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Research Article

Progesterone-Induced Blocking Factor differentially regulates trophoblast and tumour invasion by altering matrix metalloproteinase activity

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Abstract

Invasiveness is a common feature of trophoblast and tumours; however, while tumour invasion is uncontrolled, trophoblast invasion is strictly regulated. Both trophoblast and tumour cells express high levels of the immunomodulatory Progesterone-Induced Blocking Factor (PIBF), therefore, we aimed to test the possibility that PIBF might be involved in invasion. To this aim we used PIBF silenced or PIBF treated trophoblast (HTR8/Svneo, and primary trophoblast) and tumour (HT-1080, A549, HCT116, PC3) cell lines. Silencing of PIBF increased invasiveness as well as MMP-2,-9 secretion of HTR8/SVneo, and decreased those of HT-1080 cells. PIBF induced immediate STAT6 activation in both cell lines. Silencing of IL-4R α abrogated all the above effects of PIBF, suggesting that invasion-related signalling by PIBF is initiated through the IL-4R α /PIBF-receptor complex. In HTR-8/SVneo PIBF induced fast, but transient Akt and ERK phosphorylation, whereas in tumour cells PIBF triggered sustained Akt, ERK and late STAT3 activation. The late signalling events might be due to indirect action of PIBF. PIBF induced the expression of EGF and HB-EGF in HT-1080 cells. The STAT3 activating effect of PIBF was reduced in HB-EGF deficient HT-1080 cells, suggesting that PIBF-induced HB-EGF contributes to late STAT3 activation. PIBF binds to the promoters of IL-6, EGF and HB-EGF; however, the protein profile of the protein/DNA complex is different in the two cell lines. We conclude that in tumour cells PIBF induces proteins, which activate invasion signalling, while - based on our previous data - PIBF might control trophoblast invasion by suppressing invasive genes.

Keywords

PIBF – Invasion - Tumour - Trophoblast – Zebrafish

Abbreviations

BME	Basement membrane extract
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
Erk	Extracellular signal-regulated protein kinase
Fli1	Friend leukaemia integration 1
GPI	Glycophosphatidylinositol
HB-EGF	Heparin-binding EGF-like growth factor
JAK	Janus kinase
IL	Interleukin
IL-4R α	Interleukin-4 receptor alpha
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase

NK	Natural killer
PI-3K	Phosphoinositide-3-kinase
PIBF	Progesterone-induced blocking factor
PIBFR	PIBF-receptor
PKC	Protein kinase C
PIGF	Placental growth factor
sHB-EGF	soluble HB-EGF
STAT	Signal transducer and activator of transcription
SV40	Simian vacuolating virus 40
Tg	Transgene
Th	T helper
TIMP	Tissue inhibitor of metalloproteinase

Introduction

The capacity to invade basal membranes and the extracellular matrix (ECM) is a common feature of first trimester trophoblast and malignant tumours. However, while tumour invasion is a pathological process characterized by imbalanced regulation of motility and proteolysis together with unlimited metastatic capacity, trophoblast invasion is a strictly controlled physiological event, restricted in time to the implantation window, and localized in space to the endometrium and the proximal third of the myometrium. The smallest disturbances in the precise control of trophoblast invasion may lead to pathological pregnancies [1, 2].

Invasion is a multi-step process, involving detachment of primary cells, degradation of basal membranes and extracellular matrix (ECM) components and migration through the eroded stromal tissue. Degradation of ECM is initiated by proteolytic enzymes, e.g. matrix metalloproteinases (MMPs). A variety of extracellular stimuli, including cytokines, growth factors and cell-ECM interactions regulates the transcription of *MMP* genes via diverse pathways, e.g. STAT3, Akt and MAPK cascades [3].

Previously we reported that Progesterone-Induced Blocking Factor (PIBF) is secreted by maternal lymphocytes and mediates the immunological effects of progesterone during pregnancy [4, 5]. PIBF inhibits Natural Killer (NK) cell activity and enhances Th2-type cytokine production via the JAK1/STAT6 and PKC/Ca⁺⁺ pathways [6, 7], thus supports the maintenance of pregnancy.

Progesterone plays a crucial role during implantation via governing trophoblast invasion [8, 9]. Within the first trimester decidua, the expression pattern of PIBF coincides with sites of trophoblast invasion and the most intense PIBF-staining appears at the extravillous trophoblast [10]. Early detection of PIBF may be related to premature trophoblast invasion possibly into an endometrium not yet prepared for the trophoblast and might lead to early immune rejection of the fetus [11].

Recently it was shown that PIBF is up-regulated in undifferentiated proliferating cells (e.g. trophoblast cells) as well as in a set of malignancies [10-14]. Several breast cancers over-express PIBF compared to normal breast tissues [12]; moreover, MCF-7 breast carcinoma cells produce PIBF independently of progesterone [12]. The *PIBF1* gene is located on the chromosomal region 13q21-q22 which has been implicated as a common site for somatic deletions in a variety of malignant tumours [15]. Moreover, the full-length form of PIBF is

associated with the centrosome [12] as a component of the pericentriolar satellites [16], which are involved in cell cycle regulation. Furthermore, PIBF might facilitate tumour growth by suppressing local anti-tumour immune responses [17].

Based on these data, we aimed to investigate the possible involvement of PIBF in trophoblast and tumour invasion in different cell lines. Neither of the available trophoblast cell lines [18] represent the physiological conditions since these are either choriocarcinoma-derived (e.g., JEG-3, BeWo, JAR) or immortalized by transformation of human extravillous trophoblast cells with the SV40 large T antigen (e.g., HTR-8/SVneo, SGHPL-4, HIPEC65). For studying differences between trophoblast and tumour invasion, we selected the transformed HTR-8/SVneo cell line. HTR-8/SVneo possesses progenitor-like characteristics [19]. Recent data have demonstrated differences in gene expression profiles of integrins, proteases, hormones and growth factors between trophoblast cell lines, primary cytotrophoblast and extravillous trophoblast cells; furthermore, neither of the cell lines showed a close similarity to the primary trophoblast [20]. Therefore, we used freshly isolated primary trophoblast cells to confirm our findings in HTR-8/SVneo.

Here, we report a novel role of PIBF in invasion-regulation. We demonstrate for the first time that PIBF is able to directly regulate gene expression; we show that PIBF can bind the promoter region of *IL-6*, *EGF* and *HB-EGF* and induce or suppress transcription of these genes in certain tumour and trophoblast cells, respectively. The newly induced proteins in tumour cells activate signalling pathways that might be connected to altered MMP-9 and MMP-2 activity. We show that PIBF inhibits trophoblast invasiveness by down-regulating MMP-2 and MMP-9 activity while facilitating tumour invasion by inducing *MMP-2* and *MMP-9* genes, in an indirect manner.

