AT THE CROSSROAD OF PHYSIOLOGICAL AND PATHOLOGICAL INVASION: THE ROLE OF PROGESTERONE-INDUCED BLOCKING FACTOR IN TROPHOBLAST AND TUMOR INVASION

PH.D. THESES

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PÉCS

2011

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I. LIST OF ABBREVIATIONS

AEC	3-amino-9-ethylcarbazole
APC	adenomatosus polyposis coli
bFGF	basic fibroblast growth factor
BMP	bone morphogenetic protein
BRCA	breast cancer
bZIP	basic-leucine zipper
CamK	Ca^{++}/calmodulin dependent protein kinase
CBA	cytometric bead array
Cdc42	cell division control protein homolog 42
ChIP	chromatin immunoprecipitation
DABCO	1, 4-diazabicyclo [2.2.2] octane
DAG	diacyl glycerol
Dkk	Dickkopf
DMEM	Dulbecco's Modified Eagle's Medium
DTT	dithiothreitol
Dvl	dishevelled
EGF	epidermal growth factor
EGTA	ethylene glycol-bis (2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid
ERK	extracellular signal-regulated kinase
FAK	focal adhesion kinase
Fzd	frizzled
γC	common gamma chain
gp130	glycoprotein 130
GPI	glycosyl-phosphatidyl-inositol
GSK-3ß	glycogen synthase kinase 3 beta
GTPase	guanine triphosphatase
HB-EGF	heparin-binding EGF-like growth factor
hCG	human chorionic gonadotropin

HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
hPL	human placental lactogen
HRPO	horse radish peroxidase
IGF	insulin-like growth factor
IL-4Rα	interleukin-4 receptor alpha
IRS	insulin receptor substrate
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
KLF-5	Kruppel-like factor-5
LEF	lymphoid enhancer binding factor
Leu Zip	leucine zipper
LPS	lipopolisaccharide
LRP	low-density lipoprotein receptor-related protein
LTR	long terminal repeat
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
MβCD	methyl- β-cyclodextrin
MMP	matrix metalloproteinase
NK	natural killer
NLS	nuclear localization signal
ORF	open reading frame
PAK	p21 activated kinase
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PE	phycoerythrin
PHA	phytohemagglutinin
PIBF	progesterone-induced blocking factor
PIBFR	PIBF-receptor
PMSF	phenylmethylsulfonyl fluoride
PI3K	phosphoinositide 3-kinase

PIP_2	phosphatidylinositol 4,5-bisphosphate
PIP_3	phosphatidylinositol 3,4,5-triphosphate
PI-PLC	phosphatidylinositol-specific phospholipase C
PKC	protein kinace C
PlGF	placental growth factor
PLC	phospholipase C
PR	progesterone-receptor
Raf	rapidly accelerated fibrosarcoma
Ras	rat sarcoma
RhoA	Ras homolog gene family, member A
ROCK	Rho-associated protein kinase
rhuPIBF	recombinant human PIBF
RPMI	Roswell Park Memorial Institute
SDS	sodium dodecyl sulphate
SH2	src-homology 2
SOCS	suppressor of cytokine signaling
STAT	signal transducer and activator of transcription
TCF	T-cell factor
TGF	tumor growth factor
Th1/2	T-helper 1/2
TIMP	tissue inhibitor of metalloproteinases
VEGF	vascular endothelial growth factor
Wnt	Wingless and Int

II. INTRODUCTION

1. General features of trophoblast and tumor invasion

Trophoblast and tumor cells share several features **[1-4] (Table 1.)**. Both cell types have lost the capacity of contact inhibition and express telomerase activity ensuring high proliferation rate. Both tumor and trophoblast cells possess migratory and invasive properties and acquire angiogenic potential; moreover, there is an analogy in the mechanism of invasion (i.e. adhesion molecules, proteases, matrix metalloproteinases, autocrine and paracrine regulation etc.). Finally, both trophoblast and tumor cells are capable to suppress the immune system e.g. by expressing non-classical HLA molecules **[5-12]**, Th2-type cytokines **[13-16]**, immunosuppressive factors **[16-18]**, by inducing regulatory T cells **[19-28]** or by other mechanisms **[29-31]**.

TABLE 1. Similarities and differences between trophoblast and tumor cells

TROPHOBLAST	TUMOR
Common features	
Telomerase activity High proliferation rate Loss of contact inhibition Migratory and invasive properties Similarities in the mechanism of invasion Extensive vascularization Ability to suppress the immune system	
Distinctions	
Cell differentiation	
Well differentiated	Undifferentiated, transformed, immortal cells with altered morphological features
Invasion	
Physiological process	Pathological process
Regulation of invasion	
Strictly regulated in time and space	Uncontrolled invasion, metastatic capacity

The major differences between trophoblast and tumor cells reside in cell differentiation and spatio-temporal regulation of invasion.

Trophoblast cells remain well-differentiated during their lifespan; in contrast, malignancies consist of undifferentiated, transformed, immortal cells that have lost their original morphological features. Furthermore, tumor invasion is a pathological process, characterized by uncontrolled invasive behavior and unlimited metastatic capacity, while trophoblast invasion is a physiological event that is rigorously regulated, restricted in time to the first trimester of pregnancy and localized in space to the endometrium and the proximal third of the myometrium. The slightest disturbances of the fine tuning of trophoblast invasion may manifest in pathological pregnancies **[32-37] (Table 2.)**.

TABLE 2. Clincal consequences of aberrant trophoblast invasion

REDUCED TROPHOBLAST INVASION
Early pregnancy loss
Pre-eclampsia
Intrauterine growth retardation
INCREASED TROPHOBLAST INVASION
Abnormally deep attachment of the placenta
Placenta accreta
Placenta increta
Placenta percreta
Gestational trophoblastic tumors
Partial / Complete hydatidiform mole
Invasive mole
Choriocarcinoma
Placental site trophoblastic tumor
Epithelioid trophoblastic tumor

2. Invasive differentiation of trophoblast cells

Trophoblast progenitor cells residing at the basement membrane of placental villi **(Fig.1.)** give rise to distinct epithelial cell types.

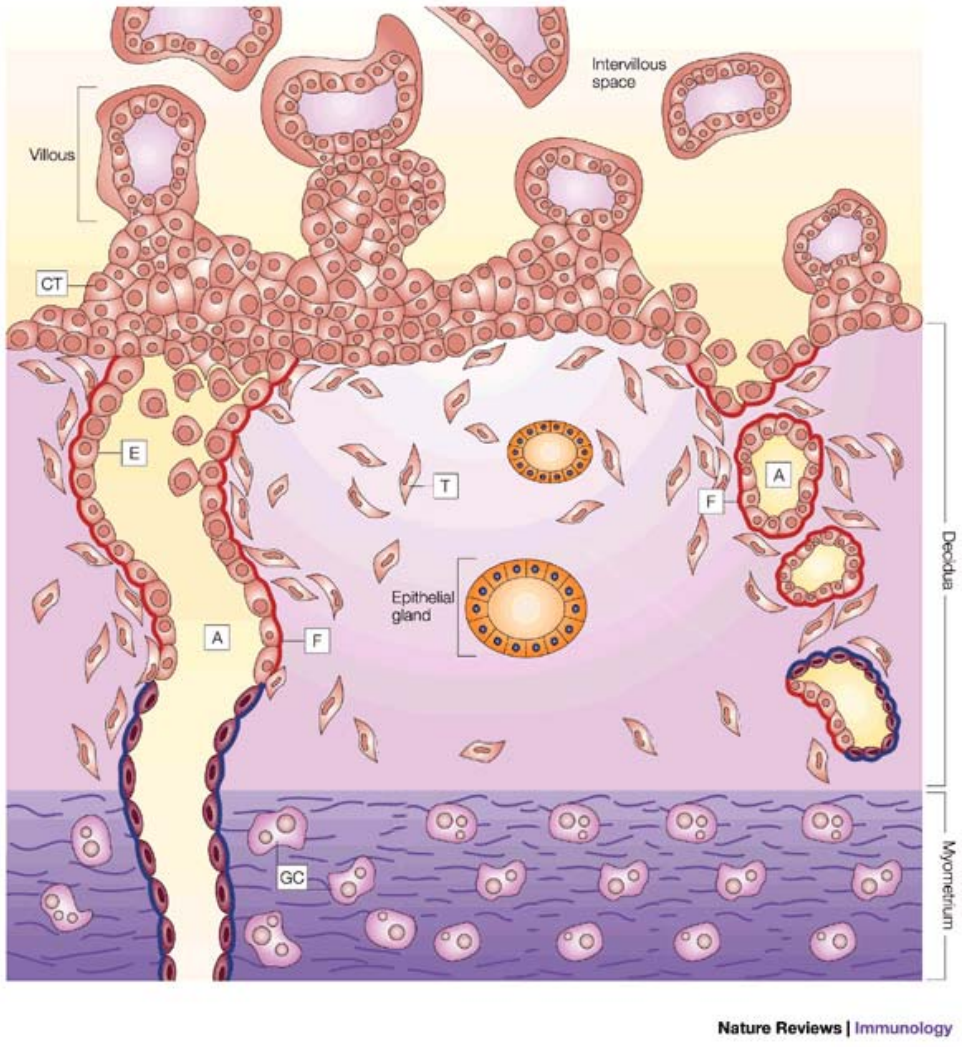

FIGURE 1. Trophoblast differentiation at the maternal-fetal interface. CT: cell column; A: spiral arteries; T: interstitial trophoblast cells; F: fibrinoid; E: endovascular trophoblast cells; GC: placental bed giant cells. **[38]**

Fusion of cytotrophoblasts generates the multinucleated syncytium which is mainly responsible for protein transport and hormone (progesterone, hCG, hPL) production.

Cytotrophoblasts of anchoring villi acquire a transiently invasive phenotype and invade the decidualized endometrium while the cytotrophoblasts of floating villi (in the extravillous space surrounded by maternal blood) remain attached to the villous basement membrane.

The inner layer of villous cytotrophoblast grows out at focal points to form cell columns. These are prominent at the anchoring villi, where attachment to the maternal decidua occurs. At the fetal–maternal boundary, the columns form a partially continuous shell. From this shell, extravillous trophoblast cells enter the decidua as interstitial trophoblast to encircle and destroy the arterial media, which is replaced by fibrinoid material. These cells interact with different maternal cell types including decidual cells, leukocytes and endothelial cells. Then, endovascular trophoblast cells move down the arteries in a retrograde manner to replace the maternal endothelial lining **[38-39]**.

A new subset of extravillous trophoblast cells, the endoglandular trophoblast **[40]**, breaks through the basement membrane of uterine glands to open their lumen towards the intervillous space. This might enable histiotrophic nutrition of the embryo during the first trimester of pregnancy prior to onset of the maternal blood flow.

The trophoblast cells move as far as the inner myometrium, where they fuse to become placental-bed giant cells.

3. Common regulators of trophoblast and tumor invasion

Several enzymes, hormones, cytokines, growth factors and extracellular matrix glycoproteins have been reported to play a role in both trophoblast and tumor invasion. Few selected molecules are addressed below.

3.1 Matrix metalloproteinases

Invasion of surrounding tissues is mediated by a set of proteolytic enzymes, among others, matrix metalloproteinases (MMPs). MMPs are zinc-dependent endopeptidases with the capacity of degrading extracellular matrix components. In humans, there are 23 MMPs and their expression is transcriptionally controlled by inflammatory cytokines, growth

factors, hormones, cell-cell and cell-matrix interactions **[41-43]**. MMP activity is also regulated by activation of the precursor zymogens (most MMPs are secreted as inactive pro-enzymes) and inhibition by the endogenous tissue inhibitors of metalloproteinases (TIMP1,-2,-3,-4). TIMPs bind to the highly conserved zinc-binding site of active MMPs.

The involvement of MMP-9 and MMP-2 – also known as gelatinases – in tumor cell migration and invasion as well as in embryo implantation is well documented. Gelatinases are capable to cleave type IV collagen, the main component of basal membranes **[44-50]**. While MMPs and TIMPs are strictly regulated during trophoblast invasion, tumor invasion and metastases occur as a result of an imbalance between MMPs and TIMPs **[51]**.

3.2 Hormones

Progesterone is one of the hormones to control trophoblast invasiveness by reducing the secretion of MMP-9 by trophoblasts **[48-50]**. A set of progesterone-induced genes (p53, Indian Hedgehog, BMP-2, galectin-1 etc. **[52-58]**) also possesses a vital role in implantation and decidualization.

Another molecule that has been implicated in control of trophoblast invasiveness is leptin; a 16-kDa peptide hormone secreted by adipose tissue, that participates in the regulation of energy homeostasis **[59]**. Leptin is synthesized by the human placenta **[60]** and its receptors are expressed in the trophoblast and endometrium during pregnancy **[61]**. Human chorionic gonadotropin (hCG) induces leptin expression in trophoblast cells probably involving the MAPK signal transduction pathway **[62-63]**. Leptin also promotes the expression of MMP-2 and MMP-9 in cultured human cytotrophoblast cells **[64]**. Leptin inhibits the secretion of progesterone by cytotrophoblast. Abnormal leptin levels in preeclampsia are associated with reduced trophoblast invasion. Overexpression of leptin-receptor was found in ovarian cancer and leptin promotes resistance to apoptosis in lung carcinoma.

3.3 Growth factors and cytokines

Growth factors and their receptors which play a central role in proliferation show a similar expression pattern in trophoblasts and malignant tumors.

Epidermal growth factor (EGF)-receptors are present in villous cytotrophoblast cells, syncytiotrophoblast and decidual cells. The ligands for EGF-receptor - EGF, TGF-α and amphiregulin - have been shown to enhance extravillous trophoblast proliferation. EGF has been demonstrated in uterine epithelial cells and decidual cells, as well as in cyto- and syncytiotrophoblast of the chorionic villi. Tumor growth factor α (TGF-α) has been detected in decidual cells and nearly all trophoblast subpopulations. Amphiregulin has been demonstrated in syncytiotrophoblast cells of the early placenta. EGF regulates early placental growth and function in an autocrine manner. Furthermore, the TGF-α-EGFR autocrine loop has been implicated in the uncontrolled proliferation of malignant trophoblast cells **[65-66]**.

EGF-like growth factors – among others heparin-binding EGF-like growth factor (HB-EGF) - are expressed throughout gestation in villous and EVT populations of the human placenta. Disruption of the EGF signaling network in human first trimester cytotrophoblast cell lines reduced, while supplementation with these growth factors increased trophoblast invasiveness **[67-68]**.

During tumor growth, angiogenesis is initiated by basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), which signal nearby vessels to send out new branches **[69-70]**. VEGF is a potent angiogenic factor which plays a role in the vascularization process and promotes invasive trophoblast proliferation during embryo implantation. Invading extravillous cytotrophoblast cells express the VEGF receptor **[71]**. VEGFR can also be bound by the trophoblast-derived placental growth factor (PlGF); a member of the VEGF family of angiogenic factors, to affect cell proliferation, invasion and/or other metabolic activities in an autocrine manner.

Interleukin-6 (IL-6) modulates the expression of genes involved in cell cycle progression, apoptosis and angiogenesis. IL-6 has been implicated in the pathogenesis of several types of tumors including lymphoma, gastric carcinoma, prostate cancer, ovarian

cancer and multiple myeloma. Trophoblast also expresses IL-6 as well as IL-6R and its associated signal transducer gp130 **[72]**.

4. Signaling cascades mediating proliferation, migration and invasion

Extracellular stimuli initiate signal transduction that controls proliferation, differentiation, migration and apoptosis through the mitogen-activated protein kinases (MAPKs), focal adhesion kinase (FAK), the phosphoinositide 3-kinase (PI3K) - Akt pathway, Signal Transducers and Activators of Transcription (STATs) **(Fig.2.)** or the Wnt pathway **(Fig.3.)**.

4.1 Mitogen-activated protein kinase (MAPK) cascades

Upon ligand binding to receptor tyrosine kinases or G-protein coupled receptors, a highly complex network of protein kinases and small GTPases (e.g. Ras) regulates the activity of MAPKs through sequential phosphorylations at critical Ser, Thr and Tyr residues. Proteins of the MAPK-kinase-kinase (MAPKKK) family such as Raf phosphorylate MAPK-kinases (MAPKKs) including MEKs. The MAPKKs then activate the four major families of MAPKs including the ERK family.

ERKs have been shown to play an important role in growth-factor-dependent regulation of trophoblast growth and migration **[73]**. In the placenta, the expression of ERK1 and ERK2 was detected in villous cytotrophoblasts, but their active phosphorylated forms were only present until the 12th week of gestation, suggesting a predominant role during early pregnancy **[74]**. Chorionic gonadotropin uses ERK pathway to facilitate trophoblast invasion and migration due to inducing MMP-2 expression **[75]**. EGF-induced trophoblast migration also requires ERK signaling cascades **[76]**. MAPK signaling is dysregulated in various malignancies **[77-79]**.

4.2 PI3K/Akt pathway

The Akt cascade is activated by receptor tyrosine kinases, integrins, cytokine receptors, G-protein coupled receptors and other stimuli that induce the phosphoinositide 3-kinase (PI3K) mediated transition of phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol 3,4,5 triphosphate (PIP_3). Elevated PIP_3 recruits and activates the protein-serine/threonine kinase Akt at the membrane. Once activated, Akt phosphorylates a number of target proteins, including direct regulators of cell growth, proliferation, survival, and migration, transcription factors and other protein kinases.

Akt regulates invasive differentiation of trophoblast cells **[73]**. In many tumors, Akt promotes metastasis **[80-82]**. EGF is a potent activator of PI3K-Akt and Akt-dependent migration of trophoblastic HTR-8/SVneo cells **[76]**. PI3K-Akt is also required for hCG- and EGF-dependent expression of MMP-2 and MMP-9 in trophoblasts **[75-76]**, Since these enzymes are also targets of ERK signaling, PI3K-Akt and MAPK signaling may have synergistic effects on protease expression and trophoblast invasion.

4.3 JAK/STAT pathway

Phosphorylation, dimerization and nuclear translocation of STAT transcriptional factors are achieved upon growth factor or cytokine-dependent activation of receptor-associated Janus kinases (JAKs). Cytokine binding induces receptor dimerization and activates associated JAK proteins which phosphorylate themselves and the receptor. Phosphorylated sites on both receptor and JAKs serve as docking sites for the SH2-containing STAT proteins. Receptor-bound STATs phosphorylated by JAKs dimerize and translocate to the nucleus to regulate target gene transcription.

The role of STAT3 in trophoblast **[83-85]** as well as in tumor invasion and metastasis has been regularly affirmed **[86-88]**. Among others, IL-6 and leptin were shown to increase STAT3 activity in cytotrophoblast cells. Dysregulation of JAK/STAT pathway leads to increased angiogenesis, enhanced survival of tumors and immunosuppression. Malignant choriocarcinoma was found to possess high level of STAT3 activity **[84]**. Xie et al reported that STAT3 upregulates the transcription of MMP-2 through direct interaction

with the MMP-2 promoter **[89]**, in addition, transfection of human melanoma cells with a dominant-negative STAT3 suppressed brain metastasis in animal models **[90]**.

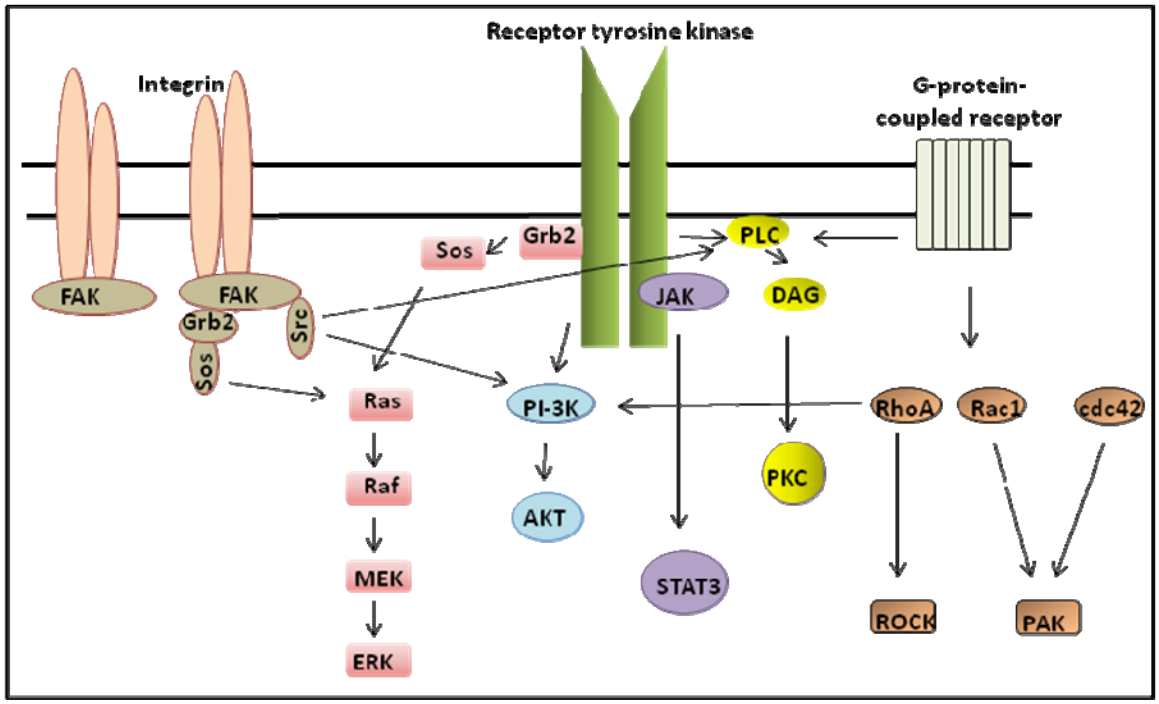

FIGURE 2. Signaling pathways in control of proliferation, differentiation, cell growth, apoptosis, migration and invasion

4.4 Wnt signaling

The Wnt proteins belong to a family of secreted morphogenes that play a key role in embryonic development and tumorigenesis. In the canonical pathway, Wnt ligands bind to the heterodimeric low-density lipoprotein receptor-related protein (LRP) and Frizzled (Fzd). Signaling from LRP/Fzd leads to activation of a cytoplasmic protein called Dishevelled (Dvl) and inhibition of the complex composed of axin, adenomatous polyposis coli (APC) and glycogen synthase kinase 3β (GSK-3β). In the absence of Wnt ligands, active GSK-3β phosphorylates β-catenin, leading to its ubiquitination and degradation. Wnt signaling results in increased β-catenin levels. β-catenin acts as a direct regulator of gene

expression by forming a complex with members of the T-cell factor (TCF)/lymphoid enhancer binding factor (LEF) family of transcription factors. To control canonical Wnt signaling, secreted Dickkopf (Dkk) members inhibit the pathway upon interaction with LRP-5/6.

Several Wnts activate β-catenin independent signaling pathways (non-canonical Wnt signaling pathways), known as the planar cell polarity pathway and Wnt/calcium pathway. Furthermore, non-canonical Wnts can antagonise the functions of canonical Wnts.

