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# Leukemia inhibitory factor triggers activation of signal transducer and activator of transcription 3, proliferation, invasiveness, and altered protease expression in choriocarcinoma cells

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*Abbreviations:* STAT, signal transducer and activator of transcription; LIF, leukemia inhibitory factor; IGF-II, insulin-like-growth-factor-II; HGF, hepatocyte growth factor; IL-6, interleukin-6; TIMP, tissue inhibitor of metalloproteinase; EMSA, electrophoretic mobility shift assay

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## 1. Introduction

Several parallels may be drawn between the state of pregnancy and cancer. The most apparent analogue is that of organ invasion, both in the morphological as well as the molecular biological setting (Murray & Lessey, 1999). Aberrant invasiveness is a hallmark of tumor malignancy. Invasion of trophoblast cells into the endometrial stroma, in contrast, is a strictly controlled process, which is initiated in the first and terminated in the third trimester of pregnancy (Garbisa et al., 1993). The fact that trophoblast cells are not only able to implant in other organs, as in ectopic pregnancies (Poliotti, 1996), but also to act as invasive xenogeneic transplants (e.g. Adams & Antczak, 2001; Poehlmann et al., 2004), implies that trophoblast cells themselves greatly contribute to the mechanism of invasion and renders them an interesting model for the control of invasive cell behavior.

A signal-transduction mediator that has been implicated in malignant cell transformation is signal transducer and activator 3 (STAT3). STAT3 has widespread functions in development and throughout the adult organism (reviewed by Levy & Lee, 2002). Physiologically, it is activated by cytokines and growth factors through receptor-mediated tyrosine phosphorylation (Heinrich, Behrmann, Müller-Newen, Schaper, & Graeve, 1998). Aberrant STAT3 activation has been reported in various cancers, e.g. of breast, skin, brain, and prostate (Dhir et al., 2002; Garcia et al., 2001; Niu et al., 2002; Schaefer, Ren, Fuller, & Schaefer, 2002), and it has also been associated with tumor metastasis (Horiguchi et al., 2002).

Recently, we have provided evidence for a correlation between trophoblast invasiveness and STAT3 activity. We could show that STAT3 activation in trophoblast cells ceases throughout pregnancy along with their loss of invasive properties (Corvinus, Fitzgerald, Friedrich, & Markert, 2003).

An abundance of cytokines and growth factors have been implicated in the regulation of trophoblast func-

tion, each having a different and specific profile in respect to its influence. Interestingly, many of these soluble factors have the capacity to trigger STAT3 by tyrosine phosphorylation, mostly, but not exclusively, through the STAT signal-transducing pathway (Bromberg, 2001). Leukemia inhibitory factor (LIF) plays a well-documented role in placentation (Stewart et al., 1992) and is additionally known to positively influence trophoblast invasion (Morrish, Dakour, & Li, 1998) migration, and proliferation (Kayisli, Selam, Demir, & Arici, 2002). Hepatocyte growth factor (HGF) is known to mediate proliferation, invasion, and differentiation in trophoblast cells (Kauma, Bae-Jump, & Walsh, 1999; Stewart, 1996) as well as motility in tumorous cells (Comoglio & Boccaccio, 2001). Immunohistological data suggested that interleukin-6 (IL-6) may be involved in the control of invasion and proliferation of villous trophoblast cells (Sengupta, Dhawan, & Ghosh, 2003), whereas insulin like growth factor (IGF)-II was reported to participate in extravillous trophoblast migration but not proliferation (McKinnon, Chakraborty, Gleeson, Chidiac, & Lala, 2001).

We are interested in defining stimuli involved in the control of STAT3 activation in trophoblast cells, and ultimately, in the promotion of invasive and proliferative properties via STAT3. Choriocarcinoma cell lines are frequently used as model systems to study mechanisms operative in trophoblasts (e.g. Helige, Hagedorfer, Smolle, & Dohr, 2001). Previously, we found that JAR cells resemble invasive, first trimester trophoblasts with respect to persistent STAT3 activation (Corvinus et al., 2003). For the purpose of this study, we chose Jeg-3 cells because of their relatively low-invasive properties and the fact that they are virtually negative for constitutive STAT3 activity. We could demonstrate that LIF was the only of the above-mentioned factors capable of evoking STAT3 tyrosine phosphorylation and DNA binding. At the same time, it was shown to drive cell proliferation and invasiveness and to evoke an alteration of the invasion-relevant protease expression pattern.

## 2. Materials and methods

### 2.1. Cell line and cell culture

Jeg-3 is an adherent human choriocarcinoma cell (HCC) line preserving several trophoblast-like capacities including production of pregnancy related hormones. It was obtained from the ATCC.

All Jeg-3 cultures were commenced at  $10^6$  cells/175-cm<sup>2</sup> flask, and maintained under standardized conditions (37 °C, 5% CO<sub>2</sub>, humidified atmosphere) in HAM's F-10 medium with L-glutamine supplemented with 10% fetal calf serum (FCS) and 2% antimycotic-antibiotic solution (AAS; Sigma, Germany). Cells were trypsinized twice a week, when confluence was estimated over 75%.

For all assays Jeg-3 cells were adjusted to  $10^5$  cells/ml and cultivated under the mentioned conditions and supplemented with various stimuli for different time spans according to the respective assay. Before addition of the stimuli, cells were starved for 2 h in medium without FCS. Cells were harvested for all analyses for immediate cell lysis, except proliferation assays, by carefully scraping the flask bottom but without trypsinization.