Materials and Methods

Cells and treatments

The immortalized human first-trimester extravillous trophoblast cell line HTR-8/SVneo was cultured in RPMI (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (FBS). The HT-1080 fibrosarcoma, HCT-116 colorectal carcinoma, A549 lung carcinoma and PC-3 prostate cancer cell lines were maintained in DMEM (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% FBS.

Primary trophoblast cells were isolated from first trimester human elective abortion material according to a method described earlier [21]. Informed consent was obtained from all participants. Experiments were performed in accordance with the ethical guidelines of Declaration of Helsinki. Approval from the Regional Ethical Committee at University of Pécs, Medical School was obtained.

After starving overnight, 6×10^5 cells were incubated at 37°C for 5, 20, 60 minutes, 6 or 24 h with medium as a control or with recombinant human PIBF at a concentration of 100 ng/ml. The recombinant human PIBF was produced in our laboratory as described earlier [22].

RNA interference

Oligonucleotides were pre-designed by Ambion (Austin, TX, USA) to interfere with PIBF, IL-4R α or HB-EGF mRNA. Diluted oligonucleotides were pre-incubated with Oligofectamine (Invitrogen Life Technologies, Carlsbad, CA, USA) for 20 min at room temperature then the mixtures were added dropwise to 3×10^5 cells in OptiMEM (Invitrogen Life Technologies, Carlsbad, CA, USA). After 4 h at 37°C, DMEM with 30% FCS was added to the cultures. Cells were lysed after 24, 48 or 72 hours for Western blot analyses.

Invasion assay

OrisTM Cell Invasion and Detection Assay (Platypus Technologies, Madison, WI, USA) was performed according to the manufacturer's instructions. Briefly; basement membrane extract (BME, 3 mg/ml) was applied on 96-well plates and the latter populated with Cell Seeding Stoppers to restrict cell seeding to the outer annular regions of the wells. Then wells were seeded with 75 000 cells. After 24 hours stoppers were removed, resulting in a 2-mm diameter un-seeded region in the centre of each well (i.e., the detection zone), and cells were overlaid with 12 mg/ml BME containing 15% FBS. After 72 hours cells were labelled with Calcein

AM and the detection zone was analysed using multi area scan by an Olympus Fluoview FV-1000 confocal microscope. Cell invasion was assessed by measuring the area of the detection zone in the scrambled and knock down images using ImageJ analysis software. Invasion was determined by area closure which was calculated as follows: invaded area of detection zone / full area of detection zone x 100.

Xenotransplantation

The *Tg(fli1:EGFP)* zebrafish (*Danio rerio*) line was maintained in compliance with the local animal care regulations and standard protocols of University of Pecs. Approval from the Regional Ethical Committee at University of Pécs, Medical School was obtained.

Fish were kept at 28°C in aquaria with day/night light cycles. Embryos were obtained using standard mating conditions. The developing embryos were kept in an incubator at constant temperatures.

Cells were stained with CM-Dil (Vybrant; Invitrogen Life Technologies, Carlsbad, CA, USA) as described elsewhere [23] and resuspended in PBS at a concentration of 10^6 cells per 50 μ l and kept on ice before microinjection. 48 hours post fertilisation zebrafish embryos were dechorionated and anesthetized with tricaine (Sigma-Aldrich, Steinheim, Germany). Approximately 100 cells were injected into the yolk sac of the embryos using an Eppendorf FemtoJet microinjector equipped with a glass capillary (1 μ m inner diameter). The approximate injection parameters were injection pressure = 140 kPa, holding pressure = 25 kPa, injection time = 0.8 sec. After injection, embryos were incubated for 1 h at 31°C and checked for cell presence at 2 hours post transplantation. Fish with fluorescent cells outside the implantation area were excluded from further analysis. All other fish were incubated at 33°C for 5 days. Disseminations of cells were monitored by an Olympus Fluoview FV-1000 confocal microscope. Cell invasion within the zebrafish embryos was analysed by using ImageJ software. Area of disseminated cells and area of cells remaining in the yolk sac was measured and invasion index was calculated as follows: area of disseminated cells / (area of disseminated cells + area of non-disseminated cells) x 100.

Substrate zymography

Cell-conditioned media from the invasion assay were subjected to electrophoresis under non-reducing conditions in a 7.5% SDS/polyacrylamide gel containing 1 mg/ml gelatine. After

electrophoresis, gels were re-natured and stained as described earlier [24]. Semi-quantification of bands was performed by densitometry using ImageJ.

Protein arrays

Cell lysates of PIBF-treated and PIBF-knock down HTR-8/SVneo and HT-1080 cells were analysed by Proteome Profiler Antibody Array (Human Angiogenesis Array Kit, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Array data on developed X-ray film were quantitated by ImageJ software.

Western blotting

Cell lysates were separated on 10% SDS-PAGE and transferred to nitrocellulose membrane at 54 mA overnight. Membranes were blocked with TBS-Tween (pH 7.4) containing 5% non-fat dried milk for an hour and incubated with rabbit polyclonal anti-human PIBF [19], rabbit polyclonal phospho-specific (Tyr-641) anti-human STAT6, or rabbit polyclonal phospho-specific (Ser473) anti-human Akt antibodies (all from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) in TBS-Tween with 3 % non-fat dried milk for an hour at room temperature, rabbit anti-human phospho-STAT3 (Tyr705), rabbit anti-human phospho-STAT3 (Ser727) or rabbit anti-human phospho-p44/42 MAPK (Thr202/Tyr204) (all from Cell Signaling Technology Inc., Danvers, MA, USA) antibodies in TBS-Tween with 5% BSA overnight at 4 °C or as control with rabbit anti-human β -actin (Sigma-Aldrich, Steinheim, Germany) in TBS-Tween with 3% non-fat dried milk for an hour. After washing, bound antibodies were detected with 1:2000 diluted horseradish peroxidase-conjugated anti-rabbit IgG (Dako, Glostrup, Denmark), followed by development with ECL reagents (Perkin Elmer Life Sciences, Waltham, MA, USA). Semi-quantification of the bands was performed by densitometry using ImageJ software.

