachment, Enhanced Proliferation and Autocrine Cytokine Production. Zur Publikation eingereicht bei *Neoplasia*

2. Fitzgerald JS, Tsareva SA, Pöhlmann T, Meissner A, Berod L, Pfitzner E, Wiederanders B, Markert UR, and Friedrich KH (2004) Leukemia Inhibitory Factor (LIF) triggers STAT3 activation, proliferation and altered protease expression in Jeg-3 choriocarcinoma cells. Zur Publikation eingereicht bei *Molecular Human Reproduction*

Kongressbeiträge, Abstracts

1. Tsareva SA, Corvinus F, Orth C, Wiederanders B, Kaufmann R, Pfitzner E, Moriggl R, and Friedrich KH: Constitutive activity of Signal Transducer and Activator of Transcription STAT3 in colorectal cancer cells, Oral presentation, 6th Meeting of the Signal Transduction Society Weimar Nov.7-9, 2002

2. Tsareva SA, Corvinus F, Orth C, Wiederanders B, Kaufmann R, Pfitzner E, Moriggl R, and Friedrich KH: Activities of oncogenic STAT 3 in colorectal cancer cells. Poster presentation, Herbsttagung der GBM und der Dt. Gesellschaft für Experimentelle und Klinische Pharmakologie, Halle/S, 2003

3. Tsareva SA, Corvinus F, Orth C, Wiederanders B, Kaufmann R, Pfitzner E, Moriggl R, and Friedrich KH: Constitutive activity of Signal Transducer and Activator of Transcription STAT3 in colorectal cancer cells coincides with elevated metalloproteinases expression. Poster presentation, VIII th International Symposium on Proteinase Inhibitors and Biological Control Brdo, Slovenia May 24-28, 2003

4. Tsareva SA , Corvinus F, Orth C, Moriggl R, Pfitzner E, Halbhuber K-J, Kaufmann R, Huber L, Zytloukal K, and Friedrich KH: Persistent STAT3 activation in colon cancer is associated with loss of cell attachment, enhanced proliferation, altered protease expression and autocrine cytokune production. Poster presentation, CellSignals Jena 03, Signaling Complexes (Ist meeting of the Joint Research Centres Sonderforschungsbereich 604 and Graduiertenkolleg 768), Jena, Germany , September 25-27th, 2003

5. Tsareva SA, Corvinus F, Orth C, Wiederanders B, Meissner A, Pfitzner E, Moriggl R, and Friedrich KH: STAT3 drives proliferation, malignant transformation and invasive behavior of colon carcinoma cells. Poster presentation, 7th Meeting of the Signal Transduction Society, Weimar, November 15-17, 2003

6. Tsareva SA , Corvinus F, Orth C, Wiederanders B, Meissner A, Pfitzner E, Moriggl R, and Friedrich KH: Konstitutive Aktivität des Transkriptionsfaktors STAT3 in Coloncarcinom Zellen ist assoziiert mit erhöhter MMP-Expression. Vortrag, „VII-Jenaer Proteolysetag“, Pössneck, Deutschland, Oktober 17, 2003

7. Fitzgerald JS, Corvinus F, Berod L, Tsareva SA, Pöhlmann T, Friedrich K, and Markert U R: Cytokine Influences on Invasiveness Related Functions of JEG-3 Choriocarcinoma Cells, Vortrag, „Deutsche Gesellschaft für Immunologie (DGfI)“, Jena, Deutschland, September 9, 2003

8. Fitzgerald JS, Corvinus F, Berod L, Tsareva SA, Poehlmann T, Friedrich K, and Markert UR (2003) Cytokine Influences on invasiveness related functions of Jeg-3 choriocarcinoma cells. *Immunobiol* **208**: 301-302

9. Pöhlmann TG, Fitzgerald JS, Meissner A, Tsareva SA, Wengenmayer T, Berod L, Corvinus F, Friedrich K, and Markert UR: Trophoblast invasiveness and intracellular STAT3 signal transduction, Oral presentation, „Joint Annual Meeting of Immunology of the Dutch and German Societies for Immunology (NVvI, DGfI)“, Maastricht, the Netherlands, October 20-23, 2004

10. Tsareva SA, Corvinus F, Wiederanders B, Meissner A, Pfitzner E, Moriggl R, and Friedrich KH: STAT3 drives proliferation, malignant transformation and invasive behavior of colon carcinoma cells. Poster presentation, Signal Transduction 2004, Luxembourg, January 25-28th, 2004

11. Corvinus F, Tsareva SA, Orth C, Moriggl R, Pfitzner E, Huber LA, Zatloukal K, and Friedrich KH: Constitutive STAT3 activity in colorectal cancer coincides with Loss of Cell Attachment, Enhanced Proliferation and Autocrine Cytokine Production. Poster presentation, Signal Transduction 2004, Luxembourg, January 25-28th, 2004

12. Tsareva SA, Orth C, Corvinus F, Meissner A, Wiederanders B, Pfitzner E, Friedrich KH: Constitutive activity of transcription factor Stat3 in colorectal cancer cells is

associated with enhanced expression of metalloproteinase-1. Oral Presentation, 21st Winter School on Proteinases and Their Inhibitors, Tiers, Italia, Februar 25-29th, 2004

13. Tsareva SA, Moriggl R, Corvinus F, Pfitzner E, Wiederanders B, Schütz A, and Friedrich K: STAT3 activation enhances invasive growth of colon carcinoma cells by transcriptional induction of matrix metalloproteinase 1 (MMP-1). Poster presentation, 8th Joint Meeting Signal Transduction, Weimar, November 4-6, 2004

Jena, den 15. Dezember 2004 Svetlana Tsareva

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10.3. Ehrenwörtliche Erklärung

Hiermit erkläre ich, dass mir die Promotionsordnung der Medizinischen Fakultät der Friedrich-Schiller-Universität bekannt ist. Die Dissertation habe ich selbst angefertigt und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben. Bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts wurde ich von Herrn PD Dr. rer. nat. Karlheinz Friedrich vom Institut für Biochemie I unterstützt. Die Hilfe eines Promotionsberaters habe ich nicht in Anspruch genommen. Dritte haben weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen, erhalten. Ich möchte hiermit noch einmal darauf hinweisen, dass die histologische Untersuchungen von Tumorproben, die der Entstehung der Abb. 20 im Kapitel 6.3.2. und Abb. 25A im Kapitel 6.3.6. zu Grunde liegen, von Herrn Dr. med. Alexander Schütz vom Institut für Pathologie (Leipzig) durchgeführt wurden. Ich erkläre außerdem, dass ich die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe und dass ich die gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung nicht bei einer anderen Hochschule als Dissertation eingereicht habe.

Jena, den 15. Dezember 2004 Svetlana Tsareva