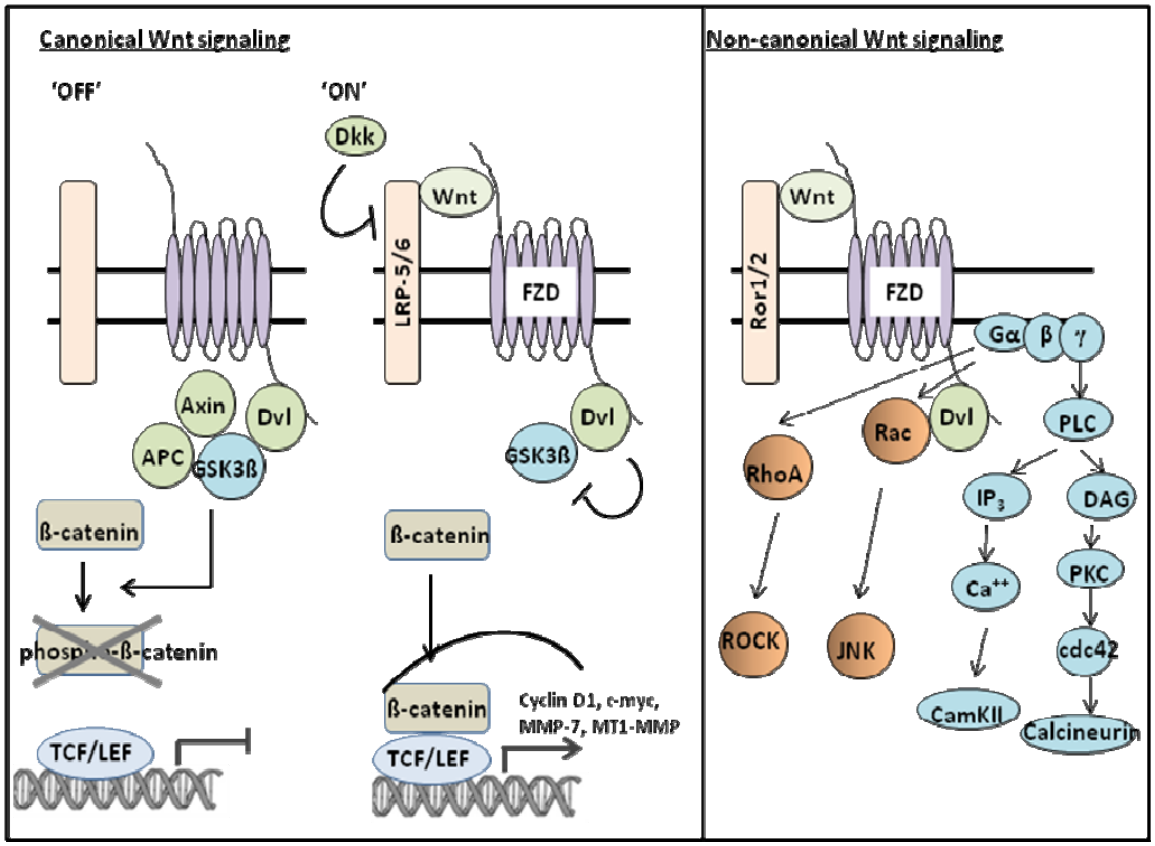

FIGURE 3. Wnt signaling. Canonical Wnt signaling pathway (left panel): In the presence of the Wnt molecule, Dvl inhibits GSK-3β and β-catenin translocates to the nucleus and induces the transcription of the target genes. Two major non-canonical Wnt signaling pathways, the planar cell polarity and Wnt/calcium pathways are shown on the right panel.

Fourteen out of 19 Wnt ligands and 8 out of 10 Fzd receptors were detectable in the human placenta, indicating that Wnt signaling might be involved in trophoblast differentiation. Wnt-dependent transcription factors were found to be predominantly expressed in p57/Kip2-positive, invasive trophoblasts **[91]**. Moreover, Wnt-3a promoted trophoblast proliferation, migration and invasion through both canonical and non-canonical signaling, the latter was manifested through activation of Akt, which is also a well-known target of the invasion-promoting factors: EGF and IGF-II in trophoblasts. In complete mole, the majority of extravillous trophoblasts were shown to contain nuclear β-catenin, suggesting that aberrant Wnt signaling could be involved in excessive trophoblast invasion.

4.5 Other pathways

Focal adhesion kinase (FAK) is a widely expressed non-receptor protein tyrosine kinase that has a growth/migration-promoting role. The activation of FAK is achieved by phosphorylation at different amino acid residues, in particular by phosphorylation at Tyr-397. FAK activity was shown to be associated with tumor progression of cancer cells towards a malignant phenotype **[66, 92]**.

FAK also has an impact on activities of Rho proteins, a family (RhoA, Racl, Cdc 42) of particular GTPases regulating diverse biological processes such as cell cycle, cell–cell/focal adhesions, polarization and cell migration **[66, 93]**. The downstream effectors of Rho include p21-activated kinase (PAK), which cross-talks to the MAPK pathway by modulating Raf and Rho-associated, coiledcoil containing protein kinase (ROCK). The functionality of the RhoA–ROCK signaling cascade has also been suggested during trophoblast migration.

5. Progesterone-dependent immunomodulation: pregnancy-protective effect of PIBF

During pregnancy, the immunological effects of progesterone are mediated by the progesterone-induced blocking factor (PIBF). Following recognition of fetal antigens,

maternal lymphocytes get activated and express progesterone receptors **[94-98]**. In the presence of progesterone these cells produce PIBF **[99-100]**. PIBF is also widely expressed in the decidua, in the placenta as well as in the amnion. **[101]**.

The gene coding for PIBF is situated on chromosome 13 between loci 13q21 and 13q22 in the neighbourhood of the homeobox gene, KLF-5 and KIAA1008 **[102]**. The *PIBF1* gene, containing 18 exons, is 2765 bp in length with an open reading frame of 2271 bp and encodes a 90-kDa protein consisting of 757 amino acids **(Fig.4.)**.

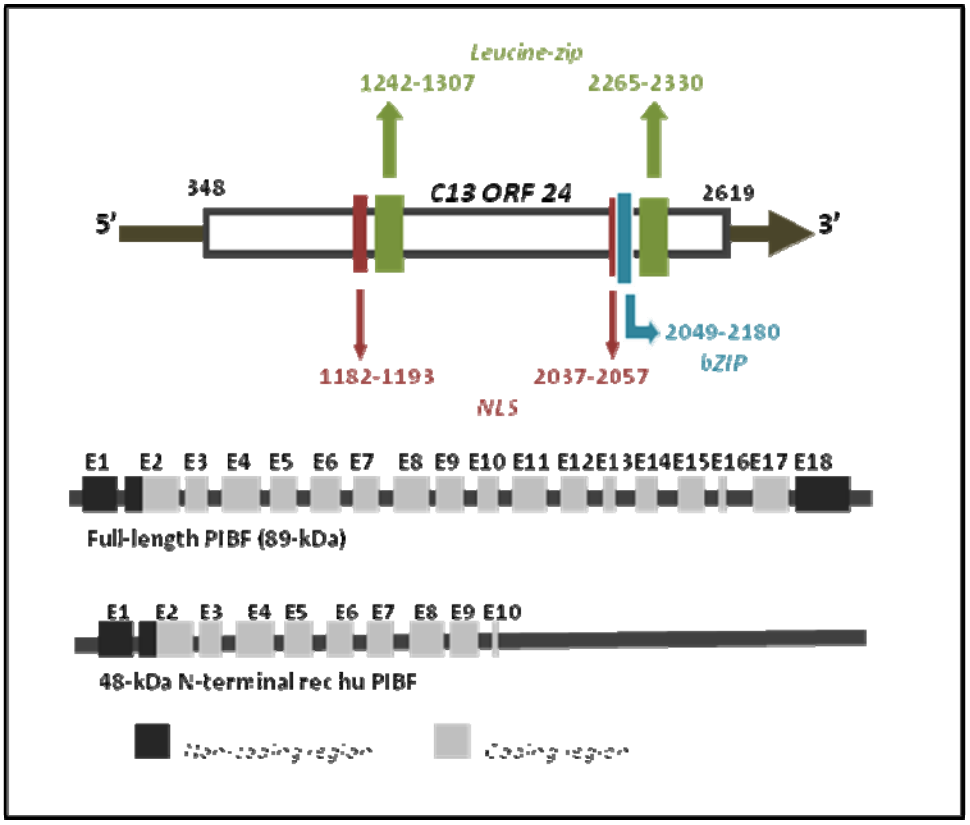

FIGURE 4. The structure of PIBF1 gene. (C: Chromosome, ORF: open reading frame, bZIP: basic-leucine zipper, NLS: nuclear localization signal, E: exon). NLS: on exon-7 and exon-13; LeuZip: on exon-8 and exon-15/16; bZip: on exon-14.

In recurrent miscarriage and in women showing clinical symptoms of threatened preterm pregnancy termination, the percentage of PIBF positive peripheral lymphocytes is

significantly lower than in healthy pregnant women **[99, 103-104]**, suggesting that PIBF production is related to the outcome of pregnancy **[105]**. This concept is further supported by the finding that neutralizing endogenous PIBF in pregnant mice results in fetal resorptions **[106]**.

PIBF supports the pregnancy via inhibiting NK activity **[106-108]**, facilitating the production of asymmetric antibodies **[109]** and altering the Th1/Th2 cytokine balance **[110-112]**. **(Fig.5.)**

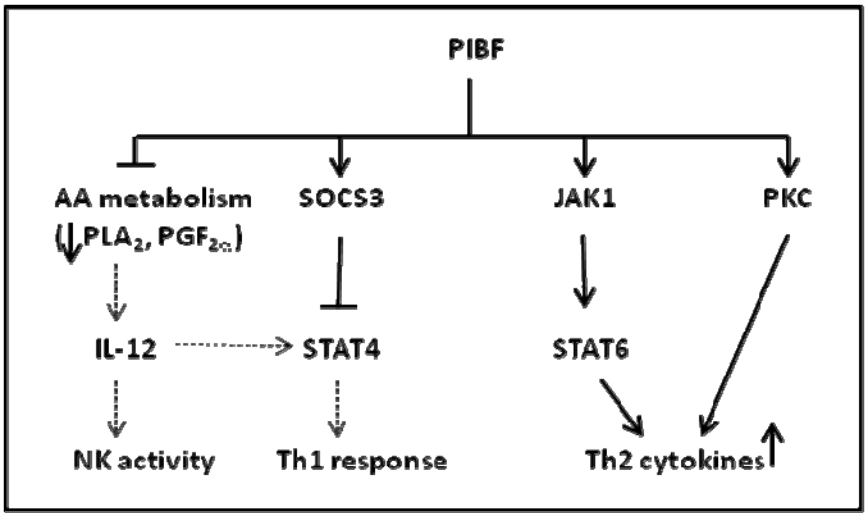

FIGURE 5. PIBF activates the Jak1/STAT6 and PKC/Ca++ - pathways which results in Th2-type cytokine production. PIBF induces SOCS3 which inhibits STAT4 phosphorylation, thus inhibits Th1 response through binding to the IL-12R. PIBF has the capacity to alter the arachidonic acid metabolism, and to inhibit NK activity.

Successful pregnancy is characterized by Th2 dominant cytokine pattern **[113-114]**. Activated lymphocytes cultured in the presence of PIBF produce increased amounts of the Th2-type cytokines IL-3, IL-4 and IL-10 **[112]**, and decrease the production of the Th-l cytokine IL-12. Based on our data, PIBF induces Th2-biased cytokine production via the Jak1/STAT6 **[115]** and PKC/Ca^{++} - pathways **[116]**.

Jak1/STAT6 pathway

PIBF induces the phosphorylation and nuclear translocation of STAT6 transcriptional factors and inhibits the phosphorylation of STAT4 molecules, which results in Th2 dominant cytokine balance.

Activation of the STAT6 pathway was known to be initiated through the IL-4 receptor uniquely, but previously we have demonstarted a novel type of IL-4R composed of the alpha chain of IL-4 receptor and the PIBF-receptor. PIBF does not bind to IL-4Rα but to its own receptor **[115]**. The STAT6 activating effect of PIBF can be abolished by blocking of IL4-Rα subunit. In addition, PIBF activates JAK1 (associated with IL-4Rα, suggesting that IL-4Rα is involved in PIBF signaling. PIBF does not phosphorylate Jak3 (associated with the common γ chain) and anti-IL-13Rα treatment has no effect on PIBF induced STAT6 activation, suggesting that the common γ-chain and the IL-13Rα subunits of IL-4 family do not take part in the signaling process of PIBF.

PKC/Ca^{2+} pathway

PIBF also affects the PKC/Ca^{2+} pathway which is involved in the regulation of Th1 and Th2 immune responses. High levels of PKC activity combined with low calcium signals favour Th2 development, while predominance of calcium signaling with low PKC activity favours Th1 development **[116]**.

In peripheral lymphocytes, PIBF induces the phosphorylation of PKCζ and PKCΘ isoforms without affecting the intracellular Ca^{2+} level supporting a Th2-type cytokine pattern.

SOCS1 and SOCS3

Suppressor of cytokine signaling (SOCS) proteins negatively regulate the signal transduction of several cytokines **[117]**. PIBF-induced SOCS-3, through binding to the IL-12R, inhibits STAT4 phosphorylation and Th1 responses in peripheral lymphocytes**[115]**.

6. A possible role for PIBF in tumor progression and in regulation of invasion

Recent literature shows that PIBF is not only expressed during pregnancy, but it is also produced by undifferentiated, proliferating cells and a set of malignant tumors. Lachmann et al. reported that various breast tumors overexpress PIBF compared to normal breast tissues both at the mRNA and protein levels. Moreover, MCF-7 mammary carcinoma cells produce PIBF in the absence of progesterone **[118]**.

In vitro data showed that human leukemia cell lines express mRNA for PIBF, and some of these cell lines also express the PIBF protein. In these cell lines PIBF production was up regulated by progesterone and down regulated by mifepristone **[119]**.

Rozenblum et al. **[120]** identified the PIBF1 gene on the chromosomal region 13q21-q22 which has been implicated as a common site for somatic deletions in a variety of malignant tumors.

Lachmann et al. **[118]** demonstrated that the full-length form of PIBF is associated with the centrosome. A number of proteins shown to be involved in tumorigenesis are associated with the centrosome. The most prominent example is the best-characterized breast cancer susceptibility gene *BRCA1* that is linked to the development of breast and ovarian cancers. Recent genetic studies indicated PIBF among the candidate genes for breast cancer predisposition and cancer progression.

The distribution of PIBF within the first trimester decidua coincides with sites of trophoblast invasion showing the strongest PIBF positivity at the extravillous trophoblast **[101]**. Check et al reported that 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 **[121]**.

Based on these data, the aim of my research project was to uncover a possible role of PIBF in regulation of invasion.

III. AIMS OF THE PROJECT

This work aims to investigate the involvement of Progesterone-Induced Blocking Factor (PIBF) in the regulation of invasion; and to identify the signaling networks via which it affects the invasive behavior of tumor cells, contributes to the success of implantation during pregnancy by regulating trophoblast invasion and participates in the pathogenesis of trophoblastic diseases.

Specific aims:

I. To confirm the involvement of PIBF in trophoblast and tumor invasion

- To analyse PIBF expression in trophoblast cells with different degrees of invasiveness;
- To investigate the effect of PIBF knock down on trophoblast and tumor invasion;
- To determine, whether PIBF has a role in matrix remodelling.

II. To analyse PIBF-induced invasion-related signaling pathways

- To characterize the PIBF-receptor;
- To uncover PIBF-induced signaling pathways involved in invasion;
- To investigate the subcellular localization of PIBF;
- To identify the function of nuclear PIBF.

IV. MATERIALS AND METHODS

1. Separation of lymphocytes

Peripheral blood mononuclear cells (PBMC) were separated from heparinized venous blood of healthy, non-pregnant volunteers by Ficoll-Hypaque discontinuous density gradient centrifugation.

2. Cell lines

The immortalized human first-trimester extravillous trophoblast cell line HTR8/SVneo was a kind gift from Charles H. Graham (Queen's University, Kingston, Ontario, Canada). HT-1080 fibrosarcoma cell line was purchased from ATCC.

Cell lines were kept at 37°C in a humidified atmosphere of 5% CO_2 under the following conditions: HT-1080 cell line was cultured in DMEM (Sigma-Aldrich) supplemented with L-Glutamine, MEM Non Essential Amino Acid Solution (Sigma-Aldrich), 10% heat inactivated fetal bovine serum (Gibco), penicillin and streptomycin (100 IU/ml and 100 μg/ml, respectively); HTR-8/SVneo cells were cultured in RPMI (Gibco) supplemented with 10% heat inactivated fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin.

3. Recombinant human PIBF and PIBF antibodies

The recombinant human PIBF was produced in our laboratory according to a method described earlier **[102]**. Briefly: Based on the previously described structure of *PIBF1* cDNA, a 1255 bp length segment of the N-terminal PIBF was ligated into a gluthatione S-transferase fusion vector (pGex-4T1), and transformed into *E. coli* BL21pLys host strain. PIBF was purified on a Glutathione-Sepharose 4B affinity column.

Fluorescein-isotiocyanate conjugated N-terminal recombinant human PIBF (PIBF-FITC) was also generated.

Polyclonal anti-PIBF antibodies and anti-exon17 were generated in our laboratory by immunizing rabbits with the 48-kDa N-terminal recombinant human PIBF or exon-17. The antibody titers were determined by ELISA. IgG was affinity purified on protein A columns.

Monoclonal anti-PIBF antibodies were generated in the Department of Immunology and Biotechnology, University of Pecs by immunizing mice with the 48-kDa N-terminal recombinant human PIBF.

4. Treatment of cells

After 4 h starving lymphocytes were incubated at 37°C for 20 minutes with the following:

- *E. coli* lysate (that had undergone the same purification procedure as the recombinant human PIBF) in a concentration of 200 ng to $5x10^7$ cells, as a control to exclude the effect of LPS contamination on cytokine production;
- 48-kDa N-terminal recombinant human PIBF at a concentration of 20, 100, 200 or 500 ng to $5x10^7$ cells;
- recombinant human IL-4 (R&D Systems Europe Ltd) at a concentration of 200 ng to $5x10^7$ cells.

After 16 h starving HTR8/SVneo and HT-1080 cells were incubated at 37°C for 5, 20 minutes, 1, 6 or 24 hours with the following:

- RPMI medium 1640 or DMEM as a control;
- *E. coli* lysate at a concentration of 100 ng to $6x10^5$ cells;
- 48-kDa N-terminal recombinant human PIBF at a concentration of 100 ng to $6x10^5$ cells.

5. Placental sections and immunohistology

Paraffin-embedded sections from hydatiform moles and choriocarcinomas were obtained from archives at the University of the Philippines (Manila, Philippines), the Gynaecology and Oncology Department of the Jagiellonian University (Krakow, Poland),

the Gynaecology and Oncology Department of the Lukaszczyk Oncological Center (Bydgoszcz, Poland) and the Clinical Gynaecology and Obstetrics Department (Rzeszow, Poland).

Five micrometer tissue sections of the formalin-fixed, paraffin-embedded blocks were mounted on gelatine-coated (Sigma-Alrich) slides. Sections were deparaffinized and rehydrated through graded alcohols to water. Endogenous peroxidase activity was blocked with 1% H_2O_2 in methanol. Antigen retrieval was carried out with 10 mM citrate buffer in a microwave oven. After pre-blocking with 1% BSA (Sigma-Aldrich) the sections were reacted with polyclonal anti-PIBF (diluted 1:100), 1:50 diluted polyclonal anti-leptin (Abcam) or 1:100 diluted monoclonal anti-leptin receptor antibodies (LifeSpan Biosciences).

Peroxidase-conjugated secondary antibodies (diluted 1:100) were applied (DakoCytomation) followed by incubation with AEC (3-amino-9-ethylcarbazole) substrate (Beckton Dickinson). The slides were counterstained with haematoxylin and coverslipped. Images were visualized with a Nikon FXA microscope. Images were taken with a digital camera (Nikon) using SPOT Advanced computer software program (SPOT Imaging Solutions).

6. Co-capping of IL-4Rα and PIBF receptor

One million lymphocytes were incubated with 5 μg of recombinant human PIBF-FITC for 30 min at 4°C. Then cells were washed twice in PBS containing 1% FCS and incubated with 5 μg of mouse monoclonal anti-human IL-4Rα or 10 μl of PE-labelled mouse anti-human CD45-RA antibody for 45 minutes at 37°. Cells were washed twice and incubated with 20 μl of PE-labelled rat anti-mouse IgG_{2A+B} or PE-labelled rat anti-mouse IgG_1 for 20 minutes at 37°C. Then cells ($1x10^5$) were plated on poly-L-lysine coated slides and incubated at 37 °C for further 10 minutes, then washed twice in PBS containing 1% FCS and fixed with freshly prepared 3% paraformaldehyde in PBS for 10 minutes at room temperature. After washing the slides were mounted with DABCO (Sigma Aldich).

To control the specificity of the capping formation, we performed all steps at 4°C.

Ligand-induced capping was also investigated. First, one million cells were incubated with 5 μg of FITC-conjugated 48-kDa rhuPIBF for 20 min at 37^{o}C. One hundred thousand cells were plated on poly-L-lysine coated slides and incubated at 37^{o}C for further 10 minutes then washed twice in PBS containing 1% FCS and fixed with freshly prepared 3% paraformaldehyde in PBS for 10 minutes at room temperature. Cells were washed twice and incubated with 2 μl of PE-labelled mouse monoclonal anti-human IL-4Rα for 45 minutes at room temperature. After washing the slides were mounted with DABCO.

Microscopic analysis was performed with a BioRad confocal microscope with 100x objective, using laser excitation at 473 nm and filters 580+/-16 nm for phycoerythrin and 522+/-17.5 nm for FITC. Images were analysed using the Adobe Photoshop 7.0 program.

7. Digestion of lymphocytes with phosphatidylinositol-specific phospholipase C (PI-PLC)

Five million PHA-pretreated PBMC was incubated with 2.5 units of phosphatidylinositol-specific phospholipase C (Sigma-Aldrich) for cleaving of the GPI-anchor in 1 ml of RPMI for 20 min at 37°C.

8. Depletion of plasma membrane cholesterol by methyl-β-cyclodextrin (MβCD)

To determine the effect of disrupting lipid rafts on PIBF-induced STAT6-activation, five million PHA-pretreated PBMC was incubated with 10mM of methyl- β-cyclodextrin (Sigma-Aldrich) in 1 ml of RPMI for 20 minutes at 37°C. The effect of MβCD on cell viability was controlled by Trypan blue. This concentration of MβCD depletes cholesterol but does not kill the cells.

9. Flow cytometry

For the detection of PIBF-receptor positive cells, lymphocytes, HTR-8/SVneo and HT-1080 cells were washed with PBS and one million cells were labeled with 1 μg FITC-

conjugated recombinant 48-kDa PIBF in 100 µl PBS supplemented with 1% FCS and 0.1% azide for 20 minutes at 4°C. After washing with PBS for 5 min, 2000 rpm at 4°C, cells were fixed with 2% paraformaldehyde.

For Annexin V staining, HTR-8/SVneo and HT-1080 cells were washed with PBS twice and one million cells were resuspended in 1X Binding Buffer (BD Biosciences). Then $1x10^5$ cells were incubated with 5 µl Annexin V-FITC for 15 min at room temperature. Finally, 200 µl of 1x Binding Buffer was added.

Labeled cells were analysed with a FACSCalibur flow cytometer (Beckton Dickinson Immunocytometry Systems) equipped with the CellQuest software program (Becton Dickinson) for data acquisition and analysis.

10. Silencing of PIBF, IL-4Rα or HB-EGF by siRNA

Oligonucleotides were pre-designed to interfere with IL-4Rα, PIBF or HB-EGF mRNA (Ambion). Cells were washed with OptiMEM (Invitrogen Life Technologies). Specific oligonucleotides or scrambled siRNA (control) were incubated for 20 min at room temperature with Oligofectamine (Invitrogen Life Technologies). This mixture was added dropwise to the cells. After 4 h incubation at 37°C, DMEM containing 30% FCS and L-glutamine was added to cultures and Western blot analyses for IL-4Rα, PIBF or HB-EGF expression were performed after 24, 48 or 72 hours subsequent to siRNA transfection.

11. Western blotting

After washing, treated cells were lysed in three volumes of high salt buffer containing 20 mM HEPES (pH 7.9), 20 mM NaF, 1mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 1mM EDTA, 1mM EGTA, 5mM DTT, 0.5mM PMSF, aprotinin (0.01µg/mL), leupeptin (0.025µg/mL), 400 mM NaCl and 20% glycerol at 4ºC. Samples were frozen and thawed three times, then incubated on ice for 20 minutes. After centrifugation at 15000 rpm at 4°C for 30 minutes the supernatants were collected and its protein content was determined.

Following SDS-PAGE (40 min, 120 V) proteins were transferred to nitrocellulose using a Hybond ECL membrane (Amersham Biosciences) 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 phospho-specific (Tyr-641) anti-human STAT6, rabbit polyclonal anti-human STAT6, rabbit polyclonal phospho-specific (Tyr-980) anti-human Jak3, rabbit polyclonal phospho-specific (Ser473) anti-human Akt antibodies (all from Santa Cruz Biotechnology Inc.) in TBS-Tween with 3% non-fat dried milk for an hour, mouse anti-β-catenin (Santa Cruz Biotechnology Inc.), rabbit anti-human phospho-STAT3 (Tyr705), rabbit anti-human phospho-STAT3 (Ser727), rabbit anti-human phospho-p44/42 MAPK (Thr202/Tyr204), rabbit anti-human Wnt5a, rabbit anti-human phospho-PKCζ/λ, rabbit anti-human phospho-PKCδ (all from Cell Signaling Technology Inc.), mouse anti-leptin-receptor (LifeSpan Biosciences) antibodies in TBS-Tween with 5% BSA overnight at 4°C or as control with rabbit anti-human β-actin (Sigma) in TBS-Tween with 3% non-fat dried milk for an hour. After washing (3x10min in TBS-Tween) bound antibodies were detected with 1:2000 diluted horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1 hour at room temperature) followed by development with ECL reagents (Perkin Elmer Life Sciences). Semi-quantification of the bands were performed by densitometry using ImageJ software.

