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PIM kinases 1, 2 and 3 in intracellular LIF signaling, proliferation and apoptosis in trophoblastic cells

Stella Mary Photini[a], Wittaya Chaiwangyen[a,b], Maja Weber[a], Boodor Al-Kawlani[a], Rodolfo R. Favaro[a,c], Udo Jeschke[d], Ekkehard Schleussner[a], Diana M. Morales-Prieto[a], Udo R. Markert[a,*,1]

[a] Placenta-Lab, Department of Obstetrics, Jena University Hospital, Am Klinikum 1, 07747 Jena, Germany
[b] School of Medical Sciences, University of Phayao, Phayao 56000, Thailand
[c] Laboratory of Reproductive and Extracellular Matrix Biology, Department of Cell and Developmental Biology, Institute of Biomedical Sciences, University of São Paulo, Brazil
[d] Ludwig Maximilians University of Munich, Department of Obstetrics and Gynecology, Maistrasse 11, 80337 Munich, Germany

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ABSTRACT

Proviral insertion in murine (PIM) lymphoma proteins are mainly regulated by the Janus Kinase/Signal Transducer Activator of Transcription (JAK/STAT) signaling pathway, which can be activated by members of the Interleukin-6 (IL-6) family, including Leukemia Inhibitory Factor (LIF). Aim of the study was to compare PIM1, PIM2 and PIM3 expression and potential cellular functions in human first and third trimester trophoblast cells, the immortalized first trimester extravillous trophoblast cell line HTR8/SVneo and the choriocarcinoma cell line JEG-3. Expression was analyzed by qPCR and immunochemical staining. Functions were evaluated by PIM inhibition followed by analysis of kinetics of cell viability as assessed by MTS assay, proliferation by BrdU assay, and apoptosis by Western blotting for BAD, BCL-XL, (cleaved) PARP, CASP3 and c-MYC. Apoptosis and necrosis were tested by flow cytometry (annexin V/propidium iodide staining). All analyzed PIM kinases are expressed in primary trophoblast cells and both cell lines and are regulated upon stimulation with LIF. Inhibition of PIM kinases significantly reduces viability and proliferation and induces apoptosis. Simultaneously, phosphorylation of c-MYC was reduced. These results demonstrate the involvement of PIM kinases in LIF-induced regulation in different trophoblastic cell lines which may indicate similar functions in primary cells.

1. Introduction

Trophoblast cells are embryonic tissue deriving from the "trophoectoderm" during early stage of pregnancy. They form the outer layer of the blastocyst, providing the nutrients to the embryo [1]. Trophoblast further proliferates and differentiates into two layers: *i*) Cytotrophoblast (CTB): Single celled, inner layer of the trophoblast [2], which further develops into extra-villous trophoblast cells (EVT) growing out from the tips of the villi, penetrating the decidualized uterus [3] and *ii*) Syncytiotrophoblast (STB): a multinucleated cell complex that covers the placental villi, separating maternal and fetal blood streams in gestation [4