Cytometric Bead Arrays

IL-6 concentrations were determined from the supernatants of PIBF knock down HTR-8/SVneo and HT-1080 cells by Cytometric Bead Array (CBA; BD Biosciences, San Diego, CA) according to the manufacturer's instructions.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

HTR-8/SVneo and HT-1080 cells were seeded on 6-well plates. After silencing of PIBF, total RNA was isolated using RNeasy Mini kit (Qiagen). One microgram of total RNA was reverse-transcribed to cDNA using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). Quantitative gene expression was performed for MMP-9 (Hs00234579_m1), TIMP-1 (Hs00171558_m1) and beta-actin (Hs99999903_m1) in a total volume of 20 μ L using TaqMan Gene Expression Assays (Applied Biosystems) according to the manufacturer's instructions. All PCRs were carried out in triplicate. Relative expression levels were calculated using the $\Delta\Delta$ CT method (25) after normalization to β -actin as endogenous control.

Confocal microscopy

Cells were plated on poly-L-lysine coated slides, then fixed and permeabilized with acetone. After blocking, cells were incubated with anti-PIBF (1:100) for one hour at room temperature, following incubation with Cy3-conjugated anti-rabbit-Ig (1:200) (Invitrogen Life Technologies, Carlsbad, CA, USA). Nuclei were stained by Hoechst 33258 (Sigma-Aldrich, Steinheim, Germany) then cells were mounted with DABCO (Sigma-Aldrich, Steinheim, Germany) and slides were closed by nail polish. Microscopic analysis was performed with an Olympus Fluoview FV-1000 confocal microscope with 60x oil immersion objective. Images were analysed using the Olympus Fluoview 1.7 software.

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation was performed according to a method described elsewhere [26]. Protein/DNA complexes were immunoprecipitated using rabbit polyclonal anti-PIBF antibodies. DNA was recovered using Qiagen PCR Purification Kit (Qiagen). PCR was performed with primers to the promoter regions of *EGF* (5'-AGCGAGTTATCTCCTCTTTGGCAGT-3' and 5'-ACAGAGCAAGGCAAAGGCTTAGAGA-3'), *IL-6* (5'-CATAATCCCAGGCTTGGGGG-3' and 5'-GGTCGTCATTGAGGCTAGCG-3'), *PIGF* (5'-GTCTGGACCTGCCGAGAG-3' and 5'-AGGTTCCCGAGCCGAGTT-3') and *HB-EGF* (5'-TCCAGAAGACCCCTGCAAAA-3' and 5'-TCCCAGGCTTCTGAGATTGG-3'). PCR products were separated on 1.5% agarose gels and visualized with ethidium bromide staining.

Statistical analysis

Statistical analysis was performed using the Student's *t*-test. Data were presented as mean± SEM and *P* values of equal or less than 0.05 were considered as statistically significant.

Results

PIBF regulates invasiveness of trophoblast and tumour cells

The invasive potential of PIBF silenced trophoblast (HTR-8/SVneo and primary first trimester human trophoblast) and tumour cells (HT-1080, HCT-116, A549, PC-3) was investigated. Quantitative analyses indicated that silencing of PIBF markedly increased the invasiveness of both HTR8/SVneo and primary 1st trimester trophoblast cells (**Fig. 1a, c**), while the same treatment significantly decreased the invasive behaviour of the tumour cell lines (**Fig. 1b, c**).

We selected the HT-1080 fibrosarcoma cell line to model tumour invasion. Since PIBF affected the invasive behaviour of the immortalized HTR-8/SVneo cells and primary trophoblast cells in the same manner, we used the HTR-8/SVneo trophoblast cells to further study the underlying molecular mechanisms.

Invasion of PIBF-silenced trophoblast and tumour cell xenografts in zebrafish embryos

Zebrafish is a favoured *in vivo* platform for studying tumour development and progression because in the immunologically immature, transparent embryos human xenografts are not rejected and dissemination of cancer cells can be easily monitored in real time [27].

To confirm the differential role of PIBF in trophoblast and tumour invasion, fluorescent labelled, PIBF-silenced trophoblast or tumour cells were microinjected into the yolk sac of two-day-old *Tg(fli1:EGFP)* transgenic zebrafish embryos, exhibiting a green fluorescent vasculature; and their invasiveness was monitored by confocal microscopy. The survival rates of the embryos were between 75% and 95% (**Table 1**). Both cell types invaded embryonic tissue, entered circulation and disseminated via the vessels within the embryos, from the yolk sac towards the tail (**Fig. 2**). When PIBF-silenced HTR-8/SVneo cells were transplanted, more cells were disseminated within the zebrafish embryos (invasion index: 46.29%) compared to the embryos injected with the control HTR-8/SVneo cells (invasion index: 20.81%) (**Fig. 2a, Table 1**), verifying that PIBF inhibits trophoblast invasion. PIBF knockdown tumour cells showed decreased invasive potential within the zebrafish embryos (invasion index: 2.03%) in contrast to the scrambled-injected control group (invasion index: 55.58%), confirming that PIBF facilitates tumour invasion (**Fig. 2b, Table 1**).

PIBF affects invasion of trophoblast and tumor cells by modulating MMP-2 and MMP-9 activity

Matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) are the key mediators of both trophoblast and tumour invasion. MMP-9 and MMP-2, named gelatinases, cleave type IV collagen, the main component of basal lamina [28, 29]. Therefore, we measured MMP-2 and MMP-9 activity in cell conditioned media from the invasion assay by substrate zymography. Silencing of PIBF resulted in increased gelatinase activity in HTR-8/SVneo cells, while PIBF knockdown HT-1080 cells showed reduced MMP-2 and MMP-9 activity (**Fig. 3a, b**).

In line with this, after 24 h PIBF treatment MMP-9 expression increased in HT-1080 cells and decreased in HTR-8/SVneo cells, whereas TIMP-1 – an inhibitor of MMP-9 – was down-regulated in tumour and up-regulated in trophoblast cells (protein array, **Fig. 3c**). We also analysed the mRNA expression of MMP-9 and TIMP-1 in HTR-8/SVneo and HT-1080 cells after silencing of PIBF for 48 hours by real-time qPCR. MMP-9 mRNA was up regulated, in PIBF silenced HTR-8/SVneo cells and down regulated in HT-1080 cells whereas TIMP-1 mRNA was down regulated HTR-8/SVneo and up regulated in HT-1080 cells (**Fig. 3d**).

PIBF induced signalling pathways in trophoblast and tumour cells

In order to reveal the underlying mechanisms, we investigated the PIBF-induced signalling pathways in HTR-8/SVneo trophoblast and HT-1080 tumour cells.

Earlier we showed that the PIBF receptor is a GPI-anchored protein, which - upon ligation - forms a temporary association with the alpha chain of IL-4 receptor [7] and activates STAT6 in peripheral lymphocytes. Therefore, we investigated the involvement of IL-4R α in PIBF regulated invasion. PIBF induced STAT6 phosphorylation (**Fig. 4a**), and silencing of IL-4R α by siRNA abrogated the effect of PIBF on invasion in both cell lines (**Fig. 4b, c**) suggesting that IL-4R α is involved in the PIBF-induced invasion-related mechanism. Therefore, we focused on IL-4R α associated signalling pathways.