12. Invasion assay

Oris^{TM} Cell Invasion and Detection Assay (Platypus Technologies) was used to detect invasion in PIBF-silenced cells **(Fig.6.)**. Basement membrane extract at the concentration of 3 mg/ml was applied on collagen-coated 96-well plates and the latter populated with Oris^{TM} Cell Seeding Stoppers which restrict cell seeding to the outer annular regions of the wells. Then wells were seeded with 75000 cells with or without siRNA (final cc. 25 nM). The stoppers were removed after 24 hours and Oris^{TM} basement membrane extract at the concentration of 12 mg/ml with 15% FBS was overlayed on the cells. Removal of the stoppers reveals a 2 mm diameter unseeded region in the center of each well, i.e., the detection zone, into which the seeded cells may then invade. The plate was incubated in a humidified chamber for 72 hours to permit cell invasion.

After 72 hours, supernatants were collected for substrate zymography then cells were labelled with Calcein AM. After applying the mask that blocks the outer seeding

region from view, images were captured using multi area scan by an Olympus Fluoview FV-1000 confocal microscope (OBJ: 20x, NA:0.45; excitation: 488 nm, detection: 500-600 nm).

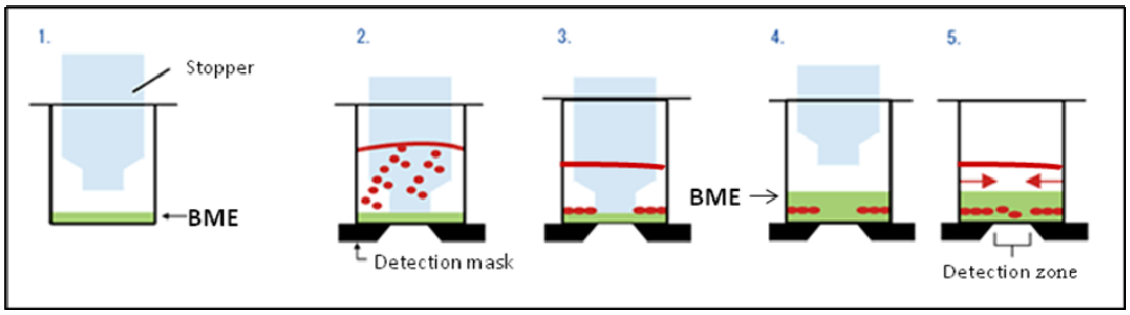

FIGURE 6. The principle of Oris™ Invasion and Detection Assay.

13. Substrate zymography

Cell-conditioned media (20 µl from the Oris™ plates) were subjected to electrophoresis under nonreducing conditions in a 7.5% acrylamide gel containing 1 mg/ml (0.01%) gelatin to detect MMP-2 and MMP-9 secretion. After electrophoresis, gels were washed twice in 2.5% Triton X-100 for renaturation and then incubated at room temperature then at 37 °C overnight in buffer containing 50 mM Tris–HCl, 5 mM $CaCl_2$ (pH 7). Thereafter, gels were stained with 0.5% (wt/vol) Coomassie Blue G-250 for 40 min and destained in 50% (vol/vol) methanol/10% (vol/vol) acetic acid until lysed bands were visible. Semi-quantification of the bands corresponding to 92-kDa gelatinase (MMP-9) and 72-kDa gelatinase (MMP-2) was performed by densitometry using ImageJ.

14. Confocal microscopy

Twenty thousand cells of HTR-8/SVneo or HT-1080 were plated on poly-L-lysine coated slides and incubated at 37°C for 20 minutes, then washed with PBS containing 1% FCS. Cells were fixed and permeabilized with acetone for 10 minutes at -20°C. Cells were

blocked with PBS containing 1% BSA and 10 mM HEPES for 20 minutes and incubated with anti-48kDa PIBF or anti-phospho-PKCδ antibody for one hour at room temperature. After washing the slides with PBS containing 1% BSA and 10 mM HEPES three times for 5 min, cells were incubated with Cy3-conjugated anti-rabbit-Ig for 30 minutes at room temperature. After washing nucleus was stained by Hoechst 33258 for 20 minutes at room temperature then cells were mounted with DABCO and slides were closed by nail polish.

Microscopic analysis was performed with an Olympus Fluoview FV-1000 confocal microscope with 60x oil immersion objective, using laser excitation at 405 nm and detection at 425-475 nm for Hoechst staining and laser excitation at 543 nm and detection at 555-655 nm for Cy3 dye. Images were analysed using the Olympus Fluoview 1.7 software.

15. Immunocytochemistry

Cytocentrifuge-prepared cultured cells of HT-1080 and HTR-8/SVneo were used to study PIBF expression. Samples were fixed in acetone at 4 °C for 10 min then washed with TBST. Peroxidase blocking reagent was added for 10 min, then rinsed twice with destilled water and twice with TBST. Non-specific binding sites were blocked with 1% BSA-TBST for 20 min. The primary antibody anti-48kDa PIBF and preimmune rabbit IgG as negative control were diluted 1:100 in 0.5% BSA-TBST for 1 h, then incubated with HRPO-conjugated anti-rabbit IgG for 30 min before addition of DAB chromogenic substrate (Dako) for 8 min. Cells were counterstained with Mayer's hematoxylin. Slides were mounted with glycerol gelatin and viewed through a Nikon microscope.

16. Protein arrays

Cells were treated with human recombinant PIBF (100ng/1x10^7 cells) in RPMI at 37°C for 20 min. As control, treatment with the lysate of *E. coli* that had undergone the same purification procedure as the recombinant human PIBF was used. Then cells were solubilized in lysis buffer (1x10^7 cells/ml) containing 1% NP-40, 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin,

and 10 μg/ml pepstatin. Samples were analysed by Proteome Profiler Antibody Array (Human Angiogenesis Array Kit, R&D Systems) according to the manufacturer's instructions. Briefly, nitrocellulose membranes bearing capture antibodies in duplicates were blocked for one hour at room temperature while samples were diluted (400 μg protein content) and mixed with a cocktail of biotinylated detection antibodies. Sample/antibody mixtures were incubated with the membranes at 4°C overnight. Following wash, Streptavidin-HRP and chemiluminescent detectiom reagents were added sequentially. Array data on developed X-ray film were quantitated by ImageJ software.

17. Isolation of nuclear fractions

$2.5x10^7$ HTR-8/SVneo and HT-1080 cells were pelleted and resuspended in 500 μl Puffer A (pH 7.2) containing 5 mM Tris-HCl (pH 8.0), 1 mM EGTA, 50 mM EDTA, protease and phosphatase inhibitors and incubated for 15 min on ice. After centrifugation at 800 g for 15 min at 4°C, supernatants were collected and frozen (cytosol fraction) while pellets were resuspended in 1 ml Puffer A supplemented with 0.5% Triton X-100 and then centrifuged with 800 g for 15 min at 4°C. Pellets were sonicated in 1 ml Buffer A on ice, then proteins were precipitated with 170 μl 72% trichloroacetic acid for 10 min on ice. After centrifugation at 13200 rpm for 10 min, pellets were resuspended in acetone (-20°C), sonicated and kept on ice for 2 min, - these steps were repeated three times. Finally, the pellet was resuspended in 1X SDS buffer, sonicated, and kept at 4°C overnight. After centrifugation, samples were boiled and subjected to SDS-PAGE and Western blot.

18. Cytometric Bead Arrays

IL-6 concentrations were determined by Cytometric Bead Array (CBA). Supernatants of PIBF silenced cells were incubated with labeled capture beads and detection reagent for 3 h in the dark at room temperature, and analyzed with flow cytometer (FACSCalibur; BD Biosciences) by using the respective CBA Analysis software (BD Biosciences).

19. Chromatin immunoprecipitation (ChIP)

$1x10^8$ HTR-8/SVneo and HT-1080 cells were grown to confluency then cross-linked with 1% formaldehyde for 10 min at room temperature. Cells were scraped into cold PBS containing protease inhibitors. Cells were pelleted for 10 min at 1500 rpm at 4°C then lysed in Cell Lysis Buffer containing 5 mM HEPES (pH 8.0), 85 mM KCl and 0.5% NP-40 for 10 min on ice. After centrifugation at 4500 rpm for 5 min, pellet was resuspended in Nuclei Lysis Buffer (1%SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.1) for further 10 min on ice. Lysate was sonicated on ice/ethanol bath to shear DNA to lengths between 200 and 1000 basepairs. Samples were centrifuged for 15 min at 13200 rpm at 4 °C and four 200 µl aliquots were diluted 10-fold with ChIP Dilution Buffer (0.01% SDS, 1.1% TritonX-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.1), 167 mM NaCl, protease inhibitors) while one 50 µl aliquot was frozen for input. After pre-clearing with 50 µl blocked (BSA and salmon-sperm DNA) Protein-G Agarose beads (Sigma) for 2 hours at 4°C, chromatin was incubated with 10 µg monoclonal anti-PIBF antibody or control IgG overnight at 4°C. Antibody/protein/DNA complex were isolated by immunoprecipitation with 60 µl of blocked Protein-G Agarose beads. After extensive washing, the pellets were treated with freshly prepared elution buffer (1% SDS; 0.1 M $NaHCO_3$) and

- the eluted protein/DNA complexes were decrosslinked with 20 µl 5 M NaCl for 6 hours at 65°C, then eluates were concentrated 4-fold by Amicon® Centricon® Centrifugal Filter Devices. 30 µl of eluates were separated on SDS-PAGE and the protein content of eluates were analysed by Western blotting; or
- the eluted protein/DNA complexes, together with the 10-fold diluted inputs were treated with 10 µl EDTA (pH 8.0), 20 µl 1 M Tris-HCl (pH 6.5) and 1 µl of 20 mg/ml Proteinase K for 2 hours at 55°C to digest the protein content, then 20 µl 5 M NaCl was added and samples were further incubated overnight at 65°C. DNA was recovered by using Qiagen PCR Purification Kit (Qiagen) and used as a template for PCR. The PCR primers for the ChIP assay were designed to correspond to the promoter regions of Wnt5a, EGF, IL-6 and FGF-1 (**Table 3.**).

TABLE 3. Sequences of PCR primers used in ChIP assays.

Gene	Chromosome	No.	Product	Position	Sequence	T_m
IL-6	Chr.7	# 1	494 bp	22766592-22767085	5'-CGCTAGCCTCAATGACGACC-3'	58,0
					5'-GAAGGCAACTGGACCGAAGG-3'	58,4
		# 2	308 bp	22766304-22766611	5'-CATAATCCCAGGCTTGGGGG-3'	58,1
					5'-GGTCGTCATTGAGGCTAGCG-3'	58,0
		# 3	238 bp	22765324-22765561	5'-CAGAGGACCACCGTCTCTGT-3'	58,5
					5'-GCTGAAACCAGACCCTTGCA-3'	58,1
PlGF	Chr.14		107 bp	75422381-75422487	5'-GTCTGGACCTGCCGAGAG-3'	57,5
					5'-AGGTTCCCGAGCCGAGTT-3'	59,0
EGF	Chr.4		290 bp	110833882-110834171	5'-AGCGAGTTATCTCCTCTTTGGCAGT-3'	59,8
					5'-ACAGAGCAAGGCAAAGGCTTAGAGA-3'	60,2
FGF-1	Chr.5	# 1	186 bp	142067460-142067645	5'-ACAGGGTTTCACAACTGGACATAA-3'	56,5
					5'-CCAGATTCCCCCCCTCCTA-3'	58,1
		# 2	166 bp	142066039-142066204	5'-GCAGGGATGCCAGATGACA-3'	57,6
					5'-TGTGTGAGCCGAATGGACTTC-3'	57,4
		# 3	177 bp	142064721-142064897	5'-TCAGGGTTTTGGTAGGGTGGTA-3'	57,8
					5'-GATGTGGGTGTGGATAGTGTATGTG-3'	57,1
Wnt5a	Chr.3	# 1	457 bp	55521340-55521796	5'-GAGAGGCGCTCCGTTTCCAA-3'	60,1
					5'-CTTGTGCGTTTTCAGCGGCA-3'	59,4
		# 2	246 bp	55502025-55502270	5'-GCCACAGTTGGCTGAGGTGA-3'	60,2
					5'-TGCAGAATGGAAACCCATGCCT-3'	59,6
		# 3	116 bp	55524169-55524284	5'-AATAAAGGTTTGTGGTTGGGTA-3'	52,3
					5'-AAGGCAGTTCGTGTAGAGGAT-3'	55,7
		# 4	109 bp	55521321-55521429	5'-AAGGTCTTTTGCACAATCACG-3'	54,0
					5'-CGCAGGCAACTGTTCCAC-3'	57,2
		# 5	149 bp	55518933-55519081	5'-CCAGCAAATGGGACTCGG-3'	56,2
					5'-AAGCGGGAAAGCAACACT-3'	55,1

V. RESULTS

1. The role of PIBF in invasiveness

1.1 Expression of PIBF in normal first trimester trophoblast and trophoblastic diseases

Controlled trophoblast invasion is a key process during human placentation and a prerequisite of successful pregnancy.

In case of gestational trophoblastic tumors, trophoblast invasion is increased. Hydatidiform mole or molar pregnancy arises when the sperm and egg join but do not develop into a fetus, however, the placental elements continue to grow forming a tissue that resembles grape-like cysts. Hydatidiform moles may be partial (containing some embryonic or fetal tissue) or complete (containing no fetal tissue). They rarely metastasize but they may develop into invasive mole or into the more aggressive, rapidly growing, metastatic choriocarcinoma.

In order to investigate the possible cross-talk between PIBF and leptin in regulation of trophoblast invasion, paraffin embedded sections from healthy first trimester placentae, partial moles, complete moles and choriocarcinomas were analysed by immunohistochemistry. Sections were reacted with PIBF, leptin or leptin-receptor specific antibodies.

PIBF was expressed in both normal first trimester villous trophoblast and in partial mole, while PIBF expression was markedly decreased in complete mole and absent in choriocarcinoma. Neither leptin, nor leptin-receptor was detected in partial mole, whereas both of these molecules were present in complete mole and choriocarcinoma **(Fig. 7.).**

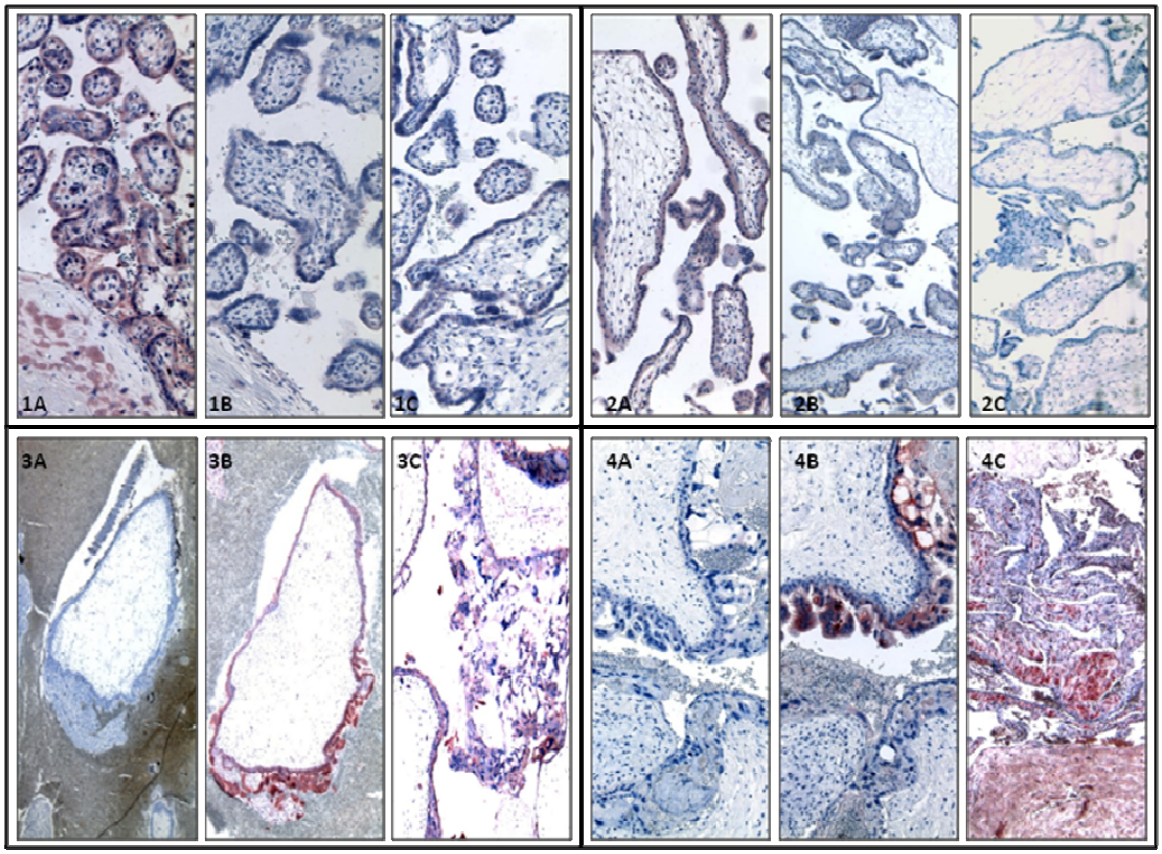

FIGURE 7. Immunohistochemical detection of PIBF (**A**), leptin (**B**) and leptin-receptor (**C**) expression in healthy 1st trimester trophoblast (**1**), partial mole (**2**), complete mole (**3**) and choriocarcinoma (**4**). (50X)

To confirm the inverse relationship between PIBF and leptin/leptin-receptor expression, PIBF deficient trophoblast cells were generated with siRNA technique and leptin-receptor was detected by Western blotting in normal and PIBF knock down cells **(Fig. 8.A.)**. Moreover, leptin expression was detected in the lysates of PIBF-treated cells with a protein array **(Fig. 8.B.)**.

Leptin-receptor expression was upregulated in PIBF deficient cells **(Fig. 8.A.)**, while leptin expression was decreased in PIBF-treated cells **(Fig. 8.B.)**, suggesting that PIBF controls the expression of leptin and its receptor in the trophoblast.

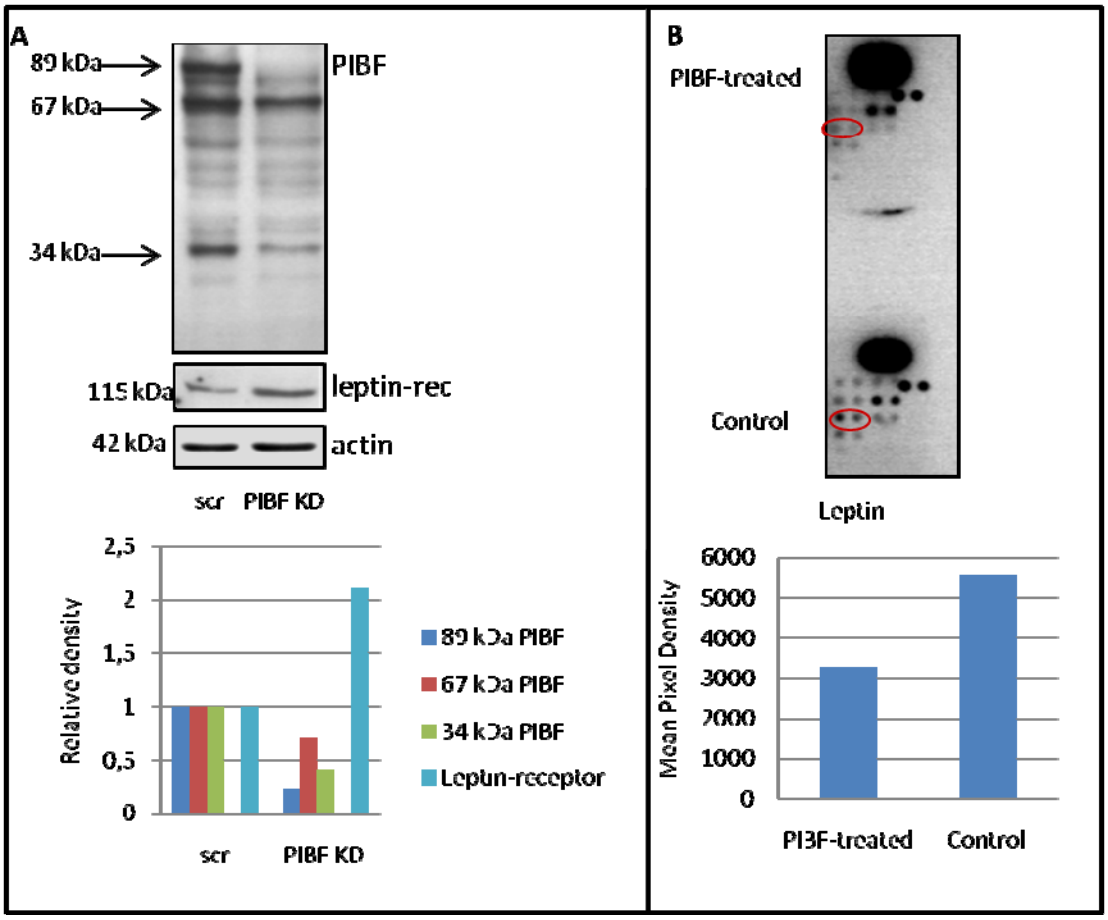

FIGURE 8. PIBF controls trophoblast invasion by down-regulating the expression of leptin-receptor (**A**) and leptin (**B**). A representative experiment of three is shown. Lysates from PIBF knock down and control (scr: scrambled siRNA) HTR-8/SVneo cells were reacted with anti-PIBF or anti-leptin-receptor antibodies in Western blotting. Equal loading was controlled with anti-β-actin antibody (**A**, upper panel). Densitometric evaluation of the presented Western blots is shown on the lower panel. **B:** Leptin expression in control and in PIBF-treated cells. (Protein array - upper panel; densitometric evaluation of the presented spots - lower panel)

1.2 The effect of PIBF-knock down on invasion of trophoblast and tumor cell lines

To further investigate the possible role of PIBF in regulating invasion, we used trophoblast and tumor cell lines, rendered PIBF deficient by siRNA technique.

To study trophoblast invasion, HTR-8/SVneo cell line was selected which had been generated by transformation of HTR-8 cells with simian virus 40 large T antigen. The

primary HTR-8 cultures were obtained after plating and outgrowth of cells from tissue pieces of human first trimester villi. The resulting cell line (HTR-8/Svneo) shares phenotypic properties with the progenitor cells **[122]** and its proliferation, migration and invasiveness are regulated by the same signaling molecules that modulate extravillous trophoblast cell responses in vitro **[123-125]**.

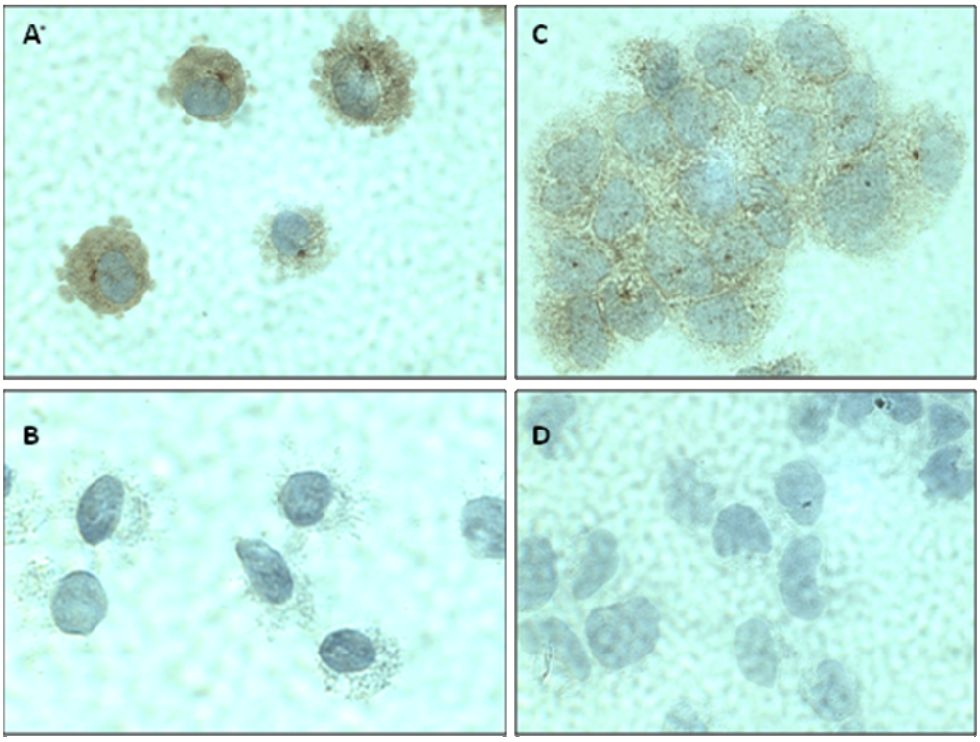

FIGURE 9. Immunocytochemical demonstration of PIBF in HT-1080 (**A**, **B**) and HTR-8/SVneo cells (**C, D**). The cells were reacted with polyclonal anti-48kDa PIBF antibody (**A, C**) or the second antibody only (**B, D**).