### 2.2. Cytokines and growth factors

The following cytokines and growth factors were used for stimulation of Jeg-3 cells at various concentrations: HGF, insulin-like-growth-factor-II (IGF-II), IL-6, LIF, transforming growth factor- $\beta$  (TGF- $\beta$ ).

### 2.3. SDS-PAGE and Western blotting

Per sample,  $2 \times 10^6$  cells were suspended in 100  $\mu$ l of lysis buffer (20 mM, HEPES; 2 mM, EGTA; 0.2 mM, EDTA; 0.12 M, NaCl; 1%, Triton) supplemented with protease inhibitors (20  $\mu$ g/ml aprotinin (trasyolol); 20  $\mu$ g/ml leupeptin; 5  $\mu$ g/ml pepstatin A; 647 ng/ml anti-pain; 10  $\mu$ g/ml bestatin; 0.1 mM PMSF; 1 mM sodium orthovanadate). Protein concentrations of cell lysates were determined using a kit based on the Bradford method (Sigma). Gel electrophoresis and Western blotting were performed as described previously (Kammer et al., 1996). Briefly, 20  $\mu$ g of whole cell extract were solubilized in gel-loading buffer (62.5 mM Tris-HCl pH 6.8; 2% SDS; 25% glycerol; 1% phe-

nol blue; 5%  $\beta$ -mercaptoethanol), boiled for 10 min and separated on 6% acrylamide SDS gels. After protein transfer, nitrocellulose membranes were blocked in NET-G buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl pH 7.5, 0.05% Triton X-100, 0.2% gelatin) for 1 h. Antibodies to STAT3 p-Tyr705 (Cell Signaling Technology) and STAT3 (c-20, Santa Cruz) were applied for 36 h at a 1:1000 dilution. For detection, peroxidase-conjugated anti-rabbit IgG (Roth) was employed at a dilution of 1:10 000 for 1 h. Visualization was performed using an enhanced chemiluminescence (ECL) detection kit (Amersham). Removal of bound antibody for re-detection with an additional antibody was carried out by treating membranes twice for 20 min with 62.5 mM Tris-HCl pH 6.7, 2% SDS, 10 mM  $\beta$ -mercaptoethanol at 68 °C and intermittent and final washing in 10 $\times$  PBS at ambient temperature.

### 2.4. Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assay for specific DNA binding of STAT3 was performed as described (Corvinus et al., 2003). Briefly, whole cell extracts obtained by repeated freeze-thawing were cleared by centrifugation and incubated with the <sup>32</sup>P-labelled STAT-binding site from the serum inducible element (SIE). Binding reactions were performed by incubating 10 000 cpm of radiolabelled probe with 20  $\mu$ g of cell lysate for 30 min at room temperature. For supershift reactions of STAT containing complexes, 2  $\mu$ g of antibodies to STAT1 (M22, Santa Cruz) or STAT3 (c-20, Santa Cruz) was added to the binding reactions before EMSA was performed. Samples were analyzed by electrophoresis through 6% native polyacrylamide gels and autoradiography.

### 2.5. Long-term cell proliferation assay

Aliquots of  $5 \times 10^5$  cells were suspended in 2 ml of medium including 10% FCS and allowed to adhere in individual wells of 6-well cell culture plates. They were grown in the absence or presence of cytokines for 3 days. At intervals, cells from individual wells were detached from the plastic surface in 0.5 ml of 0.05% trypsin/0.02% EDTA for 2 min at 37 °C before 1.5 ml of medium was added to restore the original volume; 50  $\mu$ l aliquots of cell suspensions were combined with

50  $\mu$ l of trypan blue and counted using a Neubauer chamber. Numbers of cells were extrapolated to the total volume of the culture.

### 2.6. *In vitro* cell invasion assay

Cell invasiveness was quantified by a modified Boyden chamber method using polycarbonate transwells (Corning Costar Corp., Cambridge, MA) consisting of a separate top well, a filter (pore size, 8  $\mu$ m; diameter, 6.5 mm) and a corresponding lower well. Membrane filters were coated with 200  $\mu$ l of a 1:50 Matrigel (Becton Dickinson, Bedford, MA) dilution in ice-cold serum-free RPMI 1640 medium (40  $\mu$ g/filter) and dried overnight at 37 °C;  $2 \times 10^5$  exponentially growing Jeg-3 cells in a volume of 1 ml of serum-free medium (containing no cytokine or 40 ng/ml LIF or 10 ng/ml IL-6, respectively) were seeded on top of a transwell. The lower compartment of each transwell unit contained 2.5 ml of medium supplemented with 10% fetal calf serum. After incubation for 24 h at 37 °C, medium with remaining cells and the matrigel layer were removed from the upper compartment of the transwells. Cells attached to the underside of the membranes were washed off by rinsing membranes twice with a total of 1 ml of PBS containing 0.02% (w/v) EDTA and 0.05% (w/v) trypsin. This washing solution was combined with the medium in the lower transwell compartment (containing cells that had already passed the membrane). To also include in the analysis cells that had reattached to the bottom well, the lower transwell compartment was finally treated with 200  $\mu$ l of 0.125% trypsin/0.05% EDTA in PBS for 2 min at 37 °C. The resulting suspension was added to the cell suspension described above to obtain the total of migrated cells, which were collected by 10 min centrifugation at  $1000 \times g$ . Membranes and bottom wells were checked microscopically for the virtual absence of remaining cells. Cell pellets were re-suspended in 1 ml of medium and stained with trypan blue. Migrated cells were quantified by counting of five random microscopic fields using a light microscope and a Neubauer chamber and extrapolation of the mean to the original volume of cell suspension. The percentage of invasive cells was expressed by relating the total number of migrated cells to the number of cells originally applied to the top of the transwell, which was set 100%.