Insulin receptor substrate (IRS), associated with IL-4R α , can activate the PI-3K/Akt pathway and the MAPK cascade [30, 31], that have been implicated in invasion and tumorigenesis. In trophoblast cells PIBF treatment resulted in fast, transient phosphorylation

of Akt and ERK, whereas, in the tumour cell line PIBF treatment induced sustained activation of these molecules (**Fig. 5a, b**).

Since the STAT3 pathway is important in invasion signalling [32-35], we also tested the effect of PIBF treatment on STAT3 activation in the trophoblast and tumour cells. In HTR-8/SVneo cells 6h and 24h PIBF treatment inhibited activation of STAT3; while in HT-1080 cells PIBF treatment resulted in late STAT3 Ser727- and Tyr705-phosphorylation, suggesting an indirect role of PIBF in STAT3 activation (**Fig. 5a, b**).

PIBF-induced proteins are responsible for late signalling events

The late signalling events may be attributed to indirect effects of PIBF.

IL-6 activates STAT3 [36-38], therefore, we measured secreted IL-6 levels in the supernatants of control and PIBF knock down HTR-8/SVneo and HT-1080 cells by cytometric bead array. IL-6 production of PIBF-silenced trophoblast cells increased, whereas that of PIBF knock down tumour cells was reduced after 48 hours (**Fig. 6a**). Levels of secreted IL-6 in HTR-8/SVneo cells increased from 118.54 +/-20.06 ng/ml to 200.03 +/- 21.56 ng/ml after silencing of PIBF. Levels of secreted IL-6 in HT-1080cells decreased from 288.99 +/- 35.7 ng/ml to 179.22 +/- 15.87 ng/ml after PIBF knock-down.

To further identify PIBF-regulated molecules, protein array detecting invasion and angiogenesis related proteins was performed on lysates of PIBF knock down and on those of 24 h PIBF-treated HT-1080 cells (**Fig. 6b, c**). PIBF treatment resulted in increased EGF and HB-EGF expression of the tumour cell line (**Fig. 6b**). Silencing of PIBF reduced the expression of EGF and HB-EGF in HT-1080 cells (**Fig. 6c**). Either of these might account for the late PIBF-induced signalling events.

To confirm that late STAT3 activation observed in the tumour cells was indeed due to gene induction, we tested the effect of PIBF treatment on STAT3 phosphorylation in HB-EGF knock down HT-1080 cells. In HB-EGF silenced tumour cells the STAT3-activating effect of PIBF was decreased in contrast to the PIBF-treated control (scrambled) suggesting that PIBF-induced HB-EGF might contribute to late STAT3 activation in the tumour cells (**Fig. 6d, e**).

PIBF binds to the promoter of *IL-6*, *EGF* and *HB-EGF*

The full-length PIBF contains leucine-zippers, basic leucine zipper (bZIP) domain and nuclear localization signals (NLS), which enable the molecule to bind DNA and modulate the transcription of genes [19]. To confirm that PIBF enters the nucleus, we visualized the subcellular localization of PIBF by confocal microscopy (**Fig. 7a**). PIBF is present in the cytoplasm and nuclei of both HTR-8/SVneo and HT-1080 cells.

To confirm that PIBF is capable to regulate the transcription of *IL-6*, *HB-EGF* and *EGF*, chromatin immunoprecipitation (ChIP) was performed with anti-PIBF antibody. This revealed that PIBF has the capacity to bind the promoter of *IL-6*, *HB-EGF* and *EGF* in trophoblast and tumour cells (**Fig. 7b**). However, the protein content of the protein/DNA complex immunoprecipitated by anti-PIBF antibody differed in HTR-8/SVneo and HT-1080 cells (**Fig. 7c**); in trophoblast cells the complex contained the 50-kDa and 67-kDa PIBF isoforms while in tumour cells the full-length PIBF was also included in the DNA-binding complex.

Discussion

Both trophoblast and malignant tumours are invasive, and share the same underlying signalling pathways (i.e., PI-3K/Akt, MAPK, FAK, ROCK, STAT3) and mediators (i.e., MMPs and TIMPs) [39]. However, in contrast to the unlimited invasive capacity of tumours, trophoblast invasion is under strict spatiotemporal control. We aimed to identify differences between regulatory mechanisms of invasion in tumour- and trophoblast cells. Our data indicate that PIBF activates or inhibits the transcription of invasion-related genes, in a tissue specific manner.

PIBF-mediated regulation of invasiveness was initiated through the IL-4R α /PIBF-receptor complex. Silencing of IL-4R α affected invasion of trophoblast and tumour cells in a manner similar to PIBF deficiency. Earlier data from our laboratory suggest, that PIBF inhibits trophoblast invasion by down-regulating invasion-promoting genes, e.g., leptin [40]. Furthermore, Akt and ERK, that were rapidly, but transiently phosphorylated in PIBF treated trophoblast, are both involved in promoting, rather than inhibiting invasion. Therefore, though the involvement of IL-4R α is crucial, IL-4-dependent signalling is unlikely to account for PIBF-induced down regulation of invasive genes. We hypothesize that the role of the IL-4R α /PIBF complex would be rather, to internalize [41], and allow PIBF to enter the nucleus and initiate transcription. The PIBF-induced proteins would be responsible for late signalling events, i.e. inhibition of STAT3 in trophoblast cells and induction of late Akt, ERK and STAT3 phosphorylation in tumour cells.

Various cytokines and growth factors, e.g., EGF, IL-6 signal through the PI-3K/Akt, JAK/STAT and MAPK etc. pathways. These pathways are frequently altered in different cancers and promote tumour cell invasion by inducing cell motility and altering MMP expression [34, 35, 42, 43]. Moreover, these pathways are continuously active in many transformed cells since they continuously produce both growth factors and their receptors, thereby providing an auto-stimulatory growth impulse [44, 45].

Among other proteins, IL-6, EGF and HB-EGF were differentially regulated by PIBF in trophoblast and tumour cells.