For modelling tumor invasion, the highly invasive fibrosarcoma cell line HT-1080 was selected. Both cell lines express and secrete PIBF (immunocytochemistry, **Fig. 9,** Western blotting, **10.B.**) and its receptor (Flow cytometry, **Fig. 10.A.**).

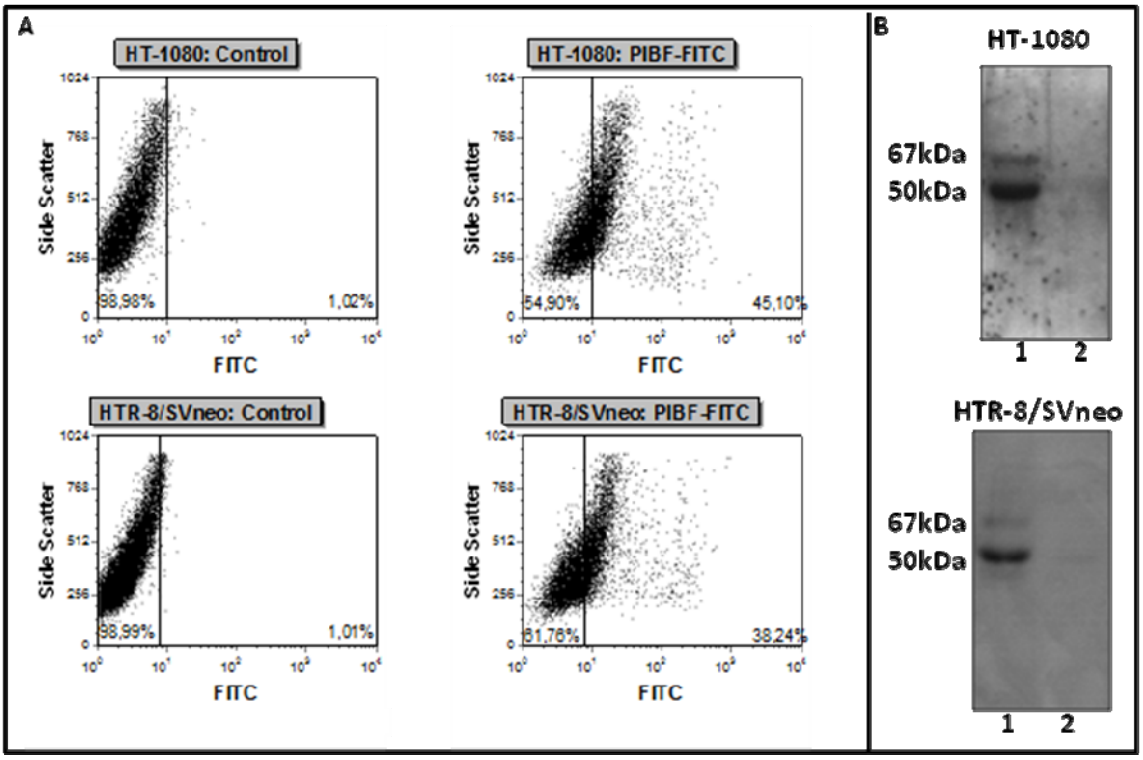

FIGURE 10. A. Flow cytometric analysis of PIBF-receptor expression on the surface of HTR-8/SVneo and HT-1080 cells. FITC-conjugated ligand was used to visualize the PIBF-receptor. **B.** The supernatants of the cultured cell lines were reacted with anti-PIBF antibody (lane 1) or second antibody for control (lane 2).

Platypus Oris cell invasion assay **(Fig. 6.)** was used to determine the invasive potential of PIBF knock down HTR-8/SVneo trophoblast and HT-1080 fibrosarcoma cells.

Silencing of PIBF increased invasivity in HTR8/SVneo cells, and decreased invasive behaviour in HT-1080 cells as shown on **Fig. 11.A.**

1.3 PIBF affects the activity of matrix metalloproteinases

Invasion of surrounding tissues is mediated by a set of proteolytic enzymes, among others, matrix metalloproteinases (MMPs). MMP-9 and MMP-2 cleave type IV collagen, (the main component of basal membranes), thus play a crucial role in trophoblast and tumor invasion.

Cell conditioned media from the invasion assay were subjected to gelatine zymography to measure MMP-2 and MMP-9 activity. In trophoblast cells, PIBF silencing resulted in increased MMP-2 and MMP-9 secretion; while PIBF knock down tumor cells showed reduced levels of secreted MMP-2 and MMP-9 matrix metalloproteinases **(Fig. 11.B.)**.

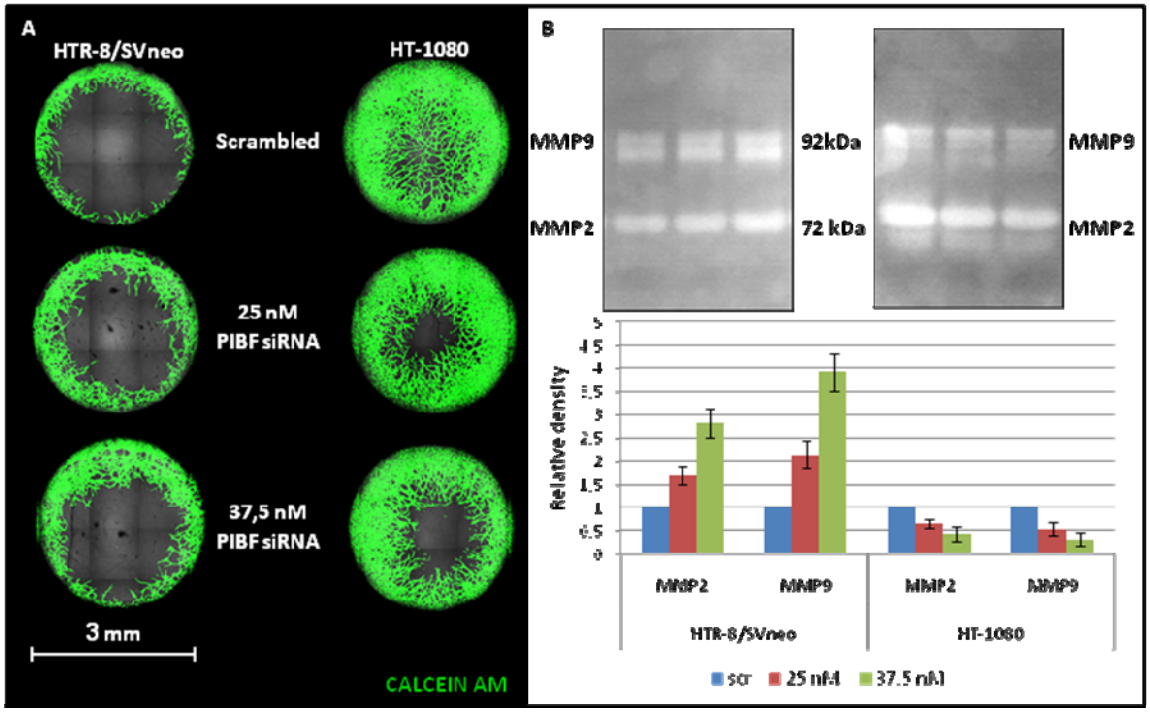

FIGURE 11. A: Invasion of HTR-8/SVneo and HT-1080 cells after silencing of PIBF. Detection zones (3 mm in diameter) in which the seeded sells invaded are shown on the confocal images 72 hours after removing the Cell Seeding Stoppers. Cells were stained with Calcein AM dye. Invasion assays were repeated at five times. (scr: scrambled siRNA) **B:** Supernatants from the Oris^{TM} Assay were subjected to gelatine zymography to detect MMP-2 and -9. A representative experiment is shown on the upper panel. Densitometric evaluation of three zymograms are shown on the lower panel. Data are represented as mean ± SEM. In trophoblast cell line HTR-8/Svneo, silencing of PIBF resulted in increased production of MMP-2 and MMP-9 and increased invasion. In tumor cell line HT-1080, knock down of PIBF had an opposite effect: production of MMP-2 and 9 as well as invasion was decreased.

Lysates of PIBF-treated and control samples were analysed by protein arrays **(Fig.12.)**. After 24 h PIBF treatment MMP-9 expression increased in fibrosarcoma cells while decreased in trophoblast cells. TIMP-1 - which has the capaity to inhibit MMP-9 - was downregulated in tumor cells while in trophoblast cells its level increased after 24 h PIBF treatment. These findings coincide well with the results of gelatin zymography.

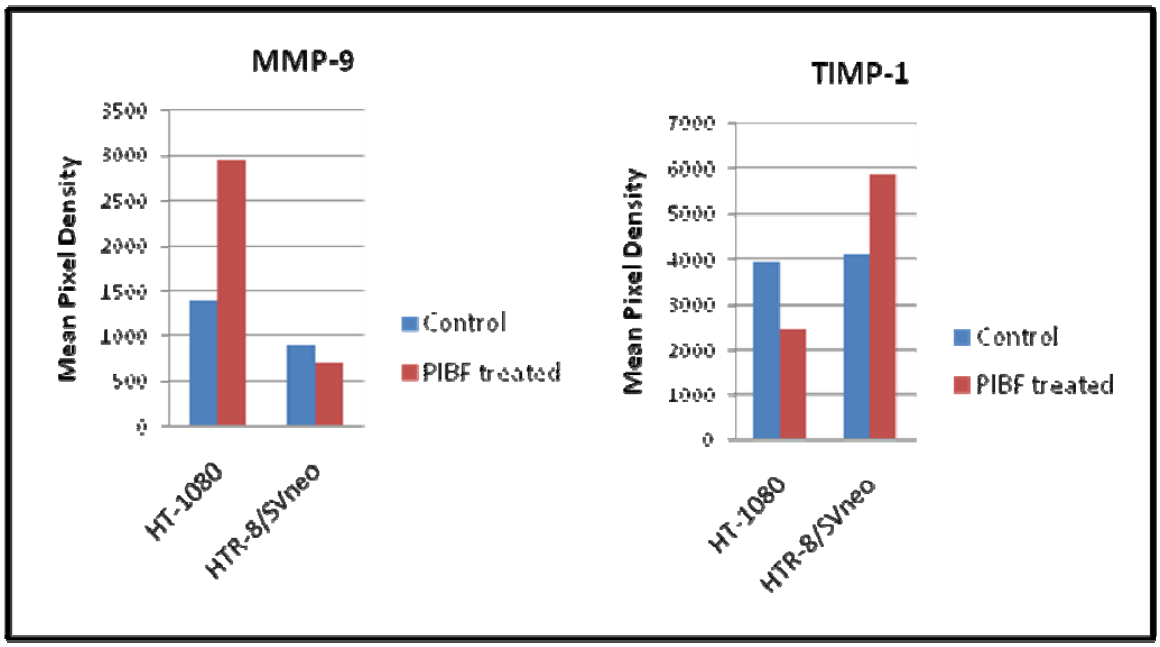

FIGURE 12. MMP-9 and TIMP-1 expression in PIBF-treated (24 h) trophoblast and fibrosarcoma cells was analysed by protein arrays. In tumor cells MMP-9 expression was increased and TIMP-1 expression was reduced after 24 h PIBF treatment whereas in trophoblast cells MMP-9 expression was decreased and TIMP-1 was increased after PIBF treatment. A representative experiment is shown.

To sum up, PIBF down-regulates MMP-2,-9 secretion, up-regulates TIMP-1 expression thus decreases invasion of trophoblast cells; while it facilitates the production of MMP-2,-9 and inhibites the secretion of TIMP-1 thus increases invasion in tumor cells.

2. PIBF-induced signaling networks and invasive behaviour

2.1 Characterization of PIBF-receptor

2.1.1. PIBF-receptor associates with IL-4Rα

Earlier we showed that the engaged PIBF receptor associates with the α-chain of the IL-4 receptor for signaling. To further verify the involvement of IL-4Rα in PIBF signaling, IL-4Rα deficient cells were created by siRNA technique. Silencing of IL-4Rα by siRNA reduced the STAT6 activating effect of PIBF in peripheral lymphocytes **(Fig. 13.)**, confirming that the α-chain of the IL-4 receptor was indeed indispensable for PIBF signaling.

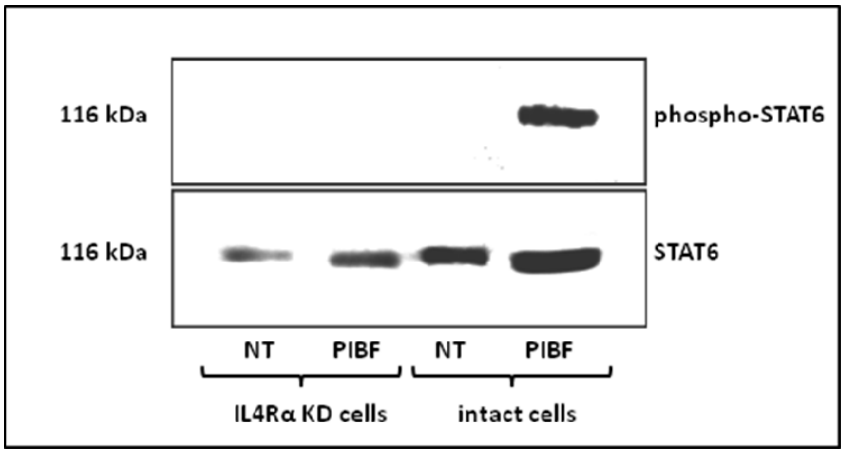

FIGURE 13. Silencing of IL-4Rα reduced the STAT6 activating effect of PIBF in lymphocytes. Lysates from IL-4Rα knock down and control cells were reacted with anti-phospho-STAT6 antibody on Western blot. Loading was controlled by reactivity with anti-STAT6 antibody. NT=untreated.

Capping experiments were performed to detect co-localization and co-capping of IL-4Rα and PIBFR on peripheral blood mononuclear cells.

When FITC-labelled PIBF is added to the cells, it binds to the PIBF-receptor, and might induce complex formation of the PIBF receptor and IL-4Rα. Receptor cross-linking by anti IL-4Rα specific antibody might also induce complex formation. To investigate these effects, both antibody- and ligand-induced cross-linking was performed.

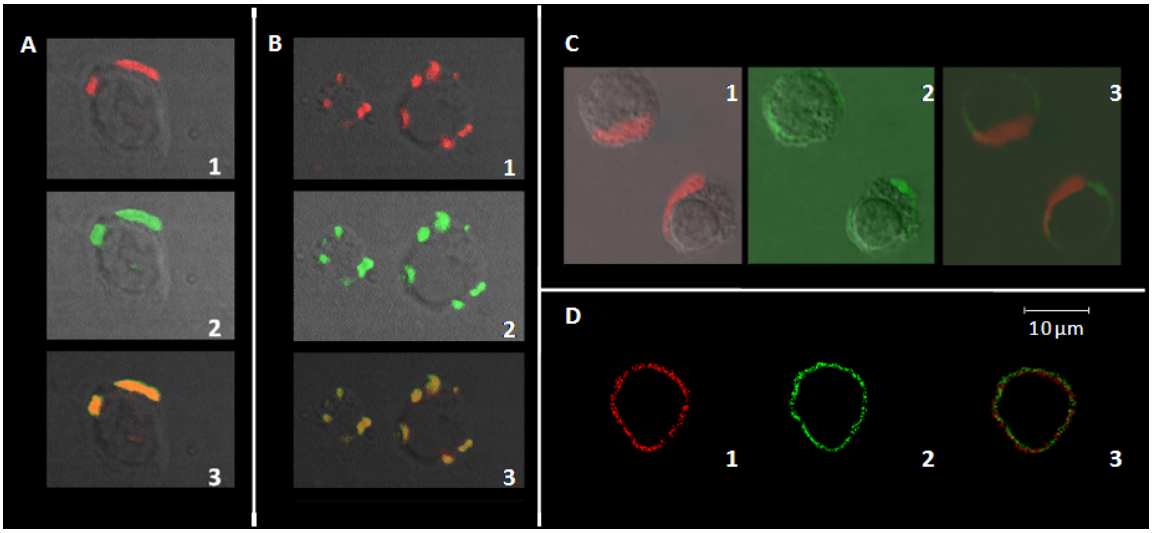

FIGURE 14. Confocal microscopy analysis to investigate the complex formation of IL-4Rα and the PIBF-receptor (100x). Both anti-IL-4Rα antibody induced cross-linking **(A)** and ligand-induced capping **(B)** resulted in co-localization and co-capping of the two binding sites. **1:** FITC-PIBF labelled PIBFR; **2:** PE-anti IL-4Rα-labelled IL-4R; **3:** merged picture. **C:** Simultaneous labelling of lymphocytes with PE-antiCD45RA (red), cross-linked by anti-mouse IgG_1-PE **(1)** and FITC-PIBF (green) **(2)**, resulted in capping of the CD45RA molecule but no co-capping of the PIBFR **(3)**. **D:** All steps were performed at 4°C. (All confocal images were taken with identical settings and at least 50 cells were counted in each slide. In each experiment more than 90% of the capped cells showed the indicated phenotypes.)

Antibody-induced cross-linking by anti-IL-4Rα and PE-conjugated anti-IgG_{2A+B} at 37ºC results in capping of the IL-4Rα and simultaneus co-capping of the PIBF receptor, as shown on **Fig. 14.A.** No capping occurred, when all steps were performed at 4°C **(Fig. 14.D.)**.

Ligand-induced cap-formation **(Fig. 14.B.)** was tested by incubating the cells with FITC-PIBF at 37°C. After ligand binding, the cells were fixed and incubated with anti-IL-4Rα-PE at room temperature. It was found that PIBF ligand induced capping resulted in co-capping of the IL-4Rα.

To test the specificity of co-capping induced by PIBF ligand or IL-4Rα specific antibody, simultaneous labelling of CD45RA and PIBF receptor was performed. Antibody-induced CD45RA crosslinking resulted in capping of the CD45RA molecule but no co-capping with the PIBFR, as shown on **Fig. 14.C**.

These data suggest that the PIBF receptor forms a complex with IL-4Rα and this process is initiated by PIBF binding.

Competition between IL-4 and PIBF was also tested. JAK3 is present on the γ-chain of the IL-4R, thus IL-4 signaling involves JAK3 phosphorylation **(Fig. 15.A.)**. In the presence of PIBF, IL-4 induced JAK3 phosphorylation was reduced in a concentration-dependent manner, confirming that the PIBF-receptor is capable of replacing the γ-chain of IL-4R **(Fig. 15.B)**.

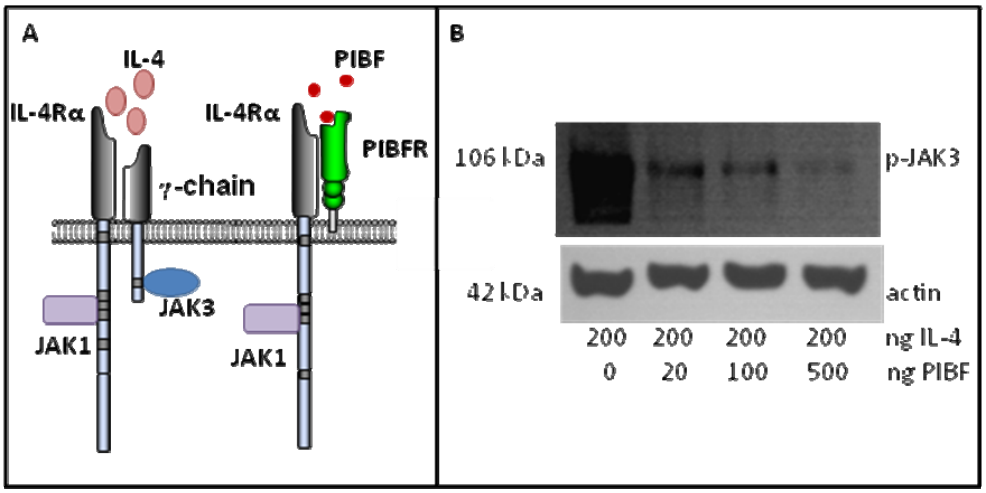

FIGURE 15. A: Type I. IL-4R consists of IL-4Rα and the common γ-chain and binds IL-4. Upon ligation, the GPI-anchored PIBF-receptor combines with IL-4Rα. **B:** PIBF-receptor replaces the γ-chain in the IL-4 receptor. The lysates of lymphocytes treated with increasing PIBF concentrations in the presence of equal concentration of IL-4 were reacted on Western blots with anti-phospho-JAK3 antibody. Loading was controlled by anti-actin reactivity. A representative experiment of three is shown.

2.1.2 The PIBF receptor is a raft-associated, GPI-anchored protein

The above findings raise the question, why IL-4Rα is needed for PIBF signaling. A plausible explanation would be that the PIBF receptor has no transmembrane and intracellular domains thus it uses the intracellular tail of IL-4Rα for signal transduction.

Assuming that the PIBF receptor is a GPI-anchored protein, we digested the putative anchoring-region with phosphatidylinositol-specific phospholipase C (PI-PLC). PIBF-induced phosphorylation of STAT6 molecules was tested by Western blotting in intact and in PI-PLC digested lymphocytes.

In PI-PLC digested lymphocytes, PIBF did not induce STAT6 phosphorylation while IL-4 retained its effect **(Fig. 16.A.)**, indicating that PIBFR is indeed a GPI-anchored molecule.

Furthermore, capping of IL-4Rα could still be induced in PI-PLC digested lymphocytes but labelling of PIBF receptor was lost **(Fig. 16.B.)**.

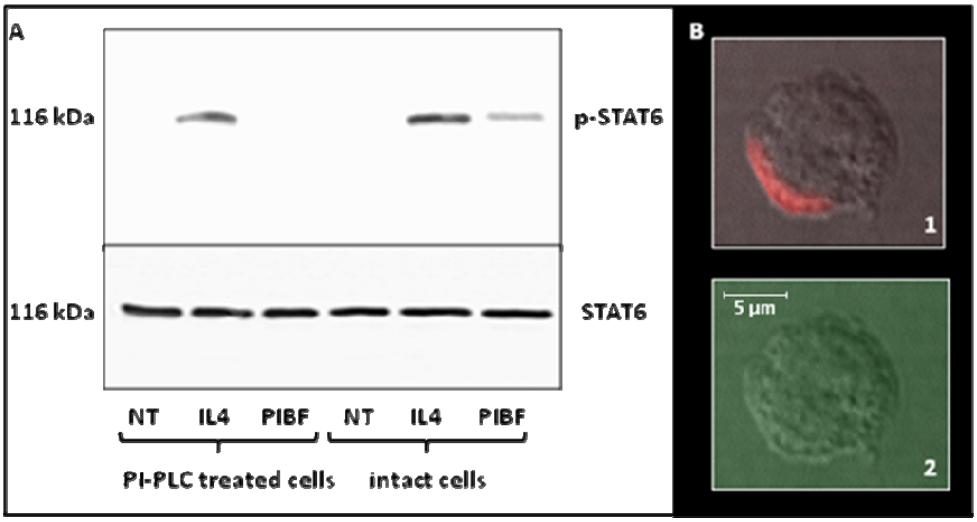

FIGURE 16. A: PIBFR is a GPI-anchored protein. Digestion with PI-PLC cleaves the anchoring regions of GPI-anchored PIBFR and results in loss of PIBF induced STAT6 activation while the effect of IL-4 remains intact. A representative experiment of three is shown. (Western blot: anti-phospho-STAT6 Ab, loading: anti-STAT6 Ab. NT: untreated cells.) **B:** Capping of the IL-4Rα **(1)** in PI-PLC digested cells in the absence of PIBF-receptor **(2)**. Confocal microscopy, 100x.

GPI-anchored proteins are enriched in the leukocyte membrane within glycosphyngolipid-cholesterol rafts. These submicron domains need cholesterol to function, therefore the hypothesis that receptors of PIBF float in lipid rafts was tested by depletion of cholesterol, using methyl-β-cyclodextrin (MβCD).

The STAT6 inducing effect of PIBF was abolished in MβCD-treated lymphocytes in a concentration-dependent manner. In MβCD treated cells neither PIBF, nor IL-4 was able to Tyr-phosphorylate STAT6 **(Fig. 17.)**, suggesting that not only the PIBF receptor but also the α chain of the IL-4 receptor might be raft-associated.