### 2.7. *Protease expression analysis by cDNA arrays*

cDNA macroarrays on the basis of nylon membranes were prepared as follows: PCR fragments of 200–500 bp in length were derived from cDNAs representing the following collection of human intra- and extracellular proteases and protease inhibitors: matrix-metalloproteinases, MMP 1, 2, 3, 7, 9, 11, 13, and 19; membrane-type matrix metalloproteinases MT1–MMP, MT2–MMP, MT3–MMP, MT4–MMP, and MT5–MMP; A disintegrin and metalloproteinases ADAM 8, 9, 10, 11, 15, 20, and 21; cathepsins B, D, F, H, K, L, S, V, W, and Z; tissue inhibitors of metalloproteinases TIMP 1, 2, 3, and 4; caspases 1, 2, 3, 4, 6, 7, 8, 9, and 10; urokinase-type plasminogen activator uPA, urokinase-type plasminogen activator receptor uPAR; integrins  $\alpha$ 2,  $\alpha$ 4,  $\alpha$ 5,  $\alpha$ 6,  $\alpha$ V,  $\beta$ 1,  $\beta$ 2,  $\beta$ 3,  $\beta$ 4,  $\beta$ 5, and  $\beta$ 7; plasminogen activator inhibitors PAI-1, and PAI-2; cystatins A, B, and C; tumor necrosis factor  $\alpha$  converting enzyme (TACE), emmprin, heparanase, tissue factor, granzyme H, and bikunin. As “house-keeping” and negative controls, probes representing the following cDNAs were included:  $\beta$ -actin, transcription factor c-ets-1, carcinoembryogenic antigen (CEA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), green fluorescent protein (GFP), ubiquitin, elongation factor EF1 $\alpha$ , and ribosomal protein S9. PCR fragments were purified by preparative agarose gel electrophoresis and concentrations were adjusted to 20 ng/ $\mu$ l in H<sub>2</sub>O. DNA solutions were heated to 95 °C for 5 min, chilled, and exposed to 100 mM NaOH for 20 min at ambient temperature. Subsequently, samples were adjusted to  $2 \times$  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 cm  $\times$  10 cm in size.

PCR primers used to generate the probes relevant in this study were Casp4-sense: 5'-TGTTCCCTATGG-CAGAAGG-3'; Casp4-antisense: 5'-TGCCAGGAA-AGAGGT AGAAAT-3'; TIMP1-sense: 5'-AGACC-ACCTTATAACCAGCG-3'; TIMP1-antisense: 5'-GAC-ACTGTGCAGGCTTCAGT-3';  $\beta$ -actin-sense: 5'-AC-CACGGCCGAGCGGGAAATC-3' and  $\beta$ -actin-antisense: 5'-GAGCCGCCGATCCACACGGAGTA-3'. Details on all other probes are available upon request. Membranes were stored at –20 °C and used for comparative analysis of cDNA obtained from non-stimulated and stimulated cell cultures.

Jeg-3 cells grown to 75% confluency in 175-cm<sup>2</sup> flask were incubated for 12 h in the absence or presence of 40 ng/ml LIF. For preparation of total RNA, cells were washed in PBS and homogenized in 1 ml of Trizol solution (Life Technologies). The RNA phase was purified by chloroform extraction and isopropanol precipitation. Quality of the RNA was evaluated by agarose gel electrophoresis.

Digoxigenin-labelled cDNA was synthesized in 30  $\mu$ l of reactions containing 5  $\mu$ g total RNA, 6  $\mu$ l of "5 $\times$  first-strand buffer" (Gibco BRL), 6.66 mM DTT, 0.5  $\mu$ g oligo(dT) 15 (Promega), 0.4 mM each dATP, dGTP and dCTP, 0.1 mM dTTP, 0.05 mM dig-11-dUTP (Roche), 30 U RNaseOUT (Gibco BRL) and 200 U SuperScript II RT (Life Technologies). Mixtures were incubated for 75 min at 42 °C and for 15 min at 72 °C. The reactions were terminated by addition of EDTA to a final concentration of 7 mM. cDNA was analyzed for quality by agarose gel electrophoresis.

Hybridization of labelled cDNAs to protease cDNA macroarrays on nylon membranes was done using Dig Easy Hyb (Roche). Membranes were pre-hybridized for 12 h at 42 °C with 150  $\mu$ g/ml hering sperm DNA. Subsequently, cDNA samples obtained as described above were denatured at 95 °C for 10 min and hybridized to the membranes in a volume of 10 ml Dig Easy Hyb for 48 h at 42 °C. Membranes were consecutively washed with 2, 1, and 0.5 $\times$  SSC/0.1% SDS at 68 °C for 15 min and finally washed three times in 0.1 $\times$  SSC/0.1% SDS at 68 °C for 45 min.