EGF is expressed by both decidual and trophoblast cells, and induces differentiation, invasion and proliferation of the latter. Qiu et al reported that EGF increases MMP-9 through the PI-3K/Akt and MAPK pathways in HTR-8/SVneo cells [46]. In JEG-3 EGF induces the expression of MMP-9 through the activation of p38MAPK and ERK signalling pathways

[47]. Haslinger et al demonstrated that in SGHPL-5 trophoblast cells the PI-3K/Akt signalling is required for trophoblast migration involving the downstream effector mTORC1 [48]. The JAK/STAT system is associated with mTOR signalling; mTORC1 mediates human trophoblast proliferation and invasion through regulation of matrix remodelling enzymes (e.g., MMP-2, -9, uPA, PAI-1) and requires STAT3 Ser-phosphorylation [49]. Based on the above mentioned data, it is plausible that PIBF - via down-regulating EGF - negatively regulates trophoblast invasion. Inhibited STAT3 phosphorylation after PIBF treatment (6h, 24h) might be the result of suppressed EGF thus inhibited PI-3K/Akt/mTOR pathway that might lead to decreased expression of MMP-2 and MMP-9.

In HT-1080 fibrosarcoma cells, EGF induces the expression of MMP-9 through a Src-dependent mechanism [50]. In line with this, PIBF induced EGF in HT-1080 cells might contribute to increased expression of MMP-9 and increased invasiveness (Fig.8).

HB-EGF is another member of the EGF-family [51] that - based on our data - is regulated by PIBF. HB-EGF has a prominent role during the peri-implantation period [52]. HB-EGF promotes differentiation of trophoblast cells to an invasive phenotype by inducing integrin-switching [53], thus required for successful invasion. HB-EGF is downregulated in preeclampsia, - a disorder associated with shallow trophoblast invasion, [53]. The membrane-anchored pro-HB-EGF is cleaved by metalloproteinases in order to release the soluble form of HB-EGF [54]. HB-EGF provokes differentiation of trophoblast cells to an invasive phenotype through coordinate activation of PI-3K/Akt, ERK, MAPK14, and JNK pathways [55]. By downregulating HB-EGF in the trophoblast, PIBF might control physiologic trophoblast invasion.

HB-EGF expression is also altered in different cancer types (e.g., breast, colon, prostate, ovarian etc.) [56]. HB-EGF binding to EGFR/ErbB1 activates downstream signalling that converges on PI-3K/Akt and ERK cascades to promote survival and proliferation [56]. Thus, we hypothesize that PIBF-induced HB-EGF might contribute to elevated Akt and ERK levels found after 6h and 24h PIBF treatments in HT-1080 cells.

In our hands, the STAT3-activating effect of PIBF was reduced in HB-EGF knock down HT-1080 cells, suggesting that - at least in part - HB-EGF was responsible for late STAT3 activation. STAT3 promotes proliferation, cell growth, invasion, metastasis formation and inhibits apoptosis in tumour cells [34, 35, 38].

The secretion of IL-6 - another activator of STAT3 signalling - was induced by PIBF in the tumour and inhibited in trophoblast cells. In primary extravillous trophoblast cells, IL-6

phosphorylates STAT3 [57] which contributes to invasiveness in this cell type [58]. Therefore, PIBF induced IL-6 might be responsible for late Akt, ERK and STAT3 phosphorylation in PIBF-treated tumour cells, while the lack of STAT3 activation in the trophoblast might be attributed to the inhibitory effect of PIBF on IL-6 production.

Recently we showed that PIBF expression in the trophoblast is inversely related to invasiveness, as well as to leptin and leptin receptor expression [40], suggesting that by suppressing invasion-related genes, PIBF might act as an intrinsic negative regulator of trophoblast invasion. In choriocarcinoma, the loss of PIBF results in increased invasive behaviour [40] since the pro-invasive genes that are normally suppressed by PIBF will be transcribed.

Our data allow the conclusion that PIBF facilitates invasion in tumour cells by activating genes of molecules, *e.g.*, *EGF* that initiate invasion signalling (Fig. 8). The secreted proteins combine with their receptors and activate signalling pathways, which in turn, might further trigger the expression of invasion promoting molecules, *e.g.* MMP-9, MMP2.

We provided evidence that PIBF binds the promoter of *IL-6*, *EGF* and *HB-EGF* genes; however, in tumour cells the protein/DNA complex includes the full-length PIBF, in addition to the 50 kDa and 67 kDa PIBF isoforms found in the trophoblast. Thus, we hypothesize that the different composition of the DNA-binding PIBF complex might explain the differential regulation of trophoblast and tumour invasion by PIBF.

Based on these data, PIBF might serve as a potential therapeutic target to control tumour invasion.

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References

1. Seckl MJ, Sebire NJ, Berkowitz RS (2010) Gestational trophoblastic disease. *Lancet* 376:717-29.
2. Cudihy D, Lee RV (2009) The pathophysiology of pre-eclampsia: current clinical concepts. *J. Obstet Gynaecol* 29:576-82.
3. Knöfler M (2010) Critical growth factors and signalling pathways controlling human trophoblast invasion. *Int J Dev Biol* 54:269-80.
4. Szekeres-Bartho J, Kilar F, Falkay G, Csernus V, Torok A, Pacsa AS (1985) The mechanism of the inhibitory effect of progesterone on lymphocyte cytotoxicity: I. Progesterone-treated lymphocytes release a substance inhibiting cytotoxicity and prostaglandin synthesis. *Am J Reprod Immunol Microbiol* 9:15-8.
5. Szekeres-Bartho J, Autran B, Debre P, Andreu G, Denver L, Chaouat G (1989) Immunoregulatory effects of a suppressor factor from healthy pregnant women's lymphocytes after progesterone induction. *Cell Immunol* 122:281-94.
6. Kozma N, Halasz M, Palkovics T, Szekeres-Bartho J (2006) The progesterone-induced blocking factor modulates the balance of PKC and intracellular Ca. *Am J Reprod Immunol* 55:122-129.
7. Kozma N, Halasz M, Polgar B, Poehlmann TG, Markert UR, Palkovics T, Keszei M, Par G, Kiss K, Szeberenyi J, Grama L, Szekeres-Bartho J (2006) Progesterone-induced blocking factor activates STAT6 via binding to a novel IL-4 receptor. *J Immunol* 176:819-26.
8. Shimonovitz S, Hurwitz A, Hochner-Celnikier D, Dushnik M, Anteby E, Yagel S (1998) Expression of gelatinase B by trophoblast cells: down-regulation by progesterone. *Am J Obstet Gynecol* 178:457-461.
9. Chen JZ, Wong MH, Brennecke SP, Keogh RJ (2011) The effects of human chorionic gonadotropin, progesterone and oestradiol on trophoblast function. *Mol Cell Endocrinol* 342:73-80.
10. Anderle C, Hammer A, Polgar B, Hartmann M, Wintersteiger R, Blaschitz A, Dohr G, Desoye G, Szekeres-Bartho J, Sedlmayr P (2008) Human trophoblast cells express the immunomodulator progesterone-induced blocking factor. *J Reprod Immunol* 79:26-36.
11. Check JH, Nazari P, Check ML, Szekeres-Bartho J, Yuan W (2002) Evidence that the adverse effect of controlled ovarian hyperstimulation on successful pregnancy outcome following embryo transfer may be related to premature trophoblast invasion. *Clin Exp Obstet Gynecol* 29:83-6.
12. Lachmann M, Gelbmann D, Kalman E, Polgar B, Buschle M, Von Gabain A, Szekeres-Bartho J, Nagy E (2004) PIBF (progesterone induced blocking factor) is overexpressed in highly proliferating cells and associated with the centrosome. *Int J Cancer* 112:51-60.