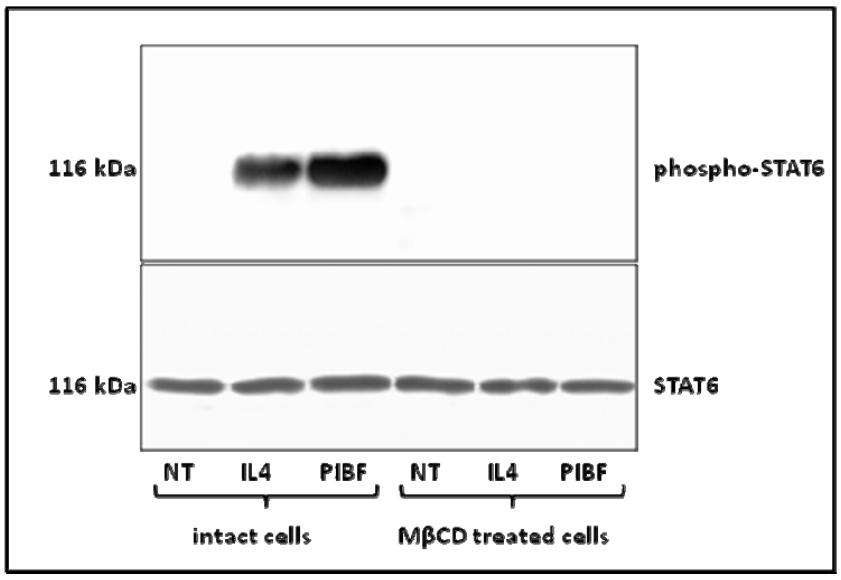

FIGURE 17. Disruption of raft integrity by MβCD abrogates PIBF and IL-4 induced Tyr-phosphorylation of STAT6, suggesting that PIBFR and IL-4Rα are associated with lipid rafts. Western blot: anti-phospho-STAT6 Ab. Loading: anti-STAT6 Ab. NT: untreated cells. The experiment was repeated at three times.

2.1.3. Internalization of PIBF-receptor

Since the GPI-anchored receptors were found to cluster and recycle at the cell surface, we examined the effect of ligand-binding on PIBFR internalization by confocal microscopy. Lymphocytes were labeled with FITC-conjugated PIBF and incubated at 37°C

for 0, 15, 30 or 60 min **(Fig. 18.)**. Internalization was detectable after 15 min and reached the maximum at 60 min.

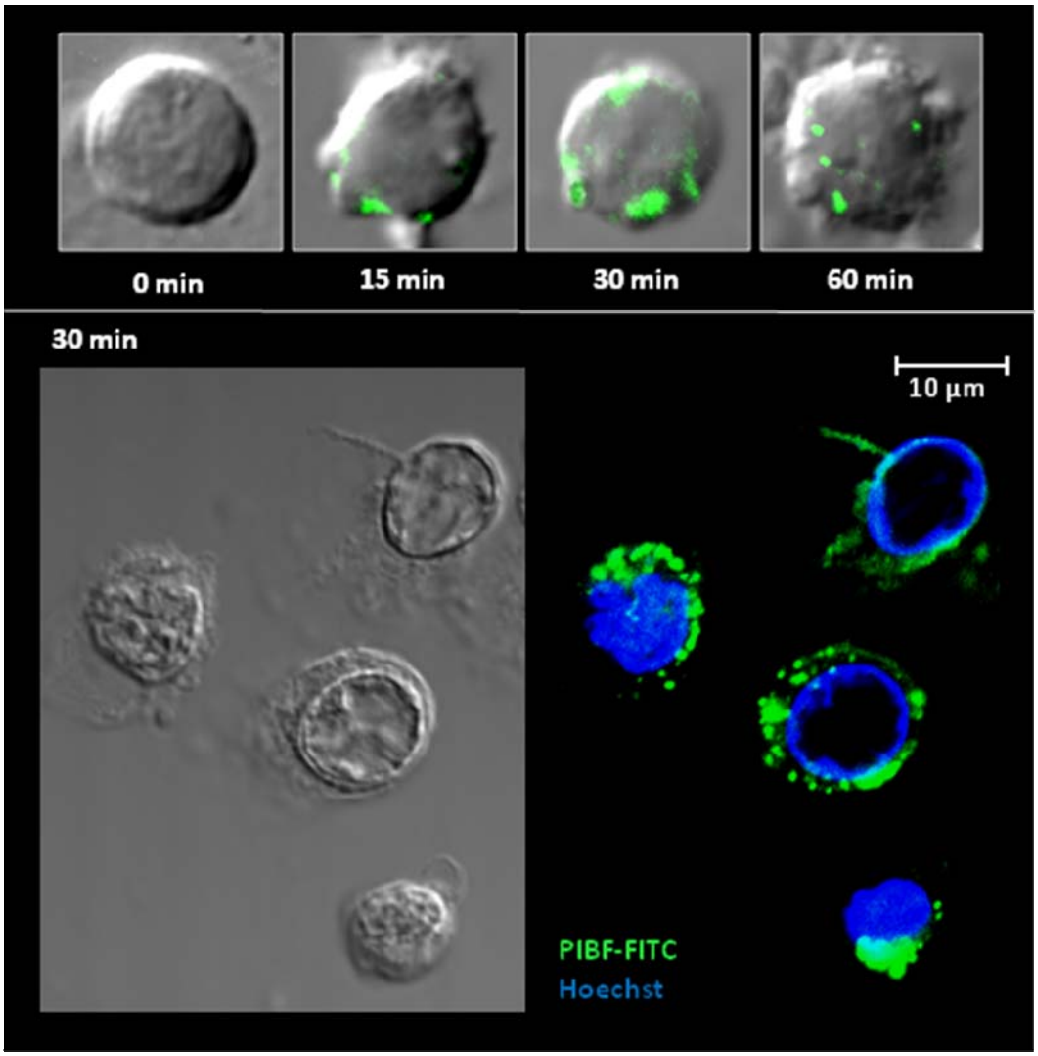

FIGURE 18. Internalization of PIBF-receptor starts within 15 minutes after adding its ligand at 37°C. Confocal microscopy, 60x oil immersion. Nuclei: Hoechst stained. PIBFR was visualized with FITC-labeled PIBF.

2.2 PIBF-induced signaling pathways which might affect invasive behaviour

Silencing of IL-4Rα abrogated the effect of PIBF on invasion in both cell lines, suggesting, that the invasion-related signaling of PIBF is initiated by the IL-4Rα/PIBFR complex **(Fig. 19.)**.

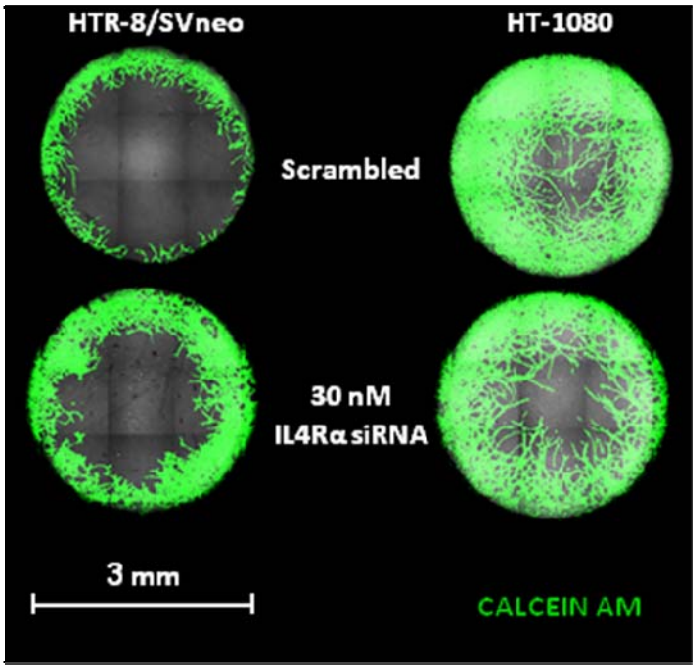

FIGURE 19. Invasive behaviour of IL-4Rα knock down HTR-8/SVneo and HT-1080 cells. IL-4Rα knock down abrogated the effect of PIBF on invasion in both cell lines. (Confocal microscopy, 20x.)

The next purpose included identifying the signaling networks that might be involved in PIBF regulated invasion. Based on the fact, that the GPI-anchored PIBF receptor forms a complex with IL-4Rα and uses its intracellular tail for signaling, we focused on IL-4Rα associated cascades that might be induced by PIBF **(Fig. 20.)**.

IRS, associated with IL-4Rα, can activate the Akt and ERK pathways. Both of these play a role in controlling invasive differentiation of trophoblast cells and promote metastatic potential of tumors. Therefore, the effect of PIBF treatment on phosphorylation of ERK and Akt were investigated in HTR-8/SVneo and HT-1080 cells by Western blotting.

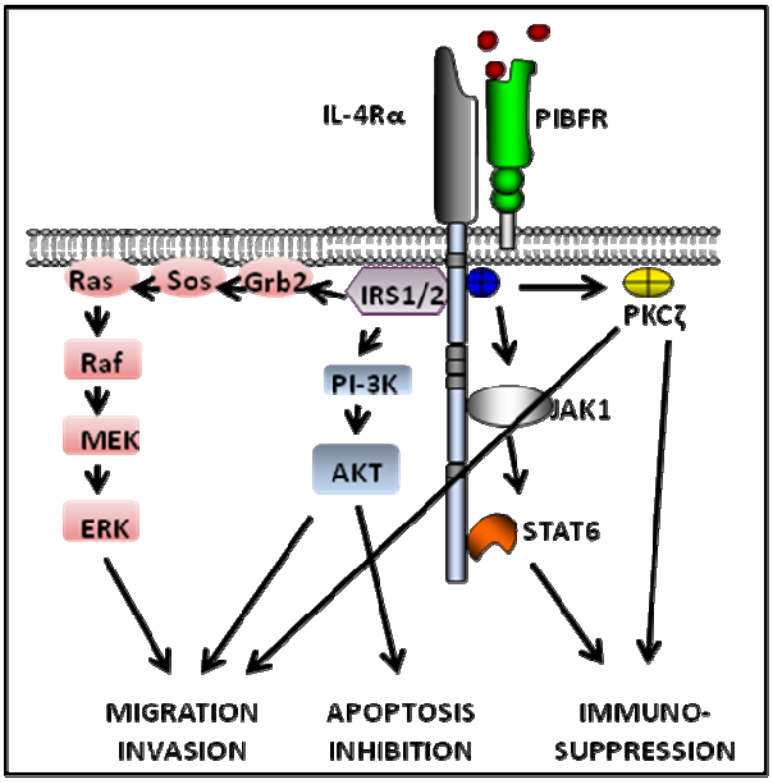

FIGURE 20. IL-4Rα associated signaling pathways that might be activated by PIBF.

In trophoblast cells PIBF phosphorylated Akt and ERK in a fast, transient way, whereas, in the tumor cell lines PIBF treatment resulted in sustained and late activation of Akt and ERK molecules **(Fig. 21, 23.)**.

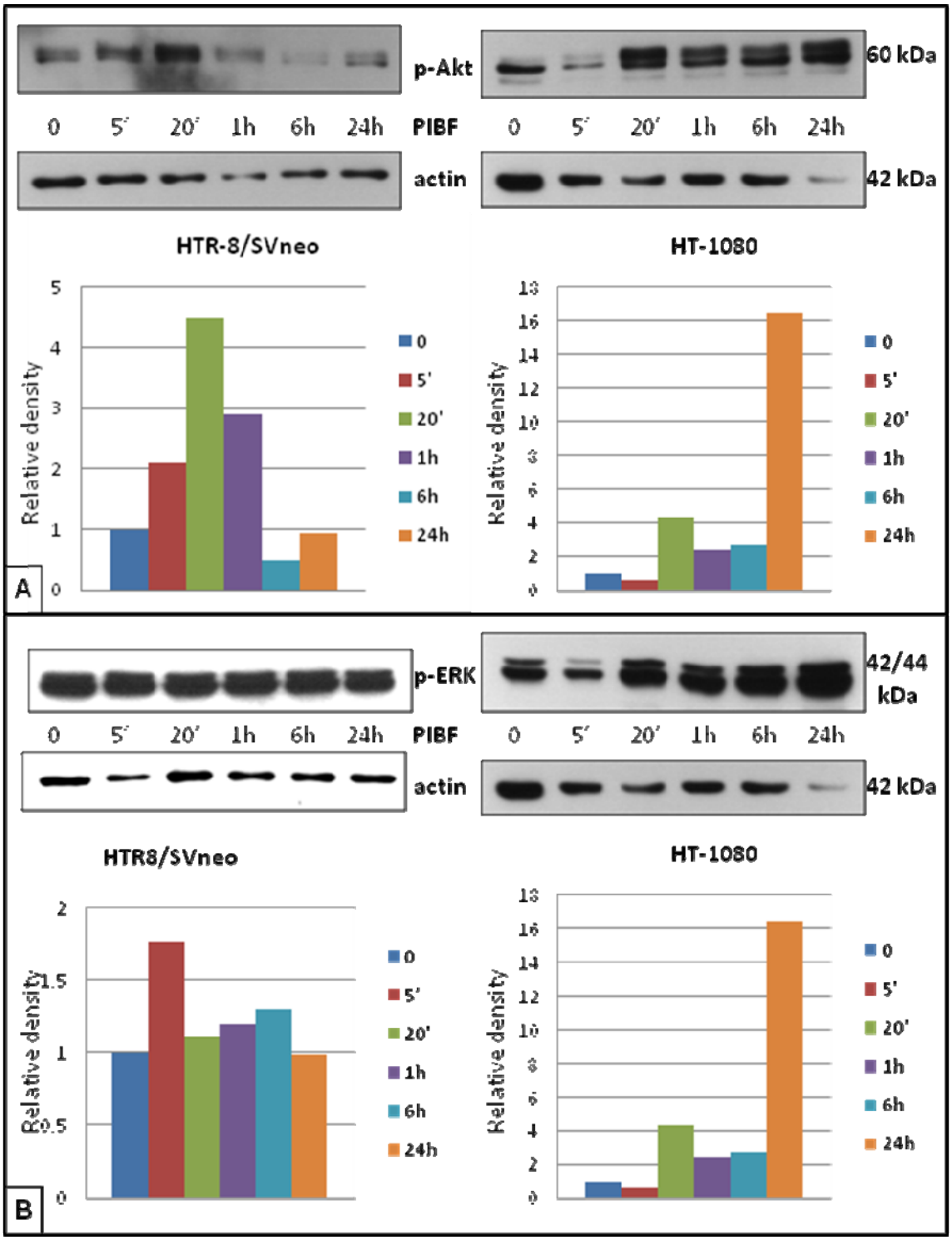

FIGURE 21. In trophoblast cells PIBF phosphorylates Akt **(A)** and ERK **(B)** in a fast, transient way while in HT-1080 fibrosarcoma cell line PIBF treatment resulted in sustained activation of these molecules. Densitograms of the presented Western blots are shown. Each sample is normalized with its actin pair.

Since many papers reported STAT3 as a key molecule in trophoblast and tumor invasion, we also investigated the effect of PIBF treatment on STAT3 Ser727 and Tyr705 phosphorylation in the same cell lines.

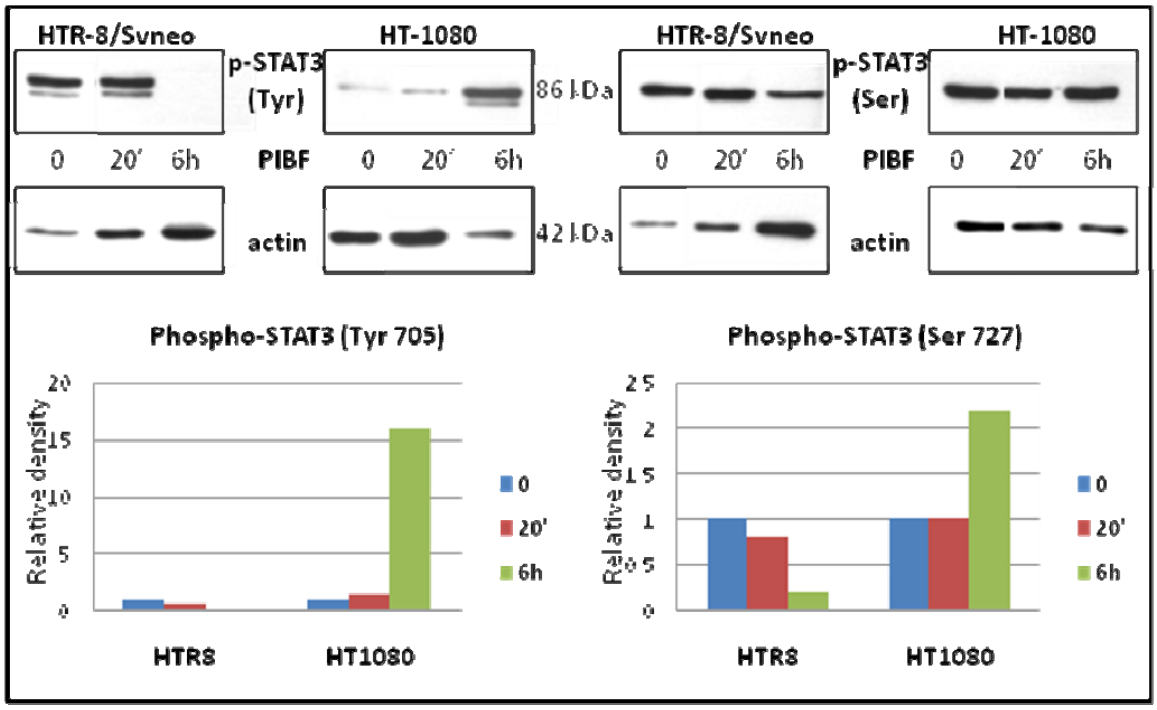

FIGURE 22. In trophoblast cells PIBF treatment decreased Ser727-phosphorylation of STAT3 and did not affect the Tyr 705 phosphorylation of STAT3. PIBF treatment resulted in late phosphorylation of both Ser727 and Tyr705 residues of STAT3 in HT-1080 fibrosarcoma cell line. Densitograms of the presented Western blots are shown. Each sample is normalized with its actin pair.

In trophoblast cells PIBF did not significantly alter the level of Tyr-phosphorylated STAT3 and decreased the amount of Ser-phosphorylated STAT3 molecules after 24 hours treatment **(Fig. 22, 23.)**. In tumor cells PIBF treatment resulted in late STAT3 Ser and Tyr phosphorylation suggesting an indirect role of PIBF in STAT3 induction.

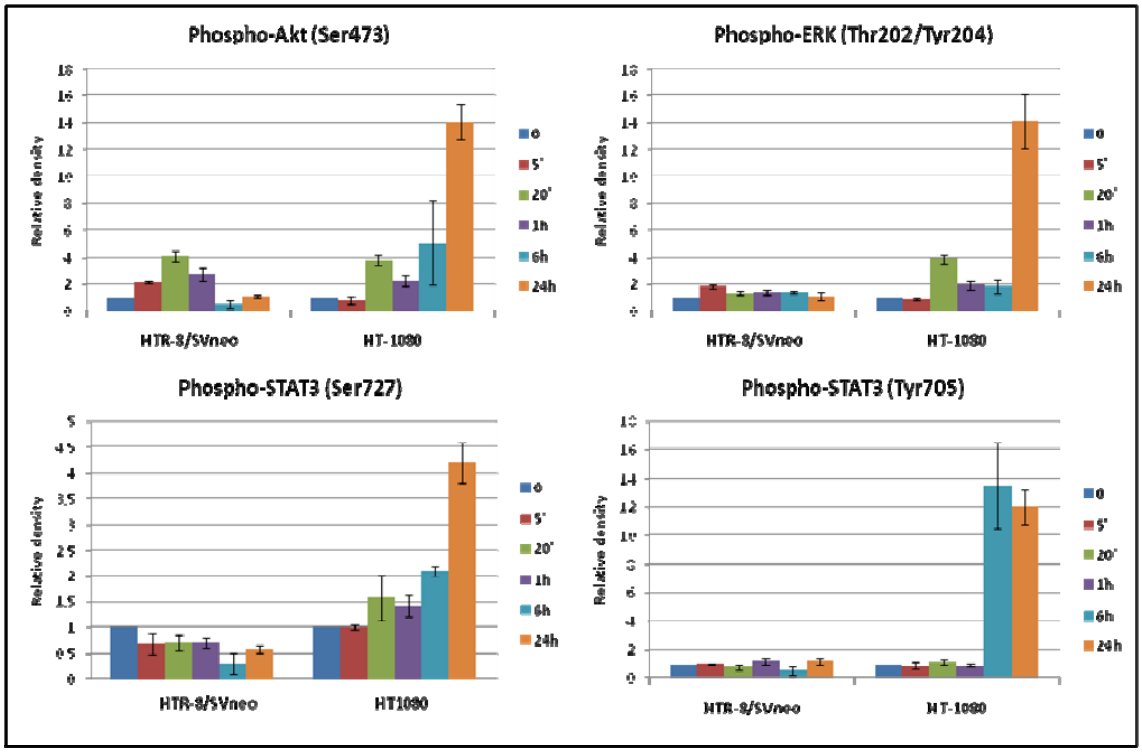

FIGURE 23. The effect of PIBF treatment on Akt, ERK and STAT3 phosphorylation in trophoblast and fibrosarcoma cells. Statistical analysis of Western blots from three different experiments are shown. Data are represented as mean relative density ± SEM.

Finally, we examined whether PIBF treatment alters the level of Wnt5a molecules which has been shown to be involved in invasion of many tumors **[126,127]**.

In HT-1080 fibrosarcoma cells PIBF induced the activation of Wnt5a molecules after 6 hours incubation thus we analysed the Wnt5a signaling pathways in tumor cells in a more detailed fashion **(Fig. 24.)**.

In fibrosarcoma cells the level of β-catenin was reduced with 60% after 6 h PIBF treatment suggesting that Wnt5a inhibits the canonical, β-catenin-mediated pathway. Moreover, after 6h PIBF treatment PKCζ and PKCδ **[128]** - the two isoforms which might be involved in the non-canonical signaling processes of Wnt5a - were also phosphorylated.

PKCδ might be associated with Wnt5a signaling or apoptosis. Nuclear localisation of PKCδ is required for its apoptotic function **[129]**, while membrane-association of PKCδ shows its involvement in Wnt5a signaling **[130]**.

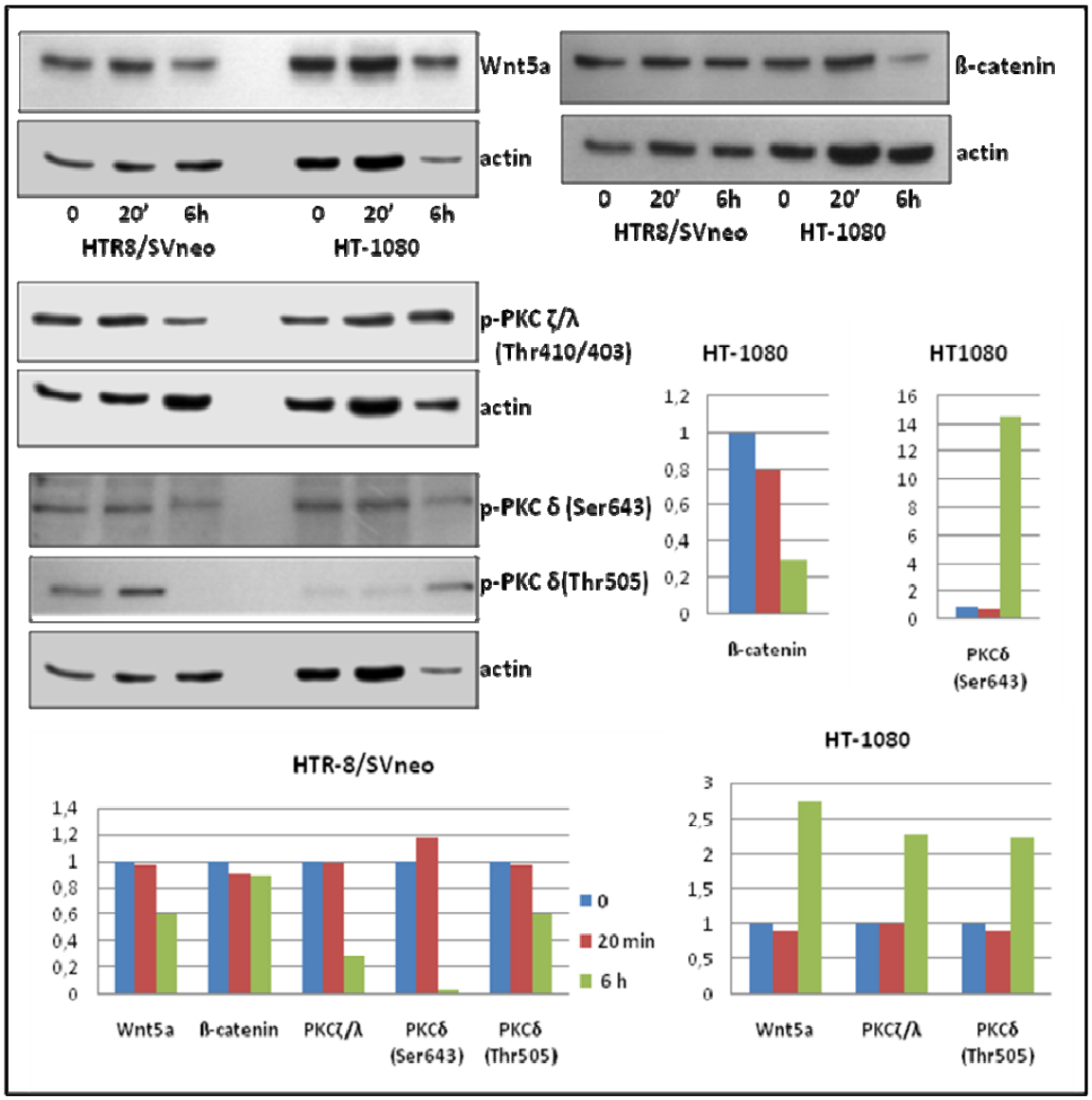

FIGURE 24. After 6 h PIBF treatment the increased Wnt5a induces the degradation of β-catenin and signals with the help of PKCζ and PKCδ. Densitograms of the presented Western blots are shown. Each sample is normalized with its actin pair. Experiments were repeated at three times.