For detection, the membranes were blocked with 5% blocking solution (Roche) for 2 h at 37 °C, incubated with a 1:10 000 dilution of anti-digoxigenin/alkaline phosphatase conjugate (Roche) in 1% blocking solution for 15 min at 37 °C, subsequently developed with CPD-Star (Roche) as recommended by the manufacturer and exposed to X-ray film.

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

## 2.8. Quantification of specific mRNAs by real-time PCR

Quantitative real-time PCR analysis was performed by using the iCycler Apparatus (Bio-Rad, Hercules, CA, USA) with sequence-specific primer pairs for caspase-4, TIMP-1, and  $\beta$ -actin (for primer sequences see Section 2.7); 25  $\mu$ l of reaction mixtures contained 3  $\mu$ l of cDNA preparation (generated as described in Section 2.7 except for the omission of dig-11-dUTP), 1 $\times$  iQ SYBR Green supermix (Bio-Rad) and 0.4  $\mu$ M of each respective primer. PCR comprised initial heating of samples to 95 °C for 3 min followed by 35 repetitions of the following cycle: 30 s, 58 °C (TIMP1 and  $\beta$ -actin) or 55 °C (Caspase-4); 30 s, 72 °C; 1 min, 95 °C; 1 min, 55 °C. Data acquisition and analysis was carried out using the iCycler iQTM software (Bio-Rad).

## 3. Results

### 3.1. LIF induces tyrosine phosphorylation of STAT3 in choriocarcinoma cells

We tested various cytokines known to activate STAT3 via their respective receptors and/or to promote invasive behavior in different cell types for their effects on STAT3 phosphorylation in Jeg-3 cells. Samples of 10<sup>6</sup> Jeg-3 cells were incubated with various concentrations of IL-6, HGF, IGF-II and for 15, 30, and 60 min and subsequently analyzed for tyrosine phosphorylation of STAT3 by immunoblot employing an antibody to STAT3 phosphorylated at Tyr 705. Fig. 1 shows that LIF stimulation evoked a specific, dose- and time-dependent phosphorylation of STAT3, reaching a maximum at 30 min stimulation with 100 ng/ml LIF. None of the other investigated factors elicited more than a marginal degree of STAT3 activity.

### 3.2. LIF elicits specific DNA-binding activity of STAT3 in choriocarcinoma cells

To further characterize the influence of cytokines on STAT3 in Jeg-3 cells, we studied specific DNA binding of STAT3 in response to stimulation with the set of cytokines described in the previous section. After

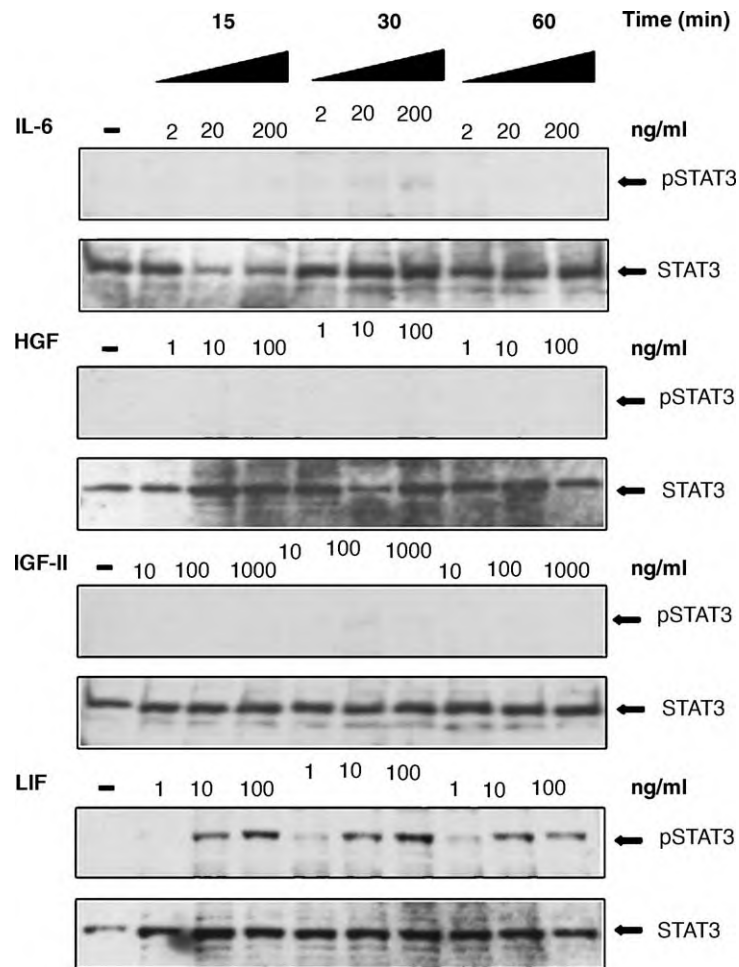


Fig. 1. Analysis of Jeg-3 cells for stimulus-dependent tyrosine phosphorylation of STAT3. Cells were either left untreated (“–”) or incubated with the indicated concentrations of cytokines or growth factors for 15, 30, or 60 min. Cells were then lysed and subjected to Western blotting. The blots were first probed with an antibody recognizing STAT3 phosphorylated at Tyr 705 (top). After washing off this antibody, the blot was re-probed with an antibody toward STAT3 to confirm protein integrity and comparable loading (bottom).