13. Srivastava MD, Thomas A, Srivastava BI, Check JH (2007) Expression and modulation of progesterone induced blocking factor (PIBF) and innate immune factors in human leukemia cell lines by progesterone and mifepristone. *Leuk Lymphoma* 48:1610-7.
14. Check JH, Dix E, Sansoucie L (2009) Support for the hypothesis that successful immunotherapy of various cancers can be achieved by inhibiting a progesterone associated immunomodulatory protein. *Med Hypotheses* 72:87-90.
15. Rozenblum E, Vahteristo P, Sandberg T, Bergthorsson JT, Syrjakoski K, Weaver D, Haraldsson K, Johannsdottir HK, Vehmanen P, Nigam S, Golberger N, Robbins C, Pak E, Dutra A, Gillander E, Stephan DA, Bailey-Wilson J, Juo SH, Kainu T, Arason A et al (2002) A genomic map of a 6-Mb region at 13q21-q22 implicated in cancer development: identification and characterization of candidate genes. *Hum Genet* 110:111–121.
16. Kim K, Rhee K (2011) The pericentriolar satellite protein CEP90 is crucial for integrity of the mitotic spindle pole. *J Cell Sci* 124:338-347.
17. Szekeres-Bartho J, Polgar, B (2010) PIBF: the double edged sword. *Pregnancy and tumor. Am J Reprod Immunol* 64:77-86.
18. Hannan NJ, Paiva P, Dimitriadis E, Salamonsen LA (2009) Models for Study of Human Embryo Implantation: Choice of Cell Lines? *Biol Reprod* 82:235-245.
19. Takao T, Asanoma K, Kato K, Fukushima K, Tsunematsu R, Hirakawa T, Matsumura S, Seki H, Takeda S, Wake N (2011) Isolation and Characterization of Human Trophoblast Side-Population (SP) Cells in Primary Villous Cytotrophoblasts and HTR-8/SVneo Cell Line. *PLoS ONE* 6:e21990.
20. Bilban M, Tauber S, Haslinger P, Pollheimer J, Saleh L, Pehamberger H, Wagner O, Knöfler M (2010) Trophoblast invasion: assessment of cellular models using gene expression signatures. *Placenta* 31:989-996.
21. Petroff MG, Phillips TA, Ka H, Pace JL, Hunt JS (2006) Isolation and Culture of Term Human Trophoblast Cells. in *Placenta and Trophoblast, Methods in Molecular Medicine Soares MJ & Hunt JS (eds) Vol 1:203-217* Totowa, New Jersey: Humana Press
22. Polgar B, Kispal G, Lachmann M, Paar C, Nagy E, Csere P, Miko E, Szereday L, Varga P, Szekeres-Bartho J (2003) Molecular cloning and immunologic characterization of a novel cDNA coding for progesterone-induced blocking factor. *J Immunol* 171:5956-63.
23. Marques IJ, Weiss FU, Vlecken DH, Nitsche C, Bakkers J, Lagendijk AK, Partecke LI, Heidecke CD, Lerch MM, Bagowski CP (2009) Metastatic behaviour of primary human tumours in a zebrafish xenotransplantation model *BMC Cancer* 9:128
24. La Rocca G, Pucci-Minafra I, Marrazzo A, Taormina P, Minafra S (2004) Zymographic detection and clinical correlations of MMP-2 and MMP-9 in breast cancer sera. *Br J Cancer* 90:1414-21.

25. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C(T)}$ method. *Methods* 25: 402–408.
26. Carey MF, Peterson CL, Smale ST (2009) Chromatin immunoprecipitation (ChIP). In *Transcriptional regulation in eukaryotes: concepts, strategies, and techniques* CSHL Press, Cold Spring Harbour, New York, USA
27. Mione MC, Trede SN (2010) The zebrafish as a model for cancer. *Dis Model Mech*; 3:517-523.
28. Mason SD, Joyce JA (2011) Proteolytic networks in cancer. *Trends Cell Biol* 21:228-37.
29. Staun-Ram E, Goldman S, Gabarin D, Shalev E (2004) Expression and importance of matrix metalloproteinase 2 and 9 (MMP-2 and -9) in human trophoblast invasion. *Reprod Biol Endocrinol* 2:59.
30. Carey GB, Semenova E, Qi X, Keegan AD (2007) IL-4 protects the B-cell lymphoma cell line CH31 from anti-IgM-induced growth arrest and apoptosis: contribution of the PI-3 kinase/AKT pathway. *Cell Research* 17:942–55.
31. So EY, Oh J, Jang JY, Kim JH, Lee CE (2007) Ras/Erk pathway positively regulates Jak1/STAT6 activity and IL-4 gene expression in Jurkat T cells. *Mol Immunol* 44:3416-26.
32. Aggarwal BB, Sethi G, Ahn KS, Sandur SK, Pandey MK, Kunnumakkara AB, Sung B, Ichikawa H (2006) Targeting signal-transducer-and-activator-of-transcription-3 for prevention and therapy of cancer: modern target but ancient solution. *Ann N Y Acad Sci* 1091:151-69.
33. Aggarwal BB, Kunnumakkara AB, Harikumar KB, Gupta SR, Tharakan ST, Koca C, Dey S, Sung B (2009) Signal transducer and activator of transcription-3, inflammation, and cancer: how intimate is the relationship? *Ann N Y Acad Sci* 1171:59-76.
34. Devarajan E, Huang S (2009) STAT3 as a central regulator of tumor metastases. *Curr Mol Med* 9:626-33.
35. Xie TX, Wei D, Liu M, Gao AC, Ali-Osman F, Sawaya R, Huang S (2004) Stat3 activation regulates the expression of matrix metalloproteinase-2 and tumor invasion and metastasis. *Oncogene* 23:3550-60.
36. Waldner MJ, Foersch S, Neurath MF (2012) Interleukin-6 – a key regulator of colorectal cancer development. *Int J Biol Sci* 8:1248-1253.
37. Wu CT, Hsieh CC, Lin CC, Chen WC, Hong JH, Chen MF (2012) Significance of IL-6 in the transition of hormone-resistant prostate cancer and the induction of myeloid-derived suppressor cells. *J Mol Med* 90:1343-1355.