To confirm that PKCδ is associated with Wnt5a signaling and not with the apoptotic pathway, confocal microscopy was performed to detect the subcellular localization of phosphorylated PKCδ after 6h PIBF treatment and cells were Annexin stained to detect early signs of apoptosis **(Fig. 25.)**. Phospho-PKCδ was associated with the membrane after

6 h treatment supporting the hypothesis that it has a role in Wnt5a signaling. Significant apoptosis was not detectable after 6 h PIBF treatment (data not shown).

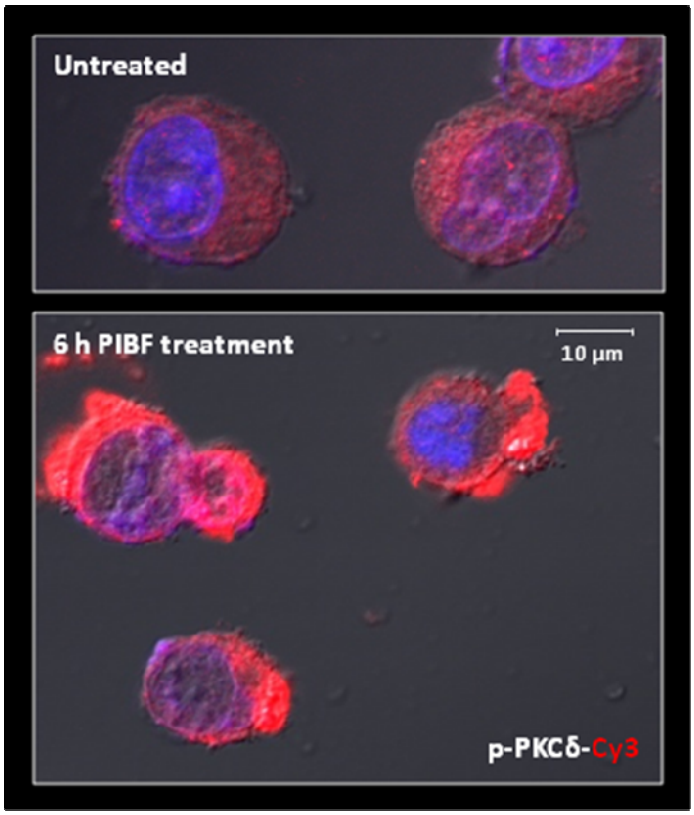

FIGURE 25. Subcellular localisation of phospho-PKCδ after 6h PIBF treatment in HT-1080 cells. Phospho-PKCδ was visualized by anti-phospho-PKCδ Ab and further stained with Cy-3-conjugated anti-rabbit Ig; nuclei were labelled by Hoechst. Confocal microscopy, 60x oil immersion.

2.3. Subcellular localization of PIBF

The late signaling events observed in tumor cells are not likely to be due to direct PIBF action, rather to that of new PIBF-induced proteins. The full-length PIBF contains leucine-zippers, nuclear localization signals and bZIP motives, which enable the molecule

to bind to DNA and regulate gene transcription **(Fig. 4.)**. If PIBF induces proteins which bind to growth factor receptors, further signal pathways might be activated.

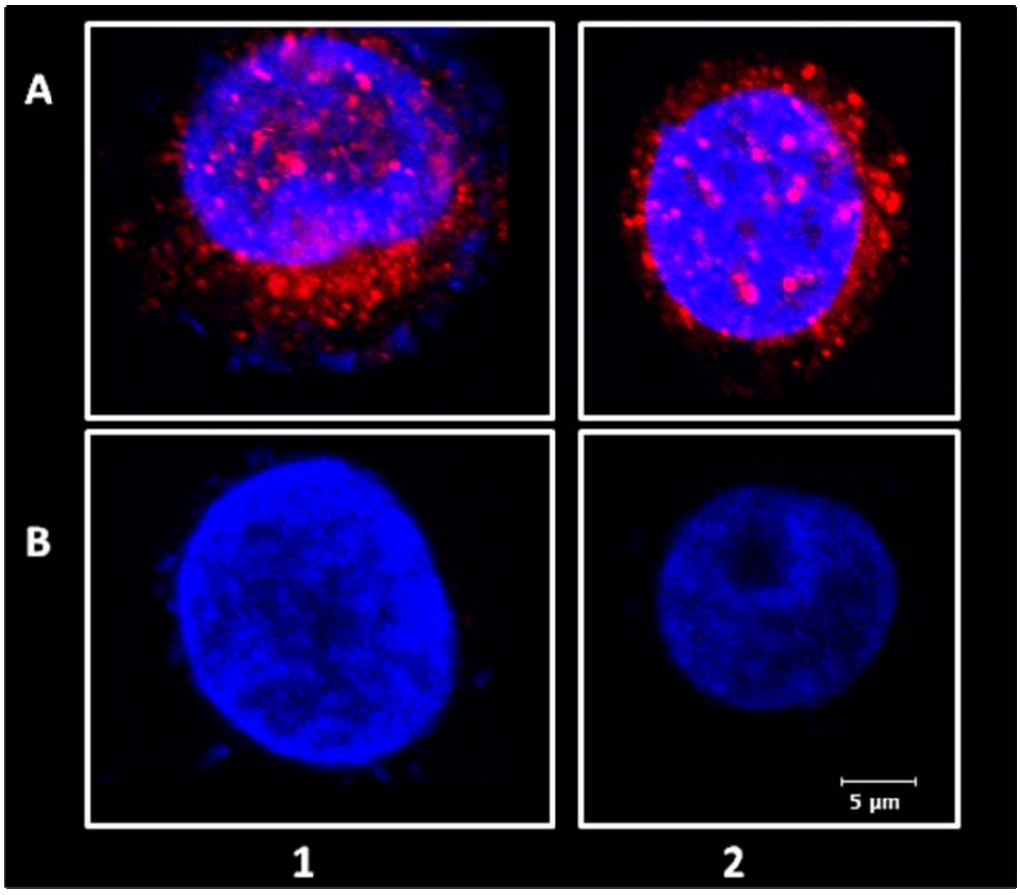

FIGURE 26. Subcellular localization of PIBF in HTR-8/SVneo (**1**) and HT-1080 (**2**) and cells by confocal microscopy. **A:** Merged images of nucleus staining (Hoechst, shown as blue) and labeling of PIBF (shown as red; 1st Ab: anti-PIBF, 2nd Ab: anti-rabbit Ig-Cy3) **B:** Merged images of nucleus staining and the secondary control without the first anti-PIBF antibody. Confocal microscopy, 60x oil immersion.

To confirm that PIBF enters the nucleus, subcellular localization of PIBF was visualized in trophoblast and tumor cell lines by confocal microscopy and in fractionated cells by Western blotting. For confocal microscopy PIBF was labelled with the fluorescent dye Cy3 and nuclei were counterstained by Hoechst **(Fig. 26.)**. PIBF reactivity was detected in the nucleus as well as in the cytoplasm of both cell lines.

Then nuclear fraction was isolated from the cells to detect PIBF by Western blotting **(Fig. 27.)**. PIBF was present in the nucleus of both cell lines, however, the ratio of nuclear versus cytoplasmic PIBF was higher in the tumor, than in the trophoblast cells.

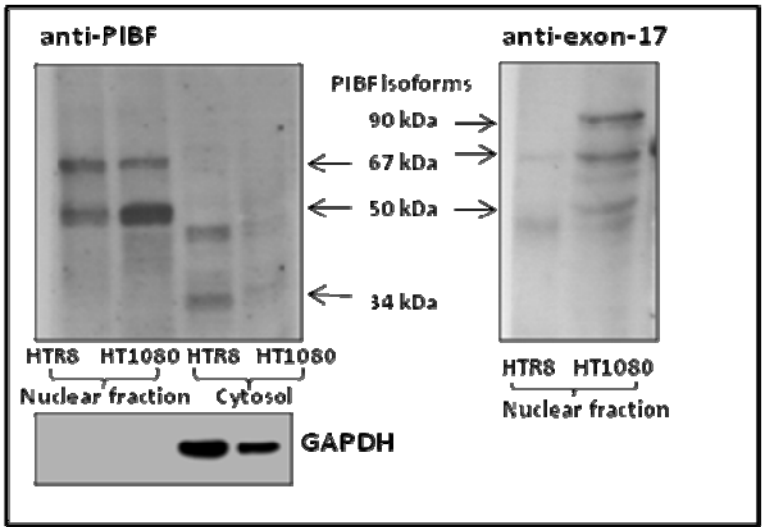

FIGURE 27. Subcellular localization of PIBF in trophoblast and tumor cell lines. Nuclear and cytosol fractions were reacted with anti-48kDa PIBF or anti-exon-17 antibodies by Western blotting. A representative experiment of three is shown.

2.4. PIBF-induced genes

To identify PIBF-induced genes, protein arrays (for detection of 55 angiogenesis- and invasion-related molecules) were performed on lysates of PIBF-silenced and 24 h PIBF-treated trophoblast and fibrosarcoma cells. In PIBF knock down tropoblast cells, no significant alteration was found, suggesting that in trophoblast cells PIBF does not induce the genes of the tested proteins and the events are due to by receptor-mediated effects of PIBF.

In fibrosarcoma cells PIBF induced the transcription of HB-EGF, PlGF, FGF-1, endothelin-1 and angiogenin **(Fig. 28.)**. Either of these can initiate the observed late signaling pathways.

To confirm this possibility, we investigated the effect of PIBF treatment on STAT3 activation in HB-EGF silenced HTR-8/SVneo and HT-1080 cells. In HB-EGF knock down fibrosarcoma cells the STAT3-activating effect of PIBF was reduced compared to the control (scrambled) suggesting that late STAT3 activation might have been caused by PIBF-induced HB-EGFF **(Fig. 29.)**.

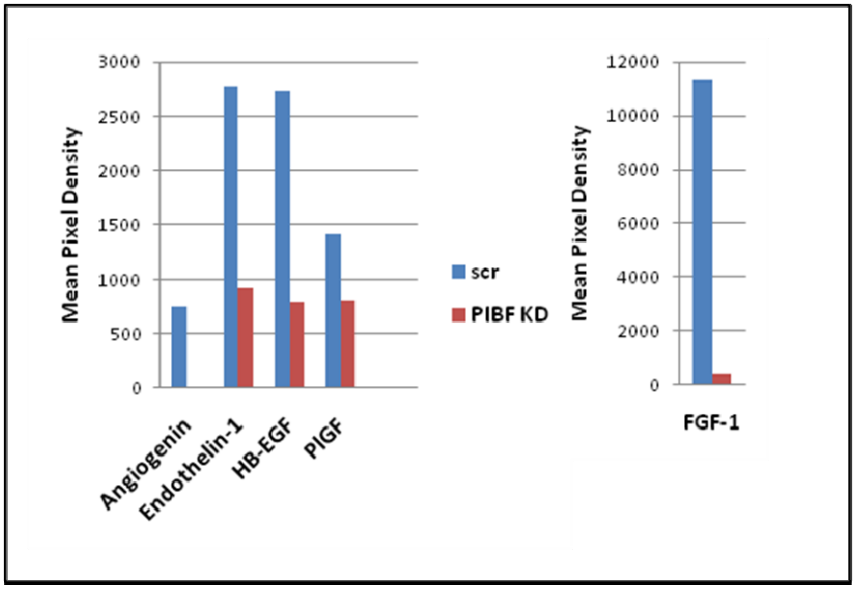

FIGURE 28. After silencing of PIBF, HT-1080 cells were analysed by protein arrays. PIBF induced angiogenin, endothelin-1, HB-EGF, PlGF, FGF-1 production. A representative experiment is shown.

IL-6 is also an important activator of STAT3 signaling pathway. Therefore, secreted IL-6 was measured in the supernatants of PIBF silenced trophoblast and fibrosarcoma cells by cytometric bead array. IL-6 production was increased in PIBF knock down trophoblast cells compared to the control sample, whereas IL-6 secretion of PIBF-silenced HT-1080 cells was reduced, suggesting that PIBF might regulate IL-6 expression **(Fig. 30.)**.

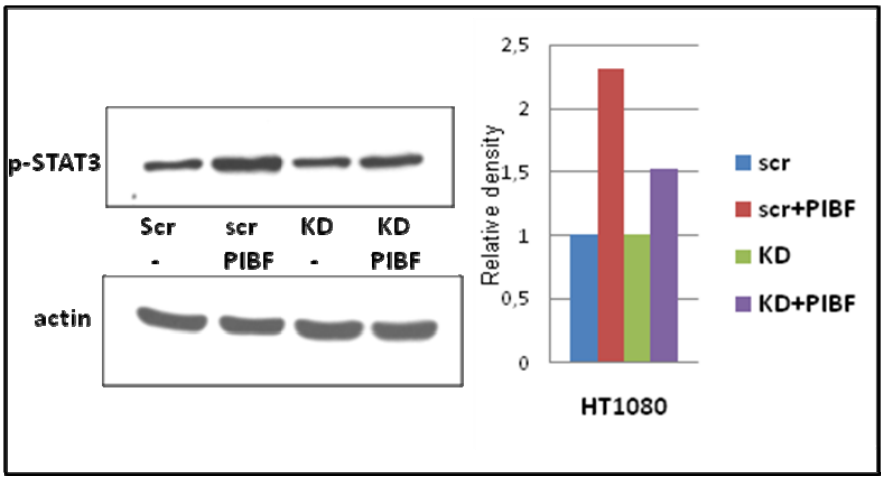

FIGURE 29. STAT3 activation by 24 h PIBF treatment in HB-EGF-silenced HT-1080 cells. Densitogram of the presented Western blots is shown. Each sample is normalized with its actin pair. A representative experiment of three is shown.

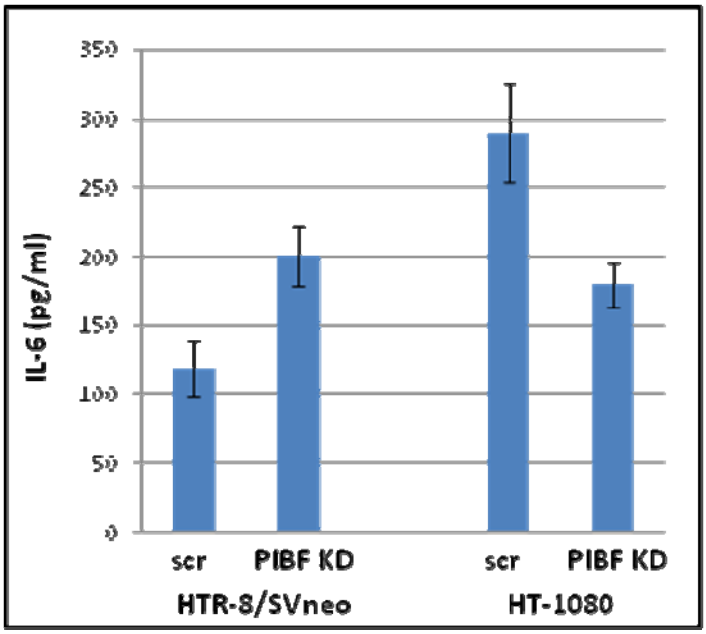

FIGURE 30. IL-6 secretion of intact and PIBF knock down trophoblast and fibrosarcoma cells. Silencing of PIBF resulted in increased IL-6 secretion in trophoblast- and decreased IL-6 secretion in tumor cells. Data are represented as mean ± SEM. (n=3/group)

We also investigated the effect of PIBF knock down on Wnt5a expression. After silencing of PIBF, the levels of Wnt5a decreased in tumor cells and increased in trophoblast cells suggesting that Wnt5a might be a PIBF-regulated gene **(Fig. 31.)**.

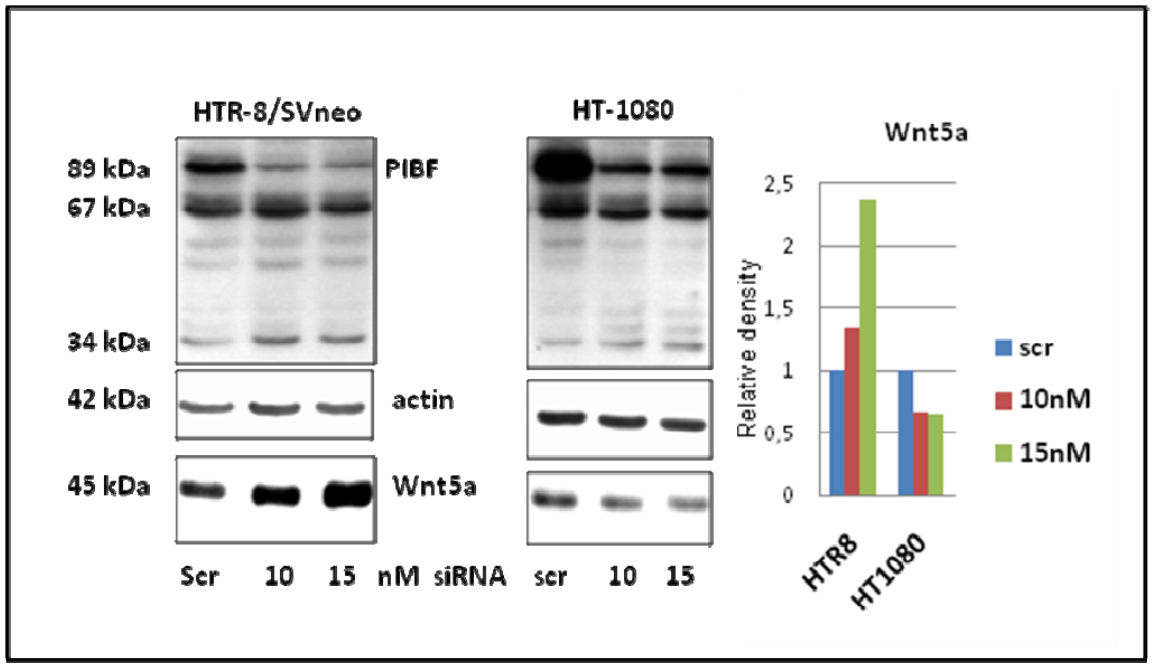

FIGURE 31. Wnt5a expression in intact and PIBF knock down trophoblast and fibrosarcoma cells. Western blots of a representative experiment are shown together with their densitogramic evaluation. Each sample is normalized with its actin pair. (scr: scrambled siRNA)

To verify that PIBF is capable to regulate the transcription of these molecules, chromatin immunoprecipitation (ChIP) was performed with anti-PIBF antibody. During testing the possible promoter-binding sites for PIBF by the primers shown in **Table 3.**, ChIP assay revealed that PIBF has the capacity to bind to the promoter of Wnt-5a and EGF both in trophoblast cells (not shown) and fibrosarcoma cells **(Fig. 32.)**.

The protein profile of the protein/DNA complex precipitated by anti-PIBF antibody was different in HTR-8/SVneo tophoblast and HT-1080 fibrosarcoma cells: in trophoblast cells the 50-kDa and 67-kDa PIBF isoforms were present in the complex while in

fibrosarcoma cells the complex also included the full-length PIBF isoform **(Fig. 33.)** suggesting that the composition of the transcription complexes are different in trophoblast and tumor cells and regulate gene expression in a tissue specific manner.

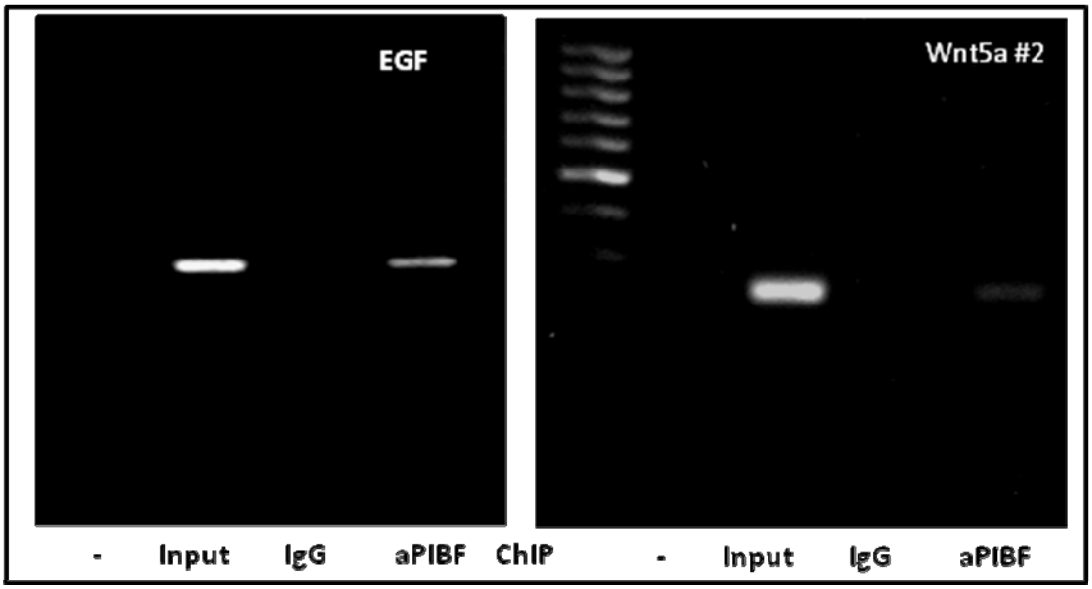

FIGURE 32. Chromatin Immunoprecipitation from HT-1080 cells shows that PIBF might bind to the promoter region of EGF and Wnt5a genes.

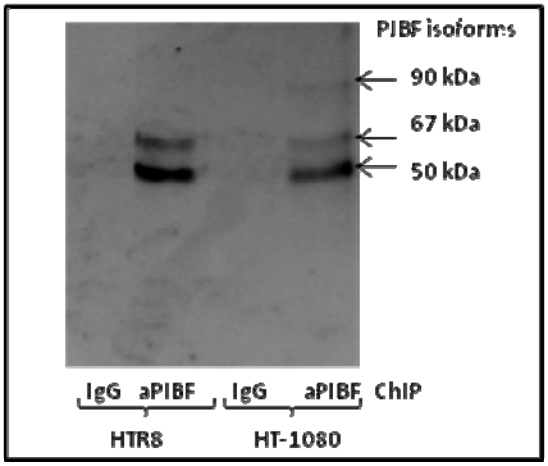

FIGURE 33. Western blot analysis of protein complex immunoprecipitated by anti-PIBF (ChIP). In trophoblast cells the complex contains the 50- and 67-kDa PIBF isoforms, while in tumor cells the 90-kDa isoform is also included in the complex.

VI. DISCUSSION

During the first trimester of pregnancy, trophoblast cells invade the uterine tissue and vessels in an invasive and eroding manner and establish the foundation of human pregnancy and foetal development. Invasive trophoblasts anchore the placenta to the uterine wall, modulate implantation as well as secretion of hormones and cytokines and most importantly play a crucial role in remodelling of maternal spiral arteries. The latter is required to increase blood flow to the placenta to provide sufficient oxygen and nutrient supply for the developing fetus **[131]**.

Invasiveness is a common feature of trophoblast and malignant tumors. Embryo implantation is similar to the growth of cancer cells. Similarly to tumor invasion of the host tissue, trophoblast invasion of the uterus is a multi-step process. It involves attachment of the trophoblast cells to the extracellular matrix (ECM) components, degradation of the ECM and migration through the eroded connective tissue. However, unlike tumor invasion, trophoblast invasion is precisely regulated. It is limited in space and only possible during the implantation window (6^{th} - 9^{th} day after fertilization in humans).

