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T.G. Poehlmann, J.S. Fitzgerald, Anja Meissner, T. Wengenmayer, E. Schleussner, K. Friedrich, U.R. Markert

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Trophoblast Invasion: Tuning Through LIF, Signalling Via Stat3

T. G. Poehlmann[a], J. S. Fitzgerald[a], A. Meissner[b,1], T. Wengenmayer[a], E. Schleussner[a], K. Friedrich[b,1] and U. R. Markert[a,*]

[a] Placenta-Laboratory, Department of Obstetrics, Friedrich-Schiller-University Jena, Bachstraße 18, 07740 Jena, Germany;
[b] Institute of Biochemistry, Friedrich-Schiller-University Jena, Nonnenplan 2, 07743 Jena, Germany

INTRODUCTION

Invasion of semi-allogeneic cytotrophoblast cells into the maternal decidua is a fascinating paradox of physiological human biology. Both the effectors of invasion and the inducers of host-immunotolerance are mimicked by a variety of tumors in all organ systems [1] with the exception that trophoblast cell invasion is performed in a controlled manner. This can be perceived by the fact that trophoblast cells stemming from term placenta possess a decreased capacity to invade. Understanding the underlying mechanisms could aid in comprehending the phenomenon of pregnancy, as well as the behaviour of malignancies.

A variety of cytokines and growth factors were proposed and discussed to induce proliferation, migration and invasion of tumor and possibly trophoblast cells. Those include mainly (granulocyte macrophage-colony stimulating factor (GM-CSF), insulin-like-growth-factor-2 (IGF-2), hepatocyte growth factor (HGF), interleukin-6 (IL-6) and leukemia inhibitory factor (LIF) (as reviewed in [2]). LIF, the factor this manuscript is focussed on, is expressed at relatively high concentrations in the endometrial tissue and the decidua, but only at low concentrations, in the chorionic villi [3–5]. LIF-receptor-beta, on the other hand, is expressed in higher concentrations in the villi [4].

One major branch of intracellular signal transduction pathways used by these factors includes representatives of the Janus Kinases (Jak) and Signal Transducers and Activators of Transcription (Stat) families [6]. One of which, Stat3, was previously shown to be related to invasiveness of tumor cells [7]. Aberrant Stat3 activation has been reported in various cancers, e.g. of breast, skin, brain and prostate [8–11] and has also been associated with tumor metastasis [12]. In previous studies we demonstrated by Western blots that Stat3 is phosphorylated to a relatively high extent in invasive first trimester trophoblast cells but not in term trophoblast cells [13].

Simultaneous results were qualitatively similar for Stat3 DNA-binding capacity as assessed by electric mobility shift assays (EMSA). The highest levels of both were detected in invasive choriocarcinoma cells [13]. In another study,

* Corresponding author. Tel.: +49 3641 933763; fax: +49 3641 933764.
E-mail addresses: tobias.poehlmann@med.uni-jena.de (T.G. Poehlmann), justine.fitzgerald@med.uni-jena.de (J.S. Fitzgerald), anja.meissner@uni-rostock.de (A. Meissner), tobias.wengenmayer@web.de (T. Wengenmayer), ekkehard.schleussner@med.uni-jena.de (E. Schleussner), khf@mti.uni-jena.de (K. Friedrich), markert@med.uni-jena.de (U.R. Markert).
[1] Tel.: +49 3641 938646.

choriocarcinoma cells were incubated with the above listed factors expected to be involved in trophoblast invasiveness to analyse potential induction of Stat3 phosphorylation and DNA-binding activity. Only LIF elicited strong tyrosine phosphorylation and specific DNA-binding activity of Stat3. Moreover, as demonstrated in cDNA-arrays and RT-PCR, LIF influenced the expression pattern of proteases and protease inhibitors with potential importance for invasiveness (upregulation of mRNA for caspase 4 and downregulation of mRNA for tissue inhibitor of metalloproteinase-1; TIMP-1) [2]. In parallel, LIF has been shown to use Stat3 for signalling in the luminal uterine epithelium at the onset of embryo implantation [14], an occurrence that is associated with organ invasion. The receptor for LIF is expressed on all extravillous trophoblast populations [15]. For the present study we aimed to investigate the possible role of Stat3 in trophoblast invasion and its regulation through LIF.

METHODS

Trophoblast isolation

Trophoblast cells were isolated from normally developed term or first trimester (legal elective interruptions) placentas by mechanical tissue disaggregation, followed by a density gradient (Percoll, Pharmacia, Sweden). For depletion of non-trophoblastic cells, magnetic activated cell separation (MACS, Miltenyi, Germany) was applied by using anti-CD3, anti-CD9, anti-CD14, anti-CD31, and anti-CD45R antibodies (modified Kliman method [13,16]).