treatment with cytokines for 30 min, cell extracts were prepared, reacted with a radiolabelled STAT-binding DNA element derived from the human *c-fos* promoter (m67) and subjected to EMSA. As shown in Fig. 2, LIF was the only factor capable of inducing the formation of specific STAT3–DNA complexes. The band doublet most probably represents complexes containing the STAT3 splice variants STAT3 $\alpha$  and  $\beta$ , respectively (Caldenhoven et al., 1996). The presence of STAT3 in the LIF-evoked complex was confirmed by including antibodies to STAT1 or STAT3, respectively, in paral-

lel reactions. The antibody to STAT1 generated a certain reduction of band intensity, leaving open the possibility of some LIF-induced STAT1 activation in Jeg-3 cells, as it has previously been observed in adipocytes (Stephens, Lumpkin, & Fishman, 1998). Much more pronounced, however, was the effect of anti-STAT3. This antibody yielded a clear bandshift, i.e. the virtual disappearance of the original DNA–protein complex and the appearance of a new, slower migrating complex. This result underscores the notion of LIF as the prime inducer of STAT3 activity in Jeg-3 cells.

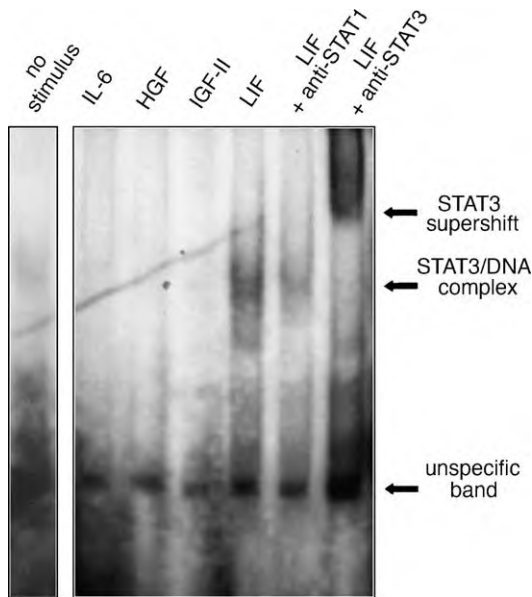


Fig. 2. Analysis of STAT3 activation in Jeg-3 cells stimulated with cytokines or growth factors by electrophoretic mobility shift assay (EMSA). Cells were left untreated or stimulated with the indicated factors for 30 min and subsequently lysed. Lysates were incubated with a double-stranded radiolabelled oligonucleotide ("m67") comprising the STAT3-binding site from the human c-fos promoter, resolved by electrophoresis and visualized by autoradiography. For specificity control, supershift experiments were performed by including antibodies to STAT1 or STAT3, respectively, in the binding reactions as indicated. Arrows mark the positions of the respective complexes.

### 3.3. LIF enhances proliferation and invasiveness of choriocarcinoma cells

LIF has been described as a promoter of proliferation in various cell types. It is also known that STAT3 exerts a positive influence on cell proliferation in certain settings. Having shown the causal connection between LIF stimulation and STAT3 activation, we, therefore, analyzed the effect of LIF on the proliferation of Jeg-3 cells. Fig. 3A shows that LIF significantly accelerated Jeg-3 proliferation in comparison to cells grown in the absence of the factor. The effect was dose-dependent, 10 ng/ml LIF was clearly more effective than 1 ng/ml. This result was consistent with the dose-dependency of LIF-induced tyrosine phosphorylation of STAT3 (comp. Fig. 1). IL-6, the only cytokine investigated in this study apart from LIF that induced a detectable, though very small degree of STAT3 tyrosine

phosphorylation (comp. Fig. 1) had only a minimal positive influence on Jeg-3 proliferation. HGF and IGF-II had no significant effect (data not shown). TGF- $\beta$ , a factor with a known negative influence on trophoblast proliferation (Graham, Lysiak, McCrae, & Lala, 1992), significantly reduced Jeg-3 multiplication. These results suggest a role of LIF-induced signaling via STAT3 in the control of choriocarcinoma cell proliferation.

Next, we investigated the influence of LIF on the invasive properties of Jeg-3 cells. Exponentially growing cells were transferred into fresh growth medium containing either no additional factor or LIF or IL-6 at different concentrations, the latter as a control for a cytokine that elicited STAT3 activation in Jeg-3 cells only to a marginal extent (comp. Fig. 1). As further controls: (i) the FCS content of the medium was increased to 20% (to test for potential non-specific promotion of invasiveness by serum constituents), and (ii) cells were stimulated with 50 ng/ml TGF- $\beta$  to monitor the effect of this potentially suppressive factor on invasiveness. Cell suspensions were seeded on top of a layer of an artificial extracellular matrix ("Matrigel"), and after 12 h of incubation, the respective fractions of cells that had migrated through this matrix were determined. Fig. 3B shows that LIF enhanced invasiveness of Jeg-3 cells by approximately the factor of 2.5. This effect was observed already at a concentration of 1 ng/ml with no substantial increase by higher concentrations. Considering the long-term character of the invasiveness assay, this result is consistent with a tendency towards higher sensitivity of STAT3 tyrosine phosphorylation at longer incubation times observed in Fig. 1. In contrast, neither IL-6 nor TGF- $\beta$  or serum had any significant effect on invasiveness. This result suggests that LIF-signaling via STAT3 is critically involved in the control of invasive behavior of Jeg-3 choriocarcinoma cells.