Among others, progesterone and progesterone-induced genes possess a crucial role during implantation via governing trophoblast invasion. Progesterone decreases invasion and gelatinase expression in early first trimester trophoblast cells **[132].**

Progesterone-Induced Blocking Factor was first described as a progesterone-induced molecule that mediates the immunological effects of progesterone. Recently, PIBF has also been found in rapidly proliferating cells, malignancies and in the trophoblast. This raised the question wheather PIBF is involved in regulation of invasion. To investigate this, we used an extravillosus trophoblast cell line (HTR-8/SVneo) and tumor cell lines (e.g. HT-1080) as model.

PIBF-silenced HTR-8/SVneo cells showed increased invasivity together with increased MMP-9, MMP-2 and decreased TIMP-1 activation while knocking down of PIBF in the tumor cell line resulted in decreased invasive potential as well as decreased MMP-9, MMP-2 and increased TIMP-1 activity **(Fig.34.)**. These findings confirm that PIBF is involved in invasion and matrix remodelling by altering the activity of gelatinases (MMP-9

and MMP-2). The data also suggest a different role of PIBF in trophoblast and tumor invasion.

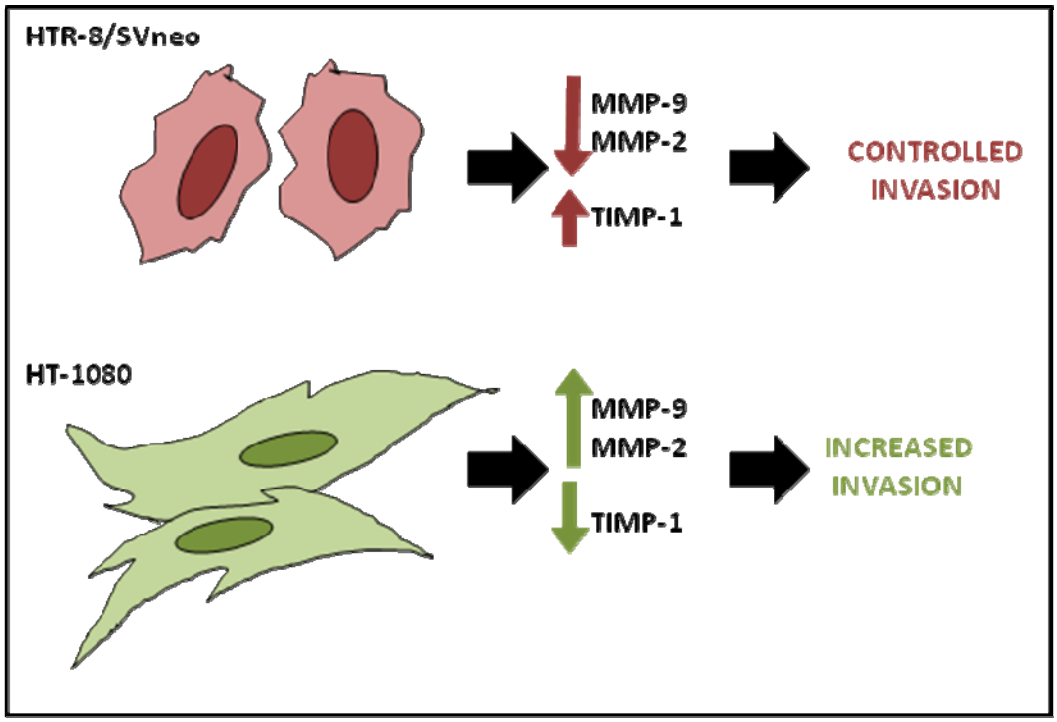

FIGURE 34. The role of PIBF in regulation of invasion. In trophoblast cells PIBF is responsible for decreased production of MMP-9, MMP-2 and increased production of TIMP-1, suggesting its role in controlling trophoblast invasion. PIBF induces MMP-9, MMP-2 activity and inhibits TIMP-1 expression in fibrosarcoma cells, thus facilitates invasion.

These data are in line with the finding that progesterone suppresses the production of MMP-9 during the implantation period. To explore the underlying mechanisms, we first characterized the PIBF-receptor.

Both HTR-8/SVneo and HT-1080 cells express PIBF-receptors on their surface and secrete PIBF, thus autocrine regulation of PIBF is possible. Many transformed cells continuously produce both growth factors and their receptors, thereby providing themselves an auto-stimulatory growth impulse. Autocrine regulation of the human trophoblast has also been described, for instance, via the VEGF/VEGF-receptor system. **[66, 72]**.

We showed that the PIBF-receptor is a GPI-anchored, lipid-raft associated protein that can form a heterodimer with IL-4Rα and uses the intracellular domain of the latter for signaling.

IL-4 is able to induce Akt **[133-135]** and ERK **[136-138]** and these molecules might be involved in invasion and tumorigenesis **[139-140]** thus we tested wheather PIBF can activate these pathways. In trophoblast cells PIBF activated Akt and ERK molecules transiently (at 20 min), while in the tested tumor cells sustained Akt and ERK phosphorylation was observed.

STAT3 phosphorylation was also investigated in these cells since it is a key player in invasion **[87-90]**. In trophoblast cells Ser-phosphorylation of STAT3 was inhibited after PIBF treatment, while in tumor cells we experienced late activation of STAT3 (both Ser and Tyr phosphorylation) transcription factors after 6 and 24 hours PIBF treatment.

Wnts have a role in cancer progression, furthermore, they are implicated in implantation and early trophoblast developement as well as in pathogenesis of trophoblastic diseases. Recent studies reported that various Wnts (Wnt5a, Wnt11) are detectable in the pre-implanting embryo and a shift was demonstrated from non-canonical signaling in the pre-implantation period towards canonical signaling in activated blastocysts during implantation **[141]**.

Dickkopf-related protein-1 (Dkk1), a major secreted Wnt signaling antagonist is up-regulated by progesterone in the endometrium during the implantation window, furthermore, progesterone-dependent induction of Dkk1 inhibited Wnt signaling **[142-143]**, suggesting that repression of the pathway plays a role in decidualisation.

Wnt5a has distinct roles in development and tissue homeostasis. Recent data point to a critical role of Wnt5a in malignant progression, but its role is controversial: Loss of Wnt5a signaling is related to development of lymphoid malignancies, whereas constitutively active Wnt5a signaling is involved in invasion or metastasis of several cancers **[127, 144]**.

Wnt5a is thought to primarily signal through non-canonical pathways. The Wnt5a/PKC pathway mediates motility and initiates an epithelial to mesenchymal transition **[145]**.

In tumor cells, 6h PIBF treatment resulted in Wnt5a activation together with reduced β-catenin levels, suggesting that the canonical Wnt pathway is inhibited. Moreover, 6 hours PIBF treatment increased PKCζ and PKCδ phosphorylation. PKCζ is known to be involved in non-canonical Wnt5a signal transduction.

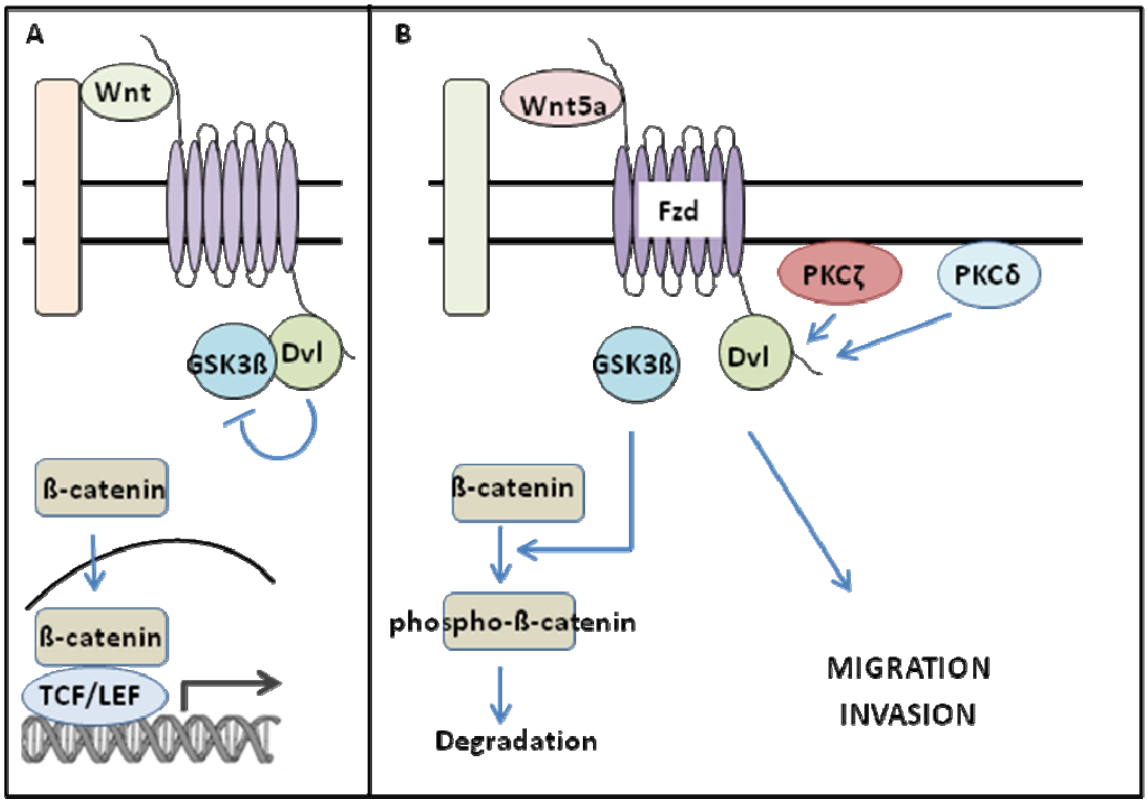

FIGURE 35. A: Canonical Wnt signaling pathway. In the presence of the Wnt molecule, Dvl inhibits GSK3β and β-catenin translocates to the nucleus and induces the transcription of the target genes. **B:** PIBF induced non-canonical Wnt5a signaling pathway. A possible signaling event in HT-1080 fibrosarcoma cells: 6h PIBF treatment induces Wnt5a and phosphorylation of PKCδ and PKCζ while β-catenin is degraded. Wnt5a supports invasion via non-canonical pathways with the help of PKCζ and PKCδ.

PKCδ might be associated with Wnt5a signaling or apoptosis. Nuclear localisation of PKCδ is required for its apoptotic function while membrane-association of PKCδ shows its involvement in Wnt5a signaling **[129, 130]**. To assess the function of PKCδ in PIBF induced signaling, PIBF-treated fibrosarcoma cells were analyzed for PKCδ localization by

confocal microscopy. After 6 h PIBF treatment, PKCδ was found to be membrane-associated suggesting its role in Wnt5a induced signaling. Moreover, 6 h PIBF treatment did not result in early signs of apoptosis (Annexin staining).

In summary, PIBF induced Wnt5a inhibits the canonical Wnt pathway by promoting degradation of β-catenin and signals via non-canonical mechanisms in which PKCζ and PKCδ are involved **(Fig.35.)**.

The sustained activation of Akt and Erk, the late STAT3 phosphorylation and Wnt5a activation in tumor cells suggest that in addition to the receptor-mediated effects of PIBF there might be other mechanisms with which PIBF participates in invasion.

Since PIBF possesses NLS, LeuZip and bZIP sequences, we analysed the subcellular localisation of PIBF. PIBF was present not only in the cytoplasm but also in the nucleus thus it is conceivable that PIBF induces genes which might be responsible for the experienced sustained and late signaling events in case of tumor cells.

Lysates of PIBF-silenced trophoblast and fibrosarcoma cells were analysed with protein arrays for 55 invasion and angiogenesis related molecules. In PIBF knock down and control trophoblast cells there were no significant alterations between the levels of the tested invasion and angiogenesis related proteins.

In PIBF silenced fibrosarcoma cells the expression of bFGF, HB-EGF, PlGF, angiogenin and endothelin-1 were reduced, supporting the hypothesis that PIBF might induce the genes of these growth factors and angiogenesis associated molecules.

In order to confirm, that late PIBF signaling was indeed due to gene induction, HB-EGF knock down fibrosarcoma cells were created. In these cells the STAT3 activating effect of PIBF was reduced compared to the control. Moreover, IL-6 secretion and Wnt5a production of PIBF knock down HT-1080 cells were reduced compared to the control sample. In contrast to this, PIBF knock down in trophoblast cells resulted in increased IL-6 production together with increased Wnt5a and leptin-receptor expression.

Finally, chromatin immunoprecipitation with anti-PIBF antibody showed that PIBF could bind to the promoter of Wnt5a and EGF both in trophoblast and tumor cells. However, while in trophoblast cells the promoter-binding complex contains the 50-kDa and 67-kDa PIBF isoforms, in tumor cells the full length PIBF is also included in the

transcription complex **(Fig.36.)**. The differential effect of PIBF might be due to this difference.

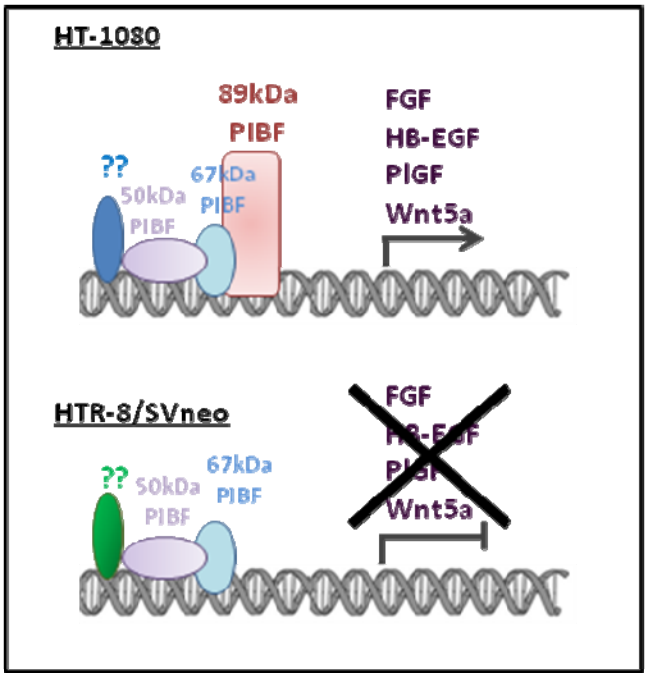

FIGURE 36. PIBF binds to the promoter regions of certain genes. In fibrosarcoma cells PIBF induces FGF, HB-EGF, PlGF and Wnt-5a while in trophoblast cells there is no gene induction.

In conclusion, in tumor – but not in trophoblast – cells PIBF promotes invasion by gene activation, e.g., that of EGF, IL-6, Wnt5a. The secreted proteins (IL-6, EGF) bind then to their own receptors and induce Akt, ERK and STAT3 phosphorylation which in turn further activates transcription of invasion promoting molecules (e.g. MMP-9, MMP2). **(Fig.37.)**

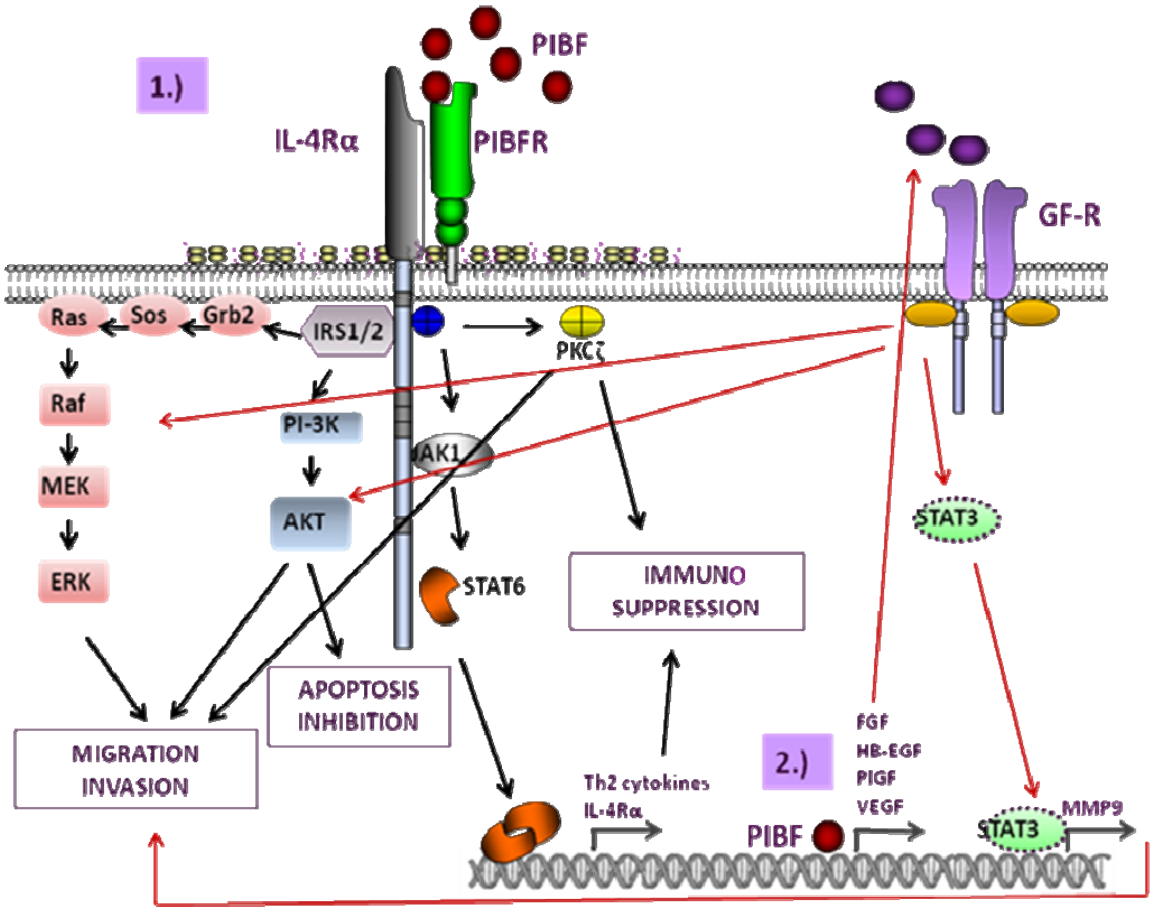

FIGURE 37. Signaling pathways that lead to PIBF-induced invasion in HT1080 fibrosarcoma cells.

Based on our findings, it can not be ruled out, that PIBF suppresses the transcription of leptin-receptor, IL-6 and Wnt5a genes in trophoblast cells and as an intrinsic control molecule negatively regulates trophoblast invasion. In line with this, in choriocarcinoma, the loss of PIBF results in increased invasive behaviour since the genes (leptin-receptor, IL-6, Wnt5a) normally suppressed by PIBF will be transcribed **(Fig.38.)**.

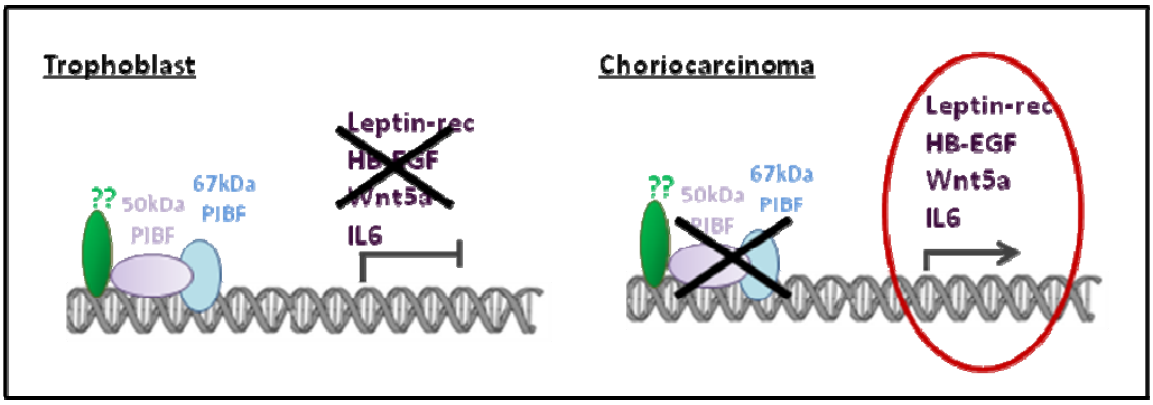

FIGURE 38. In trophoblast cells PIBF might suppress the genes of leptin-receptor, HB-EGF, Wnt5a and IL-6. In the absence of PIBF-mediated inhibition these genes will be activated.

Our data provide evidence that PIBF is not only responsible for modulating the immunological effects of progesterone but it also has a vital role in tumor invasion as well as in controlling trophoblast invasion at a physiological level. Invasion is known to be closely associated with angiogenesis. Preliminary data from our laboratory suggest that PIBF might affect the production of angiogenic factors (e.g. VEGF, angiogenin etc.) and in lymphocytes increases the expression and phosphorylation of tumor suppressor molecules (e.g. p53). The precise signaling events of these processes need to be further investigated.

These findings might be implemented in therapeutical approaches. Designing receptor agonists might be a promising anti-abortive therapy in those pregnancies which are characterized by low PIBF levels and receptor antagonists or PIBF inhibitors might be useful in the treatment of PIBF-positive tumors.

VII. SUMMARY OF THESES

I. PIBF is implicated in the regulation of both trophoblast and tumor invasion.

II. In trophoblast cells PIBF down-regulates the expression of the pro-invasive leptin and its receptor, suggesting that PIBF might be involved in the control of trophoblast invasion.

III. In the normal trophoblast → partial mole → complete mole → choriocharcinoma transition there is a shift towards uncontrolled invasive behaviour characterized by gradual loss of PIBF and an increasing leptin/leptin-receptor expression pattern, suggesting an inverse relationship between PIBF and leptin/leptin-receptor expression.

IV. In trophoblast, the lack of PIBF is associated with increased invasion, suggesting that PIBF regulates the physiological trophoblast invasion. In HT-1080 tumor cells PIBF knock down results in restricted invasion suggesting that PIBF might facilitate invasive behaviour of tumor cells.

V. PIBF takes part in matrix remodelling by altering the activity of MMP-9 and MMP-2 matrix metalloproteinases. In trophoblast cells, silencing of PIBF induces the secretion of gelatinases (i.e. MMP-9 and MMP-2) and inhibits TIMP-1 expression, while in fibrosarcoma cells downregulation of PIBF results in decreased gelatinase activity together with increased TIMP-1 level.

VI. HTR-8/SVneo and HT-1080 cells secrete PIBF and express PIBF-receptors on their surface, thus autocrine regulation of PIBF is a possibility.

VII. PIBF is capable to activate the Akt and ERK pathways via binding to the PIBF-receptor/IL-4Rα heterocomplex in both trophoblast and fibrosarcoma cells.

VIII. In trophoblast cells the effect of PIBF on Akt and ERK phosphorylation is transient, while in fibrosarcoma cells PIBF induces sustained activation of these molecules.

IX. In trophoblast cells PIBF inhibits Wnt5a activation and Ser-phosphorylation of STAT3 and it does not alter STAT3-Tyr phosphorylation.

X. In HT-1080 tumor cells PIBF induces late activation of Wnt5a and late phosphorylation of STAT3 (both Tyr and Ser) molecules.

XI. In HT-1080 cells after 6 h PIBF treatment the amount of β-catenin is reduced while PKCζ and PKCδ isoforms are activated together with Wnt5a, suggesting that Wnt5a inhibits the canonical pathway and might use these two PKC isoforms for signaling.

XII. In trophoblast and HT-1080 tumor cells PIBF is present in the nucleus.

XIII. In fibrosarcoma cells PIBF might activate molecules involved in invasion and angiogenesis (e.g. FGF-1, HB-EGF, IL-6, PlGF, endothelin and angiogenin etc.) at the gene level as a transcription factor.

XIV. PIBF is capable to bind specifically to certain promoter regions, among others, to the promoter of Wnt5a and EGF in both trophoblast and fibrosarcoma cells.

XV. Silencing of PIBF results in reduced Wnt5a and IL-6 production in fibrosarcoma cells. In contrast to this, Wnt5a and IL-6 expression is increased in PIBF knock down trophoblast cells. These data suggest that PIBF might induce the transcription of Wnt5a and IL-6 genes in fibrosarcoma cells, while PIBF might suppress the transcription of Wnt5a and IL-6 genes in trophoblast cells.

XVI. The differential effect of PIBF might reside in the composition of the protein complex which binds to the promoter region of the above mentioned genes. While in trophoblast cells the promoter-binding complex contains the 50-kDa and 67-kDa PIBF isoforms, in tumor cells the full length PIBF is also included in the transcription complex.