38. Tu B, Du L, Fan QM, Tang Z, Tang TT (2012) STAT3 activation by IL-6 from mesenchymal stem cells promotes the proliferation and metastasis of osteosarcoma. *Cancer Lett* 325:80-88.
39. Ferretti C, Bruni L, Dangles-Marie V, Pecking AP, Bellet D (2007) Molecular circuits shared by placental and cancer cells, and their implications in the proliferative, invasive and migratory capacities of trophoblasts. *Hum Reprod Update* 13:121-41.
40. Miko E, Halasz M, Jericevic-Mulac B, Wicherek L, Arck P, Arato G, Skret Magierlo J, Rukavina D, Szekeres-Bartho J (2011) Progesterone-induced blocking factor (PIBF) and trophoblast invasiveness. *J Reprod Immunol*; 90:50-7.
41. Halasz M, Polgar B, Kozma N, Berta G, Toth G, Szekeres-Bartho J (2008) Identifying the receptor-binding part of PIBF. *Am J Reprod Immunol* 60:88.
42. Kim D, Kim S, Koh H, Yoon S, Chung A, Cho KS, Chung J (2001) Akt/PKB promotes cancer cell invasion via increased motility and metalloproteinase production. *FASEB J* 15:1953-1962.
43. Reddy KB, Nabha SM, Atanaskova N (2003) Role of MAP kinase in tumor progression and invasion. *Cancer Metastasis Rev* 22:395-403.
44. Soundararajan R, Jagannadha R (2004) Trophoblast 'pseudo-tumorigenesis': Significance and contributory factors. *Reprod Biol Endocrinol* 2:15.
45. Yang SY, Miah A, Pabari A, Winslet M (2011) Growth Factors and their receptors in cancer metastases. *Front Biosci* 16:531-8.
46. Qiu Q, Yang M, Tsang BK, Gruslin A (2004) EGF-induced trophoblast secretion of MMP-9 and TIMP-1 involves activation of both PI3K and MAPK signalling pathways. *Reproduction* 128:355-363.
47. Busch S, Renaud SJ, Schleussner E, Graham CH, Markert UR (2009) mTOR mediates human trophoblast invasion through regulation of matrix-remodeling enzymes and is associated with serine phosphorylation of STAT3. *Exp Cell Res* 135:1724-1733.
48. Haslinger P, Haider S, Sonderegger S, Otten JV, Pollheimer J, Whitley G, Knofler M (2013) AKT Isoforms 1 and 3 Regulate Basal and Epidermal Growth Factor-Stimulated SGHPL-5 Trophoblast Cell Migration in Humans. *Biol Reprod* 88:54,1-11.
49. Ren HB, Jiang ZY, Sun LZ, Fan MS, Zou YF (2011) [Effect of epidermal growth factor on the expression of matrix metalloproteinase-9 and the signalling pathways involved in the trophoblast cell line JEG-3]. *Zhonghua Fu Chan Ke Za Zhi*. 46:521-526.
50. Fiorelli A, Ricciardi C, Pannone G, Santoro A, Bufo P, Santini M, Serpico R, Rullo R, Pierantoni GM, Domenico M (2011) Interplay between steroid receptors and neoplastic progression in sarcoma tumors. *J Cell Physiol* 226:2997-3003.

51. Higashiyama S, Iwabuki H, Morimoto C, Hieda M, Inoue H, Matsushita N (2008) Membrane-anchored growth factors, the epidermal growth factor family: beyond receptor ligands. *Cancer Sci* 99:214-220.
52. Jessmon P, Leach RE, Armant DR (2009) Diverse functions of HBEGF during pregnancy. *Mol Reprod Dev* 76: 1116-1127.
53. Leach RE, Kilburn B, Wang J, Liu Z, Romero R, Armant DR (2004) Heparin binding EGF-like growth factor regulates human extravillous cytotrophoblast development during conversion to the invasive phenotype. *Dev Biol* 15:223-237.
54. Miyazono K (2012) Ectodomain shedding of HB-EGF: A potential target for cancer therapy. *J Biochem* 151:1-3.
55. Jessmon P, Kilburn BA, Romero R, Leach RE, Armant DR (2010) Function-specific intracellular signalling pathways downstream of heparin-binding EGF-like growth factor utilized by human trophoblasts. *Biol Reprod* 82:921-929.
56. Yarden Y, Sliwkowski MX (2001) Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* 2:127-137.
57. Champion H, Innes BA, Robson SC, Lash GE, Bulmer JN (2012) Effects of interleukin-6 on extravillous trophoblast invasion in early human pregnancy. *Mol Hum Reprod* 18:391-400.
58. Fitzgerald JS, Poehlmann TG, Schleussner E, Markert UR (2008) Trophoblast invasion: the role of intracellular cytokine signalling via signal transducer and activator of transcription 3 (STAT3). *Hum Reprod Update* 14:335-344.

Figure Legends

Fig. 1 The effect of PIBF knock-down on invasiveness of trophoblast and tumour cells

The HTR-8/SVneo trophoblast cell line and primary trophoblast cells isolated from 1st trimester abortion material (a) as well as HT-1080 (fibrosarcoma), A549 (lung carcinoma), HCT-116 (colorectal cancer), PC-3 (prostate cancer) tumour cell lines (b) were transfected with PIBF siRNA, or scrambled oligonucleotides (scr). Wells were seeded with control (scr) or PIBF siRNA treated cells. Invasion of cells into the detection zones after 72 hours is shown. Cells were stained with Calcein AM. Images were captured using multi area scan by a confocal microscope. (Bar: 2 mm) (c) Quantification of area closure (%) calculated from measured areas of cell invasion at 72 hours. Data are presented as mean +/- SEM from 12 wells (HTR-8/SVneo, HT-1080) or 6 wells (other cell lines and primary trophoblast cells) per condition. (*: significantly different from scrambled at P<0.05) (d) The efficiency of PIBF knock down (KD) was controlled by measuring PIBF levels (90-, 67-, 50- and 34-kDa isoforms) using Western blotting at each individual experiment. Statistical analysis of Western blots are shown as mean +/- SEM; *: significantly different from scrambled at P<0.05.

Fig. 2 Xenotransplantation of PIBF-silenced trophoblast (a) and tumour (b) cells into the yolk sac of *Tg(fli1:EGFP)* zebrafish embryos

HTR-8/SVneo and HT-1080 cells were transfected with PIBF siRNA (PIBF KD) or scrambled (scr) oligonucleotides (control). Then cells were labelled with DiI (shown as red) and microinjected into the yolk sac of 48 hours post fertilisation zebrafish embryos expressing EGFP under an endothelial promoter (green vasculature). Pictures were taken 5 days post injection. Cells invaded the caudal part of zebrafish embryos. Arrows indicate distant micrometastases.