### 3.4. LIF affects the expression of a protease and a protease inhibitor involved in cell invasiveness

We were interested in approaching possible mechanistical connections between LIF-induced STAT3 activation and the invasive behavior of choriocarcinoma cells. Since cellular invasiveness coincides with proteolytic activity, we examined the effect of LIF on the expression of proteases and protease inhibitors. Arrays comprising cDNA sequences from 64 different proteases and protease inhibitors were probed

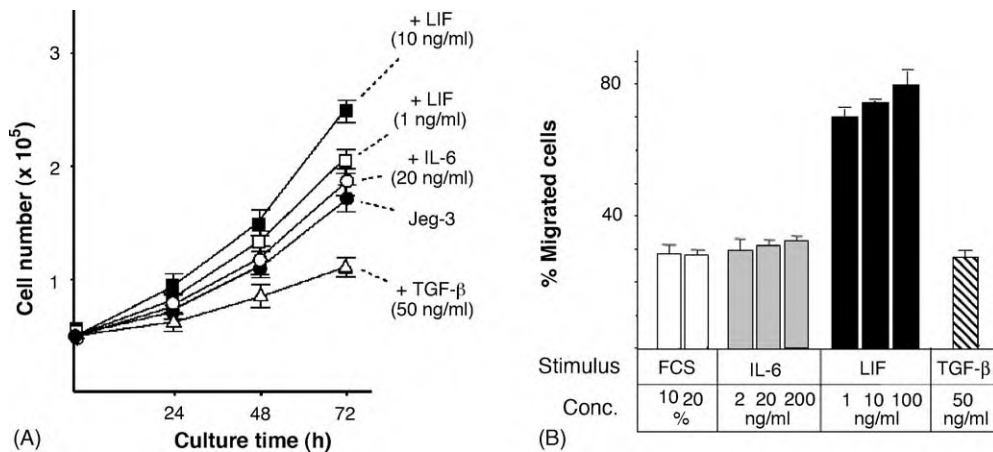


Fig. 3. Effect of LIF on proliferation and invasiveness of Jeg-3 cells. (A) Proliferation of Jeg-3 cells in response to optional stimulation with LIF and other factors. Samples of  $5 \times 10^4$  cells of each cell line were seeded into individual wells of 6-well cell culture plates in a volume of 2 ml. Medium was changed every 2 days. Cytokines or growth factors were optionally present in the medium at the indicated concentrations throughout the entire cultivation period. After 24, 48, and 72 h, aliquots were counted using a Neubauer chamber, and cell numbers were extrapolated to the total culture volume. Results are from four equivalent experiments; error bars indicate standard errors. (B) Effect of cytokine stimulation on the migration of Jeg-3 cells through matrigel layers. Samples of  $2 \times 10^5$  Jeg-3 cells were seeded onto transwells in the presence of varied concentrations of FCS, IL-6, LIF, or TGF- $\beta$  as indicated. After 12 h, the fraction of migrated cells was determined as described in Section 2. Data represent the means of three independent experiments.

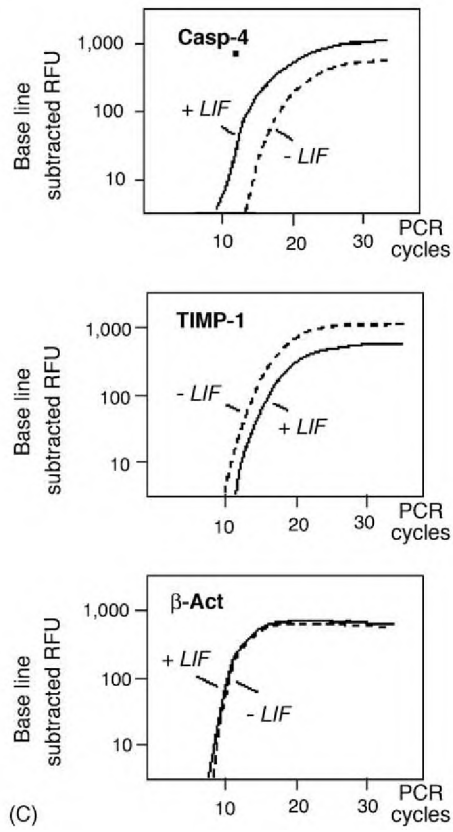
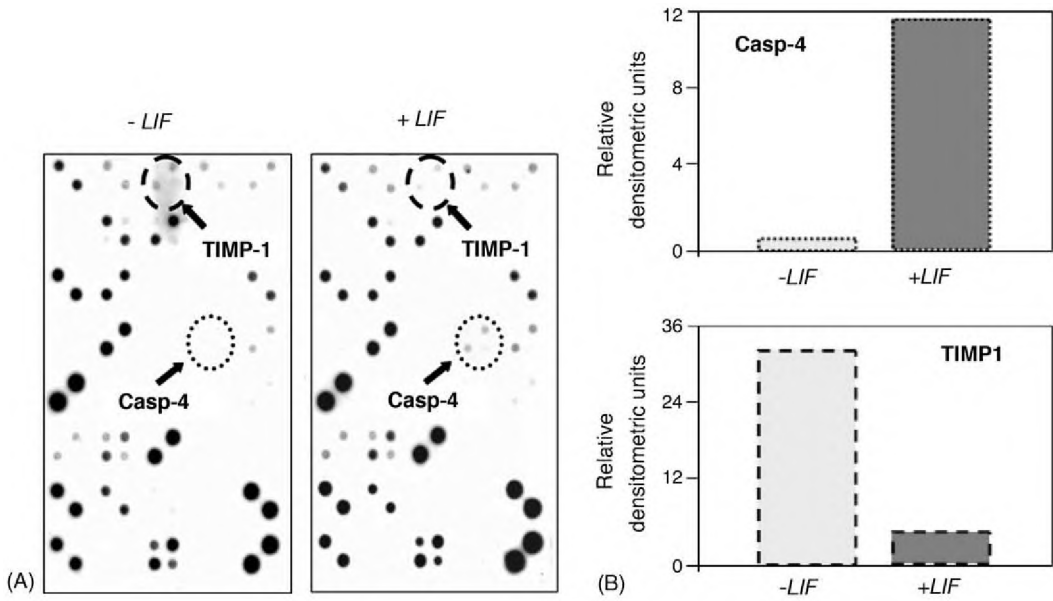
with digoxigenin-labelled cDNAs from Jeg-3 cells that had either been left untreated or stimulated with LIF (Fig. 4A and B). LIF treatment caused two significant alterations in mRNA abundance, i.e. an increase in caspase-4 expression and a downregulation in the expression of tissue inhibitor of metalloproteinase TIMP-1. To confirm the findings obtained with cDNA arrays, we analyzed LIF-dependent changes in expression of caspase-4 and TIMP-1 by quantitative real-time PCR. Fig. 4C shows that LIF clearly augments the abundance of caspase-4 mRNA in Jeg-3, whereas the abundance of TIMP-1 mRNA is downregulated by LIF stimulation. Since both proteins as well as STAT3 have been shown to contribute directly or indirectly to invasive

cell properties in certain cell types, these results are consistent with an involvement of LIF-STAT3 signaling in the manifestation of the malignant phenotype of choriocarcinoma cells.

#### 4. Discussion

Activity of signal transducer and activator of transcription 3 is correlated with the invasive behavior of trophoblasts and choriocarcinoma cells (Corvinus et al., 2003). This study provides evidence that leukemia inhibitory factor is a prime trigger of STAT3 activation and suggests that LIF/STAT3 signaling is involved

Fig. 4. Comparative analysis of protease/protease inhibitor expression in non-stimulated and LIF-treated Jeg-3 cells. (A) Nylon-based cDNA macroarrays carrying probes for some 60 extra- and intracellular proteases and protease inhibitors were hybridized with labelled cDNAs from untreated and LIF-stimulated (40 ng/ml for 12 h) Jeg-3 cells. Signals representing tissue inhibitor of metalloproteinase 1 (TIMP-1) and caspase-4 mRNAs are highlighted. (B) Comparative quantification of TIMP-1 and caspase-4 signals. Densitometrically determined signal intensities were normalized against an average of all signals from the respective membranes. The data shown are from one representative out of three independent experiments. (C) Quantitative analysis of mRNA for TIMP-1, caspase-4, and  $\beta$ -actin ("housekeeping" control) in Jeg-3 cells in dependence of LIF stimulation. cDNA preparations of unstimulated (---) or LIF-treated (40 ng/ml for 12 h (—)) Jeg-3 cells were subjected to real-time PCR measurements using specific primer pairs for caspase-4 (left), TIMP-1 (center), and  $\beta$ -actin (right) as described in Section 2. Relative fluorescence emanating from generated PCR products is plotted against the number of PCR cycles. A typical out of three independent experiments is shown.



in the control of proliferation and invasion of, at least, choriocarcinoma cells. Recent investigations in our laboratories have shown that LIF can specifically drive STAT3 phosphorylation and enhanced invasiveness also in trophoblast cells (Poehlmann et al., *in press*). In combination, these findings open up the possibility of a contribution of LIF-induced STAT3 activation in either or both implantation or maintenance of early post-implantation development.

LIF is known to play an important role during placentation in several species (Stewart, 1994). LIF-deficient mice remain infertile (though not sterile), but infertility can be restored by LIF infusion into the uterus (Stewart et al., 1992). LIF receptor knockout mice implant but die within 24 h of birth due to impaired placenta function (Ware et al., 1995). It is consistent with LIF-induced signal transduction via STAT3 in trophoblast cells that STAT3 is also essential for successful pregnancy (Takeda et al., 1997). Disruption of gp130, the STAT3-activating subunit shared by all members of the IL-6 receptor family (including the LIF receptor) leads to an identical phenotype as seen in LIF knockout mice (Ernst et al., 2001). LIF, present in high amounts at the human maternal–fetal interface, is produced by both the endometrium and the human placenta and is assumed to facilitate implantation (Cullinan et al., 1996). Indeed, LIF is maximally expressed in the human endometrium at the time of implantation (Bhatt, Brunet, & Stewart, 1991). Moreover, LIF receptors are present on trophoblasts (Kojima et al., 1995) and are, thus, probably operative in triggering the behavior of these cells.

Jeg-3 choriocarcinoma cells closely mirror a crucial feature of trophoblast cells in that they upregulate expression of HLA-G, a non-classic class I MHC molecule thought to be involved in the immune tolerance of the conceptus. Notably, they do so in a LIF-dependent fashion (Bamberger, Jenatschke, Schulte, Loning, & Bamberger, 2000).

We observed a promotion of cell proliferation in Jeg-3 cells through LIF-induced STAT3 activation. This finding is in line with various reports on the influence of STAT3 on cellular growth behavior (reviewed in Buettner, Mora, & Jove, 2002). Cell transformation by aberrant STAT3 activity in tumors probably involves upregulation of genes promoting cell cycle progression (cyclin D1, c-myc) and/or preventing apoptosis (bcl-xL; mcl-1; survivin) (Bromberg et al., 1999; Epling-

Burnette et al., 2001; Shen, Devgan, Darnell, & Bromberg, 2001).

A further question addressed in this paper was that of possible mechanisms influencing the invasive behavior of trophoblasts and choriocarcinoma cells. STAT3 appears to play an essential role in the organization of motility in various types of tumor and pluripotent cells (Boccaccio et al., 1998; Comoglio & Boccaccio, 2001; Yeung, Wang, Einstein, Lee, & Stanley, 1998; Zhang, Wang, Jove, & Vande Woude, 2002). Moreover, STAT3 has been implicated in the transcriptional regulation of proteases, a process with crucial importance for invasive cellular growth (e.g. Puricelli et al., 2002; Smola-Hess et al., 2001; Udayakumar, Stratton, Nagle, & Bowden, 2002). Our results indicate a causal connection of LIF-driven STAT3 activity, invasiveness and an altered protease expression pattern in Jeg-3 choriocarcinoma cells. Interestingly, the two proteases whose mRNA levels were influenced by LIF-dependent STAT3 activation have been described as directly or indirectly connected to invasive cell behavior or implantation; TIMP-1 expression, which we found downregulated in response to LIF, is linked to inhibition of metastasis (Bischof, 2001). In choriocarcinoma cells, its expression was found reduced due to genetic changes and this downregulation, probably resulting in an imbalance of matrix degrading proteases versus their endogenous inhibitors, was correlated with the hyperinvasive and malignant phenotype (Lala, Lee, Xu, & Chakraborty, 2002). It is conceivable to assume that TIMP-1 expression is directly influenced by STAT3, since STAT3-driven TIMP-1 regulation was also described in other cell types, such as synovial lining cells and hepatocytes (Gatsios et al., 1996; Richards et al., 1997), and the TIMP-1 promoter was shown to contain STAT3 recognition elements (Bugno et al., 1995).

Caspase-4, formerly termed interleukin-1 $\beta$ -converting enzyme homologue 2 (ICE-2) generates the bioactive form of interleukin-1 $\beta$  (Kamens et al., 1995). Its expression is low in all human tissue except in ovaries, and it is secreted by pre-implantation mouse embryos. The importance of IL-1 processing by caspase-4 in implantation is underscored by the fact that the IL-1 receptor is maximally expressed in the endometrium during the secretory phase and that the IL-1 receptor antagonist evokes a lower rate of implantation. Interestingly, immunoreactive IL-1 $\beta$  is present

in villous cytotrophoblasts, syncytiotrophoblasts and intermediate trophoblasts (Simon et al., 1994) and apparently suppresses, at least in endometrial stromal cells, the expression of TIMP-1 mRNA (Huang et al., 1998). Furthermore, TIMP-1 inhibits all MMPs in an activated form, but preferentially binds to latent and active MMP-9 (Goldberg, Strongin, Collier, Genrich, & Marmar, 1992), which has been found to be critical for cytotrophoblast invasion (Librach et al., 1991). Whether this could be indicative of an autocrine regulation requires further investigation.

LIF was also found to encourage the invasion of Jeg-3 choriocarcinoma cells in matrigel-invasion assays. Our recent work suggests that LIF also exerts influence on invasion in trophoblast cells (Poehlmann et al., submitted for publication). It has been reported that all cells of the trophoblast lineage in the human placenta express the LIF receptor, but the location of LIF expression itself is shifted from the glandular epithelium during implantation to decidual NK-cells post-implantation. Furthermore, it has been proposed that these uterine NK-cells might negatively regulate invading trophoblast cells, as they are encountered using LIF (Sharkey et al., 1999).

In this context, it should be mentioned that LIF suppresses its own effects by means of negative feedback regulation of the JAK-STAT pathway (Naka et al., 1997). This fact might explain the temporal management of STAT3 activation in the luminal epithelium, which is in its prime at the onset of uterine receptivity and embryo implantation in the mouse (Cheng, Chen, Hernandez, Alvord, & Stewart, 2001). Moreover, LIF promotes giant cell differentiation in vitro and in vivo, with giant cells being epithelial trophoblast cells that have transformed into an invasive population at the time of implantation. This differentiation is apparently contained through negative regulation via suppressor of cytokine signaling 3 (SOCS3) proteins, which suppress the JAK-STAT signal-transducing pathway and is induced by a broad spectrum of cytokines, including LIF (Starr et al., 1997; Sutherland, 2003; Takahashi et al., 2003).

Taken together, our findings indicate an involvement of LIF-driven signal transduction via STAT3 in the determination of choriocarcinoma malignancy, and along with other work from our laboratories, suggest a contribution of this pathway to the control of trophoblast invasiveness.

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