VIII. ACKNOWLEDGEMENTS

I would like to dedicate this work to my **Parents** who have supported me all through my life and made it possible to fulfil my dreams.

I would like to emphasize my gratitude to my tutor, **Professor Júlia Szekeres-Barthó** for directing this project and providing me with the opportunity to carry out this work. I gratefully acknowledge all her support, attention and time as well as the freedom in research I have got from her.

I owe my deep gratitude to **Beáta Polgár** for her continuous support, for teaching me, for always being there whenever I had a question and for her friendship.

I am grateful to **László Szereday** for all his support and useful advices.

I also thank **Nadia Halidi** and **Gergely Berta** for their precious time and assistance with the confocal microscope.

I am grateful to **Judit Pongrácz** for her useful advices in connection with Wnt5a signaling and for the anti-β-catenin antibody.

I thank **Charles H. Graham** for providing us the HTR-8/SVneo trophoblast cell line.

I also thank **Noémi Kozma** for teaching and helping me during her Ph.D. fellowship in our laboratory.

I am grateful to **Ágnes Kiss**, **Éva Molnár** and **Nóra Garamvölgyi** for introducing the cell culturing and basic immunological techniques to me when I first visited our laboratory from high school.

I am thankful to everyone who is not listed above but helped me in my research.

Finally, I am grateful to my **Family** and my **Husband** for supporting, helping and forming me.

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X. BIBLIOGRAPHY

Impact factor of published papers: ***13.074***
Impact factor of published abstracts: ***43.346***

1. Papers Related to Project

1.) Progesterone-induced blocking factor (PIBF) and trophoblast invasiveness
E Miko*, M Halasz*, B Jericevic-Mulac, L Wicherek, P Arck, G Arato, J Skret Magierlo, D Rukavina, J Szekeres-Bartho
(Joint first authors: *)
Journal of Reproductive Immunology (2011); Published online 25 May, 2011
Impact Factor: 2.519

2.) Progesterone in pregnancy: receptor-ligand interaction, signaling pathways
J Szekeres-Bartho, M Halasz, T Palkovics
Journal of Reproductive Immunology (2009); 83(1-2):60-4.
Impact Factor: 2.519

3.) The Progesterone-Induced Blocking Factor modulates the balance of PKC and intracellular Ca^{++}
N Kozma, M Halasz, T Palkovics, J Szekeres-Bartho
American Journal of Reproductive Immunology (2006); 55: 122-129.
Impact Factor: 1.743

4.) Progesterone-Induced Blocking Factor activates STAT6 via binding to a novel IL-4 receptor
N Kozma, M Halasz, B Polgar, TG Poehlmann, UR Markert, T Palkovics, M Keszei, G Par, K Kiss, J Szeberenyi, L Grama, J Szekeres-Bartho
The Journal of Immunology (2006); 176(2): 819-826.
Impact Factor: 6.293

2. Book Chapters Related to Project

1.) Immuno-Endocrine Signals
J Szekeres-Bartho, N Kozma, M Halasz, B Polgar, E Miko, T Palkovics, L Szereday
EMBIC Handbook (2006); 95-102.

3. Published Abstracts Related to Project

1.) PIBF regulates trophoblast invasion
J Szekeres-Bartho, M Halasz, E Miko, B Polgar, T Palkovics
Journal of Reproductive Immunology (2010); 86(2): 105. (IF:2.519)

2.) What harbours the cradle of life? The progesterone-dependent immunomodulation
M Halasz, B Polgar, N Kozma, T Berki and J Szekeres-Bartho
Yakhteh Medical Journal (2009); 11(Suppl.1): 34-35.

3.) The key and the lock: Characterization of the receptor-binding part of Progesterone-Induced Blocking Factor (PIBF)
M Halasz, B Polgar, N Kozma, G Berta, G Toth and J Szekeres-Bartho
European Journal of Immunology (2009); 39(Suppl.1): S710. (IF: 4.662)

4.) Identifying the receptor-binding part of PIBF
M Halasz, B Polgar, N Kozma, G Berta, G Toth, J Szekeres-Bartho
American Journal of Reproductive Immunology (2008); 60(1):88. (IF: 2.172)

5.) Expression and silencing of Progesterone Induced Blocking Factor in Jar choriocarcinoma cells
C Ermisch, M Halasz, TG Poehlmann, M Weber, J Szekeres-Bartho, UR Markert
Placenta (2008); 29(8):A61-A61. (IF: 2.775)

6.) A Molecule of Challenge: Hunting for PIBF Receptor
B Polgar, A Marki, N Halidi, M Halasz, N Kozma, T Palkovics, J Szekeres-Bartho
American Journal of Reproductive Immunology (2007); 58(3):197. (IF: 2.13)

7.) Progesterone-Dependent Immunomodulation
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8.) Genomic bases of progesterone-dependent immunomodulation.
J Szekeres-Bartho, B Polgar, E Nagy, E Miko, N Kozma, T Palkovics, O Papp, M Halasz
Tissue Antigens (2004); 64:356-357. (IF: 1.990)

4. Presentations Related to Project

4.1. Oral presentations

1.) Annual Meeting of the Austrian Society for Allergology and Immunology
Two sides of the same coin: The role of Progesterone-Induced Blocking Factor (PIBF) in trophoblast and tumor invasion
M Halasz, B Polgar, G Berta, J Szekeres-Bartho
December 3-5, 2010; Vienna, Austria

2.) 38th Congress of the Hungarian Society for Immunology
Two sides of the same coin: The role of Progesterone-Induced Blocking Factor (PIBF) in trophoblast and tumor invasion
M Halasz, B Polgar, G Berta, J Szekeres-Bartho
November 3-5, 2010; Szeged, Hungary

3.) 8th European Congress on Reproductive Immunology
PIBF regulates trophoblast invasion
J Szekeres-Bartho, M Halasz, E Miko, B Polgar, T Palkovics
November 11-13, 2010; Munich, Germany

4.) International Symposium for Immunology of Reproduction (ISIR)
Progesterone and immune-endocrine cross-talk in pregnancy
J Szekeres-Bartho, M Halasz, B Polgar
August 28-29, 2010; Icho Kaikan, Osaka, Japan

5.) Royan International Twin Congress
10th Congress on Reproductive Biomedicine, 5th Congress on Stem Cell Biology and Technology
What harbours the cradle of life? The Progesterone-Dependent Immunomodulation
M Halasz, B Polgar, N Kozma, T Berki, J Szekeres-Bartho
Sep 23-25, 2009; Tehran, Iran

6.) 37th Congress of the Hungarian Society for Immunology
Tales of the unexpected: Expression of the PIBF receptor
B Polgar, M Halasz, G Berta, A Marki, G Toth, T Palkovics, J Szekeres-Bartho
Oct 29-31, 2008; Budapest, Hungary

7.) 36th Congress of the Hungarian Society for Immunology
A Molecule of Challenge: Hunting for PIBF Receptor
B Polgar, A Marki, N Halidi, M Halasz, N Kozma, T Palkovics, J Szekeres-Bartho
Oct 17-19, 2007; Hajduszoboszlo, Hungary

8.) 5th European Congress of Reproductive Immunology
Progesterone-Dependent Immunomodulation
J Szekeres-Bartho, B Polgar, M Halasz, N Kozma, E Miko, T Palkovics, A Barakonyi, L Szereday
Aug 30 – Sep 2, 2007; Berlin, Germany

9.) 5th European Congress of Reproductive Immunology
A Molecule of Challenge: Hunting for PIBF Receptor
B Polgar, A Marki, N Halidi, M Halasz, N Kozma, T Palkovics, J Szekeres-Bartho
Aug 30 – Sep 2, 2007; Berlin, Germany

10.) X. International Congress of Reproductive Immunology
PIBF signaling in decidual cells
J Szekeres-Bartho, A Bogdan, M Halasz, N Kozma, U Markert, E Miko, T Palkovics, B Polgar, T Poehlmann
June 10-14, 2007; Opatija, Croatia

11.) 35th Congress of the Hungarian Society for Immunology
Is PIBF the ligand of a novel type of Il-4 receptor?
M Halasz, N Kozma, B Polgar, N Halidi, L Grama, J Szekeres-Bartho
Oct 19-22, 2005; Sopron, Hungary

12.) 13th Symposium on Signals and Signal Processing in the Immune System
PIBF is the ligand of a novel type of IL-4-receptors
M Halasz, N Kozma, B Polgar, NHalidi, L Grama, M Nyitrai, B Somogyi, J Szekeres-Bartho
Sep 7-11, 2005; Balatonoszod, Hungary

13.) 1st Embic Summer School: Embryo implantation: from basics to clinics.
PIBF is the ligand of a novel type of IL-4 receptors
M Halasz, N Kozma, B Polgar, T Palkovics, N Halidi, L Grama, M Nyitrai, B Somogyi, J Szekeres-Bartho
June 4-10, 2005; Malinska, Croatia

14.) 1st Embic Summer School: Embryo implantation: from basics to clinics.
Role of progesterone in the immuno-endocrine control of successful pregnancy
J Szekeres-Bartho, M Halasz, N Kozma, E Miko, T Palkovics, B Polgar, L Szereday, P Varga
June 4-10, 2005; Malinska, Croatia

15.) 26th Students' International Scientific Conference
PIBF is the third type of IL-4 receptors
April 26, 2005 - Jessenius Faculty of Medicine, Comenius University, Martin, Slovakia

16.) XXVII. Students' National Scientific Conference
PIBF (Progesterone Induced Blocking Factor) as a ligand of a third type IL-4 receptor
March 22, 2005 - Medical School, University of Szeged, Szeged, Hungary

17.) 1st International Conference on Basic and Clinical Immunogenomics
Genomic bases of progesterone-dependent immunomodulation
J Szekeres-Bartho, B Polgar, E Nagy, E Miko, N Kozma, T Palkovics, O Papp, M Halasz
Oct 3-7., 2004; Budapest, Hungary

4.2. Poster presentations

1.) 2nd European Congress of Immunology
The key and the lock: Characterization of the receptor-binding part of Progesterone-Induced Blocking Factor (PIBF)
M Halasz, B Polgar, N Kozma, G Berta, G Toth, J Szekeres-Bartho
Sep 13-16, 2009; Berlin, Germany

2.) 37th Congress of the Hungarian Society for Immunology
Identifying the receptor-binding part of PIBF
M Halasz, B Polgar, N Kozma, G Berta, G Toth, J Szekeres-Bartho
Oct 29-31, 2008; Budapest, Hungary

3.) 14th Meeting of the International Federation of Placental Associations/12th Meeting of the European Placenta Group (IFPA/EPG)
Expression and silencing of Progesterone Induced Blocking Factor in Jar choriocarcinoma cells
C Ermisch, M Halasz, TG Poehlmann, M Weber, J Szekeres-Bartho, UR Markert
Sep 10-13, 2008; Seggau Castle, Austria

4.) 4th EMBIC Summer School: Advances in embryo implantation and pregnancy.
Tales of the unexpected: The mysterious PIBF receptor
B Polgar, M Halasz, G Berta, A Marki, G Toth, T Palkovics, J Szekeres-Bartho
June 2-6, 2008; Barcelona, Spain

5.) 4th EMBIC Summer School: Advances in embryo implantation and pregnancy.
Analysis of PIBF expression in Jar cells and its silencing by RNA interference
C Ermisch, M Halasz, M Weber, TG Poehlmann, J Szekeres-Bartho, UR Markert
June 2-6, 2008; Barcelona, Spain

6.) 4th EMBIC Summer School: Advances in embryo implantation and pregnancy.
Identifying the receptor-binding part of PIBF
M Halasz, B Polgar, N Kozma, G Berta, G Toth, J Szekeres-Bartho
June 2-6, 2008; Barcelona, Spain

7.) 1st Joint Meeting of European National Societies of Immunology
16th European Congress of Immunology
The GPI-anchored PIBF-receptor activates the JAK/STAT pathway via forming a complex with IL-4Ralpha
M Halasz, N Kozma, B Polgar, N Halidi, L Grama, J Szekeres-Bartho
Sep 6-9, 2006; Paris, France

8.) 1st Joint Meeting of European National Societies of Immunology
16th European Congress of Immunology
JAK/STAT and PKC/Calcium pathway mediating the effects of PIBF
N Kozma, M Halasz, B Polgar, T Palkovics, G Par, M Keszei, J Szekeres-Bartho
Sep 6-9, 2006; Paris, France

9.) 2nd EMBIC Summer School: Molecular mechanisms of implantation.
The GPI-anchored PIBF-receptor invokes the interleukin-4 receptor alpha for signaling
M Halasz, N Kozma, B Polgar, N Halidi, L Grama, J Szekeres-Bartho
July 1-4, 2006; Pecs, Hungary

10.) 2nd EMBIC Summer School: Molecular mechanisms of implantation.
The role of JAK/STAT and PKC/Calcium pathways in PIBF signalling
N Kozma, M Halasz, B Polgar, T Palkovics, G Par, M Keszei, J Szekeres-Bartho
July 1-4, 2006; Pecs, Hungary

11.) 35th Congress of the Hungarian Society for Immunology
PIBF effects on the JAK/STAT and PKC/Ca++ pathways
N Kozma, M Halasz, B Polgar, T Palkovics, G Par, M Keszei, J Szekeres-Bartho
Oct 19-22, 2005; Sopron, Hungary

12.) 13th Symposium on Signals and Signal Processing in the Immune System
PIBF effects on the JAK/STAT pathway depend on the IL-4 receptor
N Kozma, G Par, B Polgar, M Halasz, M Keszei, Cs Szalai, A Falus, J Szekeres-Bartho
Sep 7-11, 2005; Balatonoszod, Hungary

13.) 1st Embic Summer School: Embryo implantation: from basics to clinics.
PIBF effects on the JAK/STAT pathway depend on the IL-4 receptor
N Kozma, G Par, B Polgar, T Palkovics, M Halasz, M Keszei, Cs Szalai, A Falus, J Szekeres-Bartho
June 4-10, 2005; Malinska, Croatia

5. Awarded Lectures

1.) **2nd Prize**
Ph.D. Conference organized by the Regional Committee of the Hungarian Academy of Sciences at Pecs
2010, Pecs, Hungary

2.) **1st Prize, Royan Award in Female Infertility**
Royan International Twin Congress
10th Congress on Reproductive Biomedicine, 5th Congress on Stem Cell Biology and Technology
What harbours the cradle of life? The Progesterone-Dependent Immunomodulation
M Halasz, B Polgar, N Kozma, T Berki, J Szekeres-Bartho
Sept 23-25, 2009; Tehran, Iran

3.) **2nd Prize, Best Abstract in Basic Immunological Research by Sigma-Aldrich**
37th Congress of the Hungarian Society for Immunology
Identifying the receptor-binding part of PIBF
M Halasz, B Polgar, N Kozma, G Berta, G Toth, J Szekeres-Bartho
Oct 29-31, 2008; Budapest, Hungary

4.) **Top Selected Abstract**
4th EMBIC Summer School: Advances in embryo implantation and pregnancy.
Identifying the receptor-binding part of PIBF
M Halasz, B Polgar, N Kozma, G Berta, G Toth, J Szekeres-Bartho
June 2-6, 2008; Barcelona, Spain

5.) **Young Signaling Researcher of 2005**
13th Symposium on Signals and Signal Processing in the Immune System
PIBF is the ligand of a novel type of IL-4-receptors
M Halasz, N Kozma, B Polgar, NHalidi, L Grama, M Nyitrai, B Somogyi, J Szekeres-Bartho
Sept 7-11, 2005; Balatonoszod, Hungary

6. Additional Papers

1.) Immunology of HCV infection: the causes of impaired cellular immune response and the effect of antiviral treatment
G Par, T Berki, L Palinkas, P Balogh, L Szereday, M Halasz, J Szekeres-Bartho, A Miseta, G Hegedus, Gy Mozsik, B Hunyady, A Par
Orvosi Hetilap (2006); 147(13):591-600.

2.) Cloning of Metallothionein: A Senescence Associated Gene in Arabidopsis thaliana
M Halasz
Students' Scientific Reports (2000); p: 63-68.

7. Additional Published Abstracts

1.) *Altered surface expression of inhibitory KIR2DL3 and activating CD160, NKG2D receptors on NK and cytotoxic T cells in chronic HCV hepatitis*
G Par, L Szereday, T Berki, M Halasz, A Miseta, J Szekeres-Bartho, G Hegedus, Gy Mozsik, B Hunyady, A Par
Journal of Hepatology (2008); 48:S51. (IF:7.056)

2.) *Cytokine profiles of peripheral blood monocytes may predict rapid virological response in chronic hepatitis C*
G Par, T Berki, L Palinkas, M Halasz, L Szereday, A Miseta, Zs Faust, G Hegedus, Gy Mozsik, B Hunyady, A Par
Folia Hepatologica (2007); 11:S24.

3.) Increased Th1 cytokine production may predict rapid virological response in chronic HCV hepatitis
G Par, T Berki, L Palinkas, M Halasz, L Szereday, A Miseta, Zs Faust, G Hegedus, Gy Mozsik, B Hunyady, A Par
Hepatology International (2007); 1:137.

4.) *Pretreatment increased T helper 1 type cytokine production of peripheral blood monocytes may predict rapid virological response to PEG-IFN+RBV therapy in patients with chronic hepatitis C*
G Par, T Berki, L Palinkas, M Halasz, L Szereday, A Miseta, Zs Faust, G Hegedus, Gy Mozsik, B Hunyady, A Par
Journal of Hepatology (2007); 46:S174. (IF: 6.642)

5.) *Rapid virological response is associated with increased pretreatment Th1 type cytokine production of Toll-like receptor 4 stimulated monocytes in HCV1 patients*
G Par, T Berki, L Palinkas, M Halasz, L Szereday, A Miseta, Zs Faust, G Hegedus, Gy Mozsik, B Hunyady, A Par
German Journal of Gastroenterology (2007); 44:440. (IF: 0.632)

6.) *Transforming growth factor-beta 1 downregulates NKG2D killer activator receptor expression on cytotoxic cells in patients with chronic HCV hepatitis*
G Par, T Berki, L Palinkas, L Szereday, M Halasz, A Miseta, G Hegedus, Zs Faust, Gy Mozsik, B Hunyady, A Par
Liver International (2006); 26(S1):8. (IF: 2.344)

7.) *Increased TGFβ1 secretion via down-regulating NKG2D killer activator receptor expression results in impaired natural killer cell activity in patients with chronic HCV hepatitis*
G Par, T Berki, L Palinkas, L Szereday, M Halasz, J Szekeres-Bartho, A Miseta, G Hegedus, Zs Faust, Gy Mozsik, B Hunyady, A Par
Journal of Hepatology (2006); 44(S2): S164-165. (IF: 6.073)

8.) *Th-1 type cytokine production of the macrophages may predict the virological response of IFN in chronic hepatitis C*
G Par, A Par, T Berki, L Palinkas, Zs Faust, M Halasz, Gy Mozsik, B Hunyady
German Journal of Gastroenterology (2005); 43:505. (IF: 0.800)

9.) *Pretreatment T-helper1/T-helper2 cytokine profile may predict virological response in chronic hepatitis C patients and the effect of IFN plus ribavirin treatment*
G Par, A Par, T Berki, L Palinkas, M Halasz, Zs Faust, G Hegedus, B Hunyady
Canadian Journal of Gastroenterology (2005); 19 (Suppl C): R0746. (IF: 1.421)

8. Additional Presentations

1.) 43rd Annual Meeting of the European Association for the Study of the Liver
Altered surface expression of inhibitory KIR2DL3 and activating CD160, NKG2D receptors on NK and cytotoxic T cells in chronic HCV hepatitis
G Par, L Szereday, T Berki, M Halasz, A Miseta, J Szekeres-Bartho, G Hegedus, Gy Mozsik, B Hunyady, A Par
April 23-27, 2008; Milan, Italy

2.) 49th Annual Meeting of Hungarian Society of Gastroenterology
Rapid virological response is associated with increased pretreatment Th1 type cytokine production of Toll-like receptor 4 stimulated monocytes in HCV1 patients
G Par, T Berki, L Palinkas, M Halasz, L Szereday, A Miseta, Zs Faust, G Hegedus, Gy Mozsik, B Hunyady, A Par
June 1-6, 2007; Tihany, Hungary

3.) 6th Congress of European Federation of Internal Medicine (EFIM)
Cytokine profiles of monocytes predict rapid virological response to PEG-IFN+RBV therapy in patients with chronic hepatitis C
G Par, T Berki, L Palinkas, M Halasz, L Szereday, A Miseta, Zs Faust, G Hegedus, Gy Mozsik, B Hunyady, A Par
May 23-26, 2007; Lisboa, Portugal

4.) 42nd Annual Meeting of European Association of the Study of the Liver (EASL)
Pretreatment increased T helper 1 type cytokine production of peripheral blood monocytes may predict rapid virological response to PEG-IFN+RBV therapy in patients with chronic hepatitis C
G Par, T Berki, L Palinkas, M Halasz, L Szereday, A Miseta, Zs Faust, G Hegedus, Gy Mozsik, B Hunyady, A Par
April 11-15, 2007; Barcelona, Spain

5.) Congress of Asian and Pacific Association of the Study of the Liver (APASL)
Increased Th1 cytokine production may predict rapid virological response in chronic HCV hepatitis
G Par, T Berki, L Palinkas, M Halasz, L Szereday, A Miseta, Zs Faust, G Hegedus, Gy Mozsik, B Hunyady, A Par
March 26-29, 2007; Kyoto, Japan

6.) Congress of the Hungarian Society for Hepatology
Cytokine profiles of peripheral blood monocytes may predict rapid virological response in chronic hepatitis C
G Par, T Berki, L Palinkas, M Halasz, L Szereday, A Miseta, Zs Faust, G Hegedus, Gy Mozsik, B Hunyady, A Par
Feb 20-24, 2007; Bukfurdo, Hungary

7.) 4th Central European Gastroenterology Meeting
TGF-beta 1 downregulates NKG2D killer activator receptor expression on cytotoxic cells in patients with chronic hepatitis C
G Par, T Berki, L Palinkas, L Szereday, M Halasz, A Miseta, G Hegedus, Zs Faust, Gy Mozsik, B Hunyady, A Par
June 29 - July2, 2006; Visegrad, Hungary

8.) 41st Annual Meeting of European Association of the Study of the Liver (EASL)
Increased TGF-beta1 secretion via down-regulating NKG2D killer activator receptor expression results in impaired natural killer cell activity in patients with chronic HCV hepatitis
G Par, T Berki, L Palinkas, L Szereday, J Szekeres-Bartho, M Halasz, A Miseta, Zs Faust, G Hegedus, Gy Mozsik, B Hunyady, A Par
26-30 April 2006; Wien, Austria

9.) World Congress of Gastroenterology
Pretreatment T-helper1/T-helper2 cytokine profile may predict virological response in chronic hepatitis C patients and the effect of IFN plus ribavirin treatment
G Par, A Par, T Berki, L Palinkas, M Halasz, Zs Faust, G Hegedus, B Hunyady
Sep 10-13, 2005; Montreal, Canada

10.) 52nd Annual Meeting of the Hungarian Society for Internal Medicine
IFN és ribavirin terápia hatása a Th1/Th2 cytokin profilra krónikus C hepatitisben
G Par, T Berki, L Palinkas, M Halasz, Zs Faust, G Hegedus, Gy Mozsik, A Par, B Hunyady
June 23-25, 2005; Bukfurdo, Hungary.

11.) 47th Annual Meeting of the Hungarian Society of Gastroenterology
Th-1 type cytokine production of the macrophages may predict the virological response of IFN in chronic hepatitis C
G Par, A Par, T Berki, L Palinkas, Zs Faust, M Halasz, Gy Mozsik, B Hunyady
June 7-11, 2005; Balatonaliga, Hungary

XI. PAPERS

Paper 1

Progesterone -induced blocking factor (PIBF) and trophoblast invasiveness
E Miko*, M Halasz*, B Jericevic-Mulac, L Wicherek, P Arck, G Arato, J Skret Magierlo, D Rukavina, J Szekeres-Bartho
(Joint first authors: *)
Journal of Reproductive Immunology (2011); Published online 25 May, 2011
Impact Factor: 2.519

Paper 2

Progesterone in pregnancy: receptor-ligand interaction, signaling pathways
J Szekeres-Bartho, M Halasz, T Palkovics
Journal of Reproductive Immunology (2009); 83(1-2):60-4.
Impact Factor: 2.519

Paper 3

Progesterone-Induced Blocking Factor activates STAT6 via binding to a novel IL-4 receptor
N Kozma, M Halasz, B Polgar, TG Poehlmann, UR Markert, T Palkovics, M Keszei, G Par, K Kiss, J Szeberenyi, L Grama, J Szekeres-Bartho
The Journal of Immunology (2006); 176(2): 819-826.
Impact Factor: 6.293