Cell line

Jeg-3 is an adherent human choriocarcinoma cell line preserving several trophoblast-like capacities including production of pregnancy related hormones. This cell line was purchased from ATCC (USA).

Cell cultures

All cultures were commenced at 10^6 cells/175 cm^2 flask, and maintained under standardized conditions (37°C, 5% CO_2, humidified atmosphere) in DMEM medium (Cambrex, U.K.) with L-glutamine supplemented with 10% foetal calf serum (FCS) and 2% antimycotic–antibiotic solution (AAS, Sigma, Germany). In the case of choriocarcinoma cells, cells were trypsinized twice a week when confluence was estimated to be over 75%. Isolated primary trophoblast cells were allowed to recuperate under the above-mentioned conditions for 48–72 h, which facilitated cell confluence.

RNAinterference (RNAi)

Oligonucleotides were hand-designed to interfere exclusively with Stat3 mRNA. As a negative control, the very same nucleotides were scrambled to form a non-genomic combination (Ambion Ltd., U.K., Table 1). This was assured by a Pubmed blast. These 21-nucleotide small interfering RNA (siRNA) duplexes were applied at a concentration of 66 nM.

Table 1. Oligonucleotide sequences used for Stat3 RNAi

Target sequence Stat3	AAT GTT CTC TAT CAG CAC AAT
Target sequence scrambled	AAG CCA CTT ATA AAT TCG TTC

For negative control, a non-genomic sequence was designed by scrambling the very same oligos.

At 50% confluence, cells were washed with Optimem media (Invitrogene, Germany) twice. Annealed oligos were mixed with Optimem. Oligofectamine (Invitrogene, Germany) was used for transfection. After 4 h, DMEM containing 30% FCS was added to cultures. Western blots for Stat3 expression were performed 24 h subsequent to siRNA transfection (for a detailed protocol, see [17,18]).

Western blots

Cells were suspended in 100 µl lysis buffer (20 mM HEPES, 2 mM EGTA, 0.2 mM EDTA, 0.12 M NaCl, 1% Triton) supplemented with protease inhibitors (5 mM beta-glycerophosphate, 3 mM $MgCl_2$, 20 µg/ml aprotinine (trasylol), 20 µg/ml leupeptine, 5 µg/ml pepstatine A, 647 ng/ml antipain, 10 µg/ml bestatine, 0.1 mM PMSF, 1 mM sodium orthovanadate). Protein concentrations of cell lysates were determined using a kit based on the Bradford method (Sigma). Briefly, 20 µg of whole cell extract were solublized in gel loading buffer (62.5 mM Tris/HCl pH 6.8; 2% SDS; 25% glycerol; 1‰ phenol blue; 5% β-mercaptoethanol), denatured for 10 min and separated on 10% acrylamide SDS gels. After protein transfer, nitrocellulose membranes were blocked in milk powder (2%) buffer for 1 h. Antibodies against Stat3 (New England Biolabs, USA) were applied for 12 h at 1:1000 dilution. For detection, peroxidase-conjugated anti-rabbit IgG (New England Biolabs) was employed at a dilution of 1:10,000 for 1 h. Visualization was performed using a LumiGlo Chemiluminescence reagent (New England Biolabs). Protein loading control of Western blots was performed by Ponceau S (Roth) staining of the nitrocellulose membrane or alternatively by application of anti-actin antibodies (Sigma) following the same detection procedure as described for Stat3. Density of Western blot bands was analyzed with Scion Image software.

Poly(ADP-ribose) polymerase (PARP) cleavage assay

To exclude possible induction of apoptosis through the downregulation of Stat3, a PARP cleavage assay was performed by using anti-cleaved PARP antibodies (New England Biolabs, USA) in flow cytometry 48 h after transfection. Experiments were repeated three times.

Matrigel assay

Twenty-four hours following transfection, 200,000 cells were settled per well, stimulated (or not) with 10 ng/ml LIF or

20 ng/ml IL-6 (Strathmann Biotec, Germany), and following another 24 h, trespassed cells were counted. Results were expressed as a ratio. All experiments were repeated at least three times.

RESULTS

Applying the described siRNA method and oligonucleotide sequence, the cellular content of Stat3 was almost completely eliminated in Jeg-3 cells when 66 nM relevant oligos, but not when scrambled oligos, were used as analyzed by Western blotting (Figure 1). Using the same method, Stat3 was also strongly reduced in first trimester and term trophoblast cells as assessed by a density analysis of Western blot bands (Figure 2).

To exclude that Stat3 knock-down induces apoptosis, a PARP assay was performed. The ratio of apoptotic trophoblast cells did not differ in controls and siRNA transfected cells (Figure 3).

To assess whether LIF affected the invasion of cells, matrigel invasion assays were performed. Here, the invasion of Jeg-3 choriocarcinoma, first trimester and term trophoblast cells increased significantly under the influence of LIF (Figure 4). For comparison, Jeg-3 cells were stimulated with IL-6, but alteration of invasion was always below 12% compared to controls and not significant (data not shown). Stat3 was knocked down in all cell types to analyse its significance for invasiveness. The stimulating effects of LIF, but also the differences of spontaneous invasion among the three cell types could be completely prevented when Stat3 was knocked down (Figure 4).

DISCUSSION

The present, in combination with previous results, emphasise the invasion regulating role of LIF and Stat3. LIF provides a soluble extracellular signal that stimulates invasion in trophoblast and Jeg-3 choriocarcinoma cells. Loss of LIF-mediated invasion in these cells subsequent to Stat3 knock-down strongly suggests that Stat3 plays a crucial role in mediating this invasion.

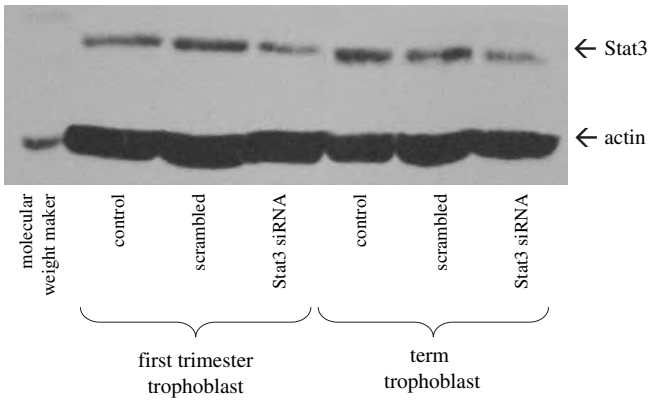

Figure 2. Representative Western blot for Stat3 expression in first trimester and term trophoblast cells. Cells were treated with siRNA for Stat3 or a scrambled non-genomic siRNA combination of the same nucleotides. Loading control was performed with anti-actin antibodies.

LIF is present in high amounts at the human maternal–foetal interface, is produced by both the endometrium and the human placenta and is assumed to facilitate implantation [19]. In the decidua, where concentrations are higher than in the villi, it is produced mainly in leukocytes, which were suggested to be predominantly NK cells [3,15]. Decidual LIF production was found throughout the entire pregnancy including at term [20]. LIF was reported earlier to be responsible for trophoblast differentiation mediated by human chorionic gonadotropin (hCG) [21], but is also responsible for regulation of hCG expression [20]. HCG is inversely able to regulate LIF expression, but these mechanisms vary during different stages of pregnancy [20]. Considering the present results, LIF may be the principle regulator of invasiveness. Stat3, which is involved in the intracellular signal cascade of a wide range of cytokines, including LIF, transduces the LIF signal from the receptor and binds phosphorylated and dimerized to specific DNA sequences, thus ultimately acting as a promoter and thereby influencing invasiveness related

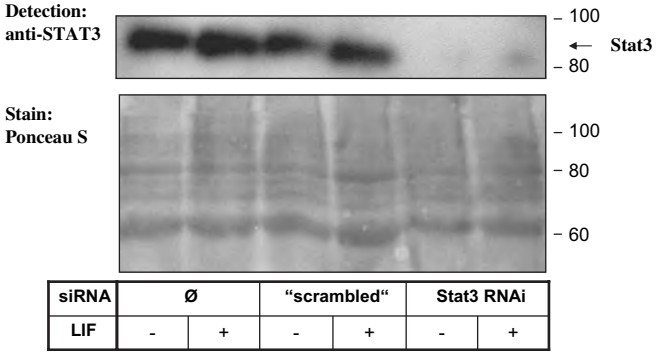

Figure 1. Above: representative Western blot for Stat3 expression in Jeg-3 choriocarcinoma cells. Cells were treated with various combinations of siRNA for Stat3, scrambled non-genomic siRNA and 10 ng/ml LIF 24 h after transfection. Below: loading control of the respective membrane (Ponceau S staining).

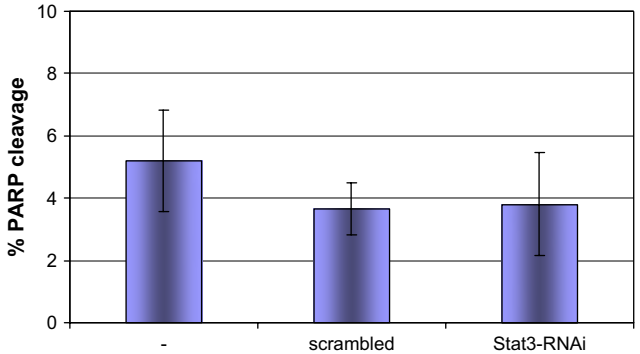

Figure 3. Trophoblast cells were treated with siRNA for Stat3 or a scrambled non-genomic siRNA combination of the same nucleotides. Apoptosis rate was assessed by a flow cytometric, intracellular PARP cleavage assay. Error bars indicate standard deviation. No significant differences were detected ($n = 3$).

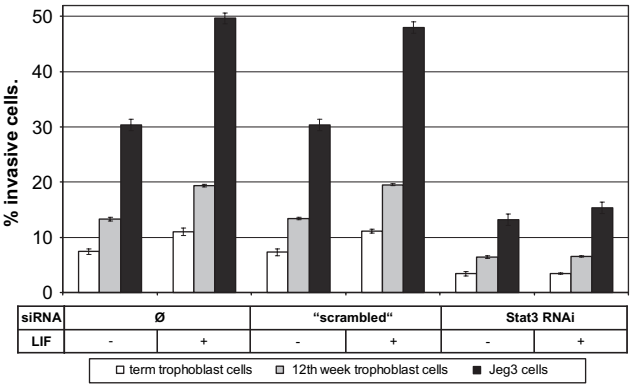

Figure 4. Invasiveness of Jeg-3 choriocarcinoma and trophoblast cells from human placentae. Cells were incubated for 24 h on a matrigel chamber and treated with various combinations of siRNA for Stat3, scrambled non-genomic siRNA and 10 ng/ml LIF. Invasion capacity was assessed as percentage of trespassed cells on total cells. Error bars indicate standard deviation ($n = 4$; 2 independent experiments with 2 parallel assays). For all controls (including those with scrambled oligos), but not the Stat3 knock-down samples, LIF-induced invasion was significantly increased ($p < 0.01$). For all Stat3 knock-down samples, invasion was significantly ($p < 0.01$) decreased compared to the respective controls (all assessed by a Student's *t*-test).

proteases and protease inhibitors, such as the downregulation of TIMP-1 expression, a protein which decreases MMP-9 activity [2].

The literature on LIF regulation of expression and activation of MMP-9, and also MMP-2, a further major inducer of trophoblast invasion [22], appears inconsistent. MMP-9 secretion has been reported to be decreased in laminin receptor alpha 6 beta 4 positive cytotrophoblast subsets following LIF stimulation, but not in fibronectin receptor alpha 5 beta 1 positive cells [23]. Other authors report an LIF-induced stimulation of MMP-9 secretion in mouse blastocysts [24] or even in trophoblasts (as reviewed in [25,26]). In this context, it has to be considered that secretion of MMPs does not necessarily include activation. An LIF-induced elevation of MMP-9 activation, though, has been demonstrated as well (as reviewed in [27]). Furthermore, when human endothelial cells were treated with LIF, LIF increased the capacity of their conditioned medium to induce stromal cells to activate MMP-2 [28].

It also needs to be considered that the role of MMPs for invasiveness is discussed controversially, and that several MMPs, including MMP-9, may even serve to inhibit invasion via generation of angiostatin (as reviewed in [29]). This coincides with the finding that MMP-2 and MMP-9 expression in trophoblast cells does not necessarily correlate with their invasive capacities [30].

This might indicate that although secretion of MMP-9 is decreased, overall activation of MMP-9 is enhanced, possibly through TIMP-1 downregulation. A decisive role in the adjustment of TIMP-1, TIMP-2, MMP-2 and MMP-9 balance for trophoblast invasion has been recently described [31,32].

Although LIF has been described to be involved in regulation of invasion of trophoblast cells [25], and Stat3 is known for its strong correlation with invasive malignancies [12], thus far, their joint venture in invasion was never demonstrated. The physiological role of this cooperation stresses that pathologies frequently abuse physiological mechanisms. The understanding of invasion regulation may be a guide to new diagnostic and therapeutic approaches, in pregnancy and infertility, as well as in malignant diseases.

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