Fig. 3 PIBF modulates MMP-2 and MMP-9 activity

(a) Supernatants from the invasion assay were subjected to gelatine zymography to detect MMP-2 and MMP-9 activity. (b) Densitometric evaluation of three separate zymograms. Data

are represented as mean +/- SEM (* significantly different from the control at $P < 0.05$). (c) Changes in relative expression of MMP-9 and TIMP-1 in PIBF-treated (24 h) HTR-8/SVneo trophoblast and HT-1080 fibrosarcoma cells (protein array, $n=2$). (d) The expression of MMP-9 and TIMP-1 was also analysed at mRNA level in PIBF knock down (KD) cells by real-time PCR. Cells were analysed at 48 hours post transfection with siRNA. Changes in relative mRNA levels are shown. Data are represented as mean +/-SEM.

Fig. 4 PIBF signals via the IL-4R α /PIBFR complex in trophoblast and tumour cells

(a) Serum-starved HTR-8/SVneo and HT-1080 cells were treated with PIBF (100 ng/ml) for the indicated time points. Total STAT6 and phosphorylated STAT6 levels were detected by Western blotting. (b) Invasion of control (scrambled, scr), IL-4R α or PIBF siRNA (KD) treated HTR8/SVneo and HT1080 cells. Invasion of cells into the detection zones (2 mm) after 72 hours is shown. Cells were stained with Calcein AM. Images were captured using multi area scan by a confocal microscope. (c) Quantification of area closure (%) calculated from measured areas of cell invasion at 72 hours. Data are presented as mean +/- SEM from 12 wells per condition. (*: significantly different from scrambled at $P < 0.05$) (d) Efficiency of PIBF or IL-4R α knock down (KD) was determined by Western blotting. Statistical analysis of Western blots are shown as mean +/- SEM; *: significantly different from scrambled at $P < 0.05$.

Fig. 5 PIBF induced signalling in trophoblast and tumour cells

(a) Serum-starved HTR-8/SVneo and HT-1080 cells were treated with PIBF (100 ng/ml) for the indicated time points. Phospho(Ser473)-Akt, total Akt, phospho(Thr202/Tyr204)-ERK, total ERK, phospho(Ser727)-STAT3, phospho(Tyr705)-STAT3 and total STAT3 levels were detected by Western blotting. (b) Densitometric evaluation of the Western blots. The bars indicate mean +/-SEM of three separate experiments (* $P < 0.05$).

Fig. 6 PIBF-regulated proteins

(a) IL-6 secretion of PIBF knock down HTR-8/SVneo and HT-1080 cells (cytometric bead array). Supernatants were analysed at 48 hours post transfection with siRNA. Data are

represented as mean +/- SEM of 3 separate experiments (* P<0.05) (b) EGF and HB-EGF expression in control and 24h PIBF treated HTR-8/SVneo and HT-1080 cells in a protein array (n=2) (c) EGF and HB-EGF expression in control (scrambled) and PIBF knock down HT-1080 cells in a protein array (n=2). (d) The effect of 24 h PIBF treatment on STAT3 activation in control (Scr) and HB-EGF knock down HT-1080 cells. Upper panel: Western blot for phospho-STAT3 (Tyr). Lower panel: Mean+/-SEM of densitograms from three separate experiments (* P<0.05). (e) Efficiency of HB-EGF knock down was analysed by Western blotting. Statistical analysis of Western blots are shown as mean +/- SEM; *: significantly different from scrambled at P<0.05.

Fig. 7 PIBF binds to the promoter of IL-6, HB-EGF and EGF

(a) Subcellular localization of PIBF in HTR-8/SVneo and HT-1080 cells. (1) Cells were labelled with anti-PIBF antibody, and reacted with anti-rabbit Ig-Cy3 (shown as red). Nuclei were counter stained with Hoechst, (shown as blue). Merged images taken by confocal microscope. (2) Control, without anti-PIBF antibody. (b) PCR of non-immunoprecipitated (input) and PIBF or control IgG immunoprecipitated DNA from ChIP assay of HTR-8/SVneo and HT-1080 cells, using primers to the promoter of *IL-6*, *HB-EGF*, *EGF* and *PIGF*. *PIGF* ChIP was used as a negative control. (c) Detection of PIBF isoforms by Western blotting on the eluted protein/DNA complex immunoprecipitated by anti-PIBF.

Fig. 8 Transcriptional regulation of tumour invasion by PIBF

PIBF activates not only the Jak1/STAT6 pathway but also the PI-3K/Akt and ERK cascades through the IL-4R α /PIBFR complex. Moreover, PIBF is able to enter the nucleus where binds the promoter regions of *EGF*, *IL-6* and *HB-EGF* and induces their transcription. The expressed and secreted EGF then binds its own receptor and further triggers the PI-3K/Akt, MAPK and Jak/STAT3 pathways resulting in proliferation, survival and increased MMP-9 expression, thus increased invasive behaviour. (ECM: extracellular matrix)

Table Legends

Table 1 *In vivo* invasion assay: xenotransplantations of HTR-8/SVneo and HT-1080 cells into 2-day old zebrafish embryos

Number of embryos micro-injected and analysed is shown in the table. If 2 hours post injection fluorescent cells were detected, embryo was excluded from further analysis. After micro-injection with scrambled HT-1080 cells, one embryo developed yolk sac oedema thus was not analysed. When HTR-8/SVneo cells were injected, survival rates of embryos were 95% (scrambled, scr) and 90% (PIBF knock down, KD); when HT-1080 cells were transplanted, survival rates of embryos were 75% (scr) and 90% (PIBF KD) Invasion index was calculated in live embryos at 5 days post injection as follows: area of disseminated cells / area of total cells x 100.

Table 1 *In vivo* invasion assay: xenotransplantations of HTR-8/SVneo and HT-1080 cells into 2-day old zebrafish embryos

Microinjections		Number of injected embryos (Analysed) †: Dead	Area of non-disseminated cells (Mean, mm ²)	Area of disseminated cells (Mean, mm ²)	Invasion index	P-value
HTR-8/Svneo	scr	20 (18) †1	0.01227	0.003225	20.81%	0.0034
	PIBF KD	20 (17) †2	0.008497	0.007323	46.29%	
HT-1080	scr	20 (14) †5	0.01815	0.03071	55.58%	0.0024
	PIBF KD	20 (17) †2	0.0476	0.000984	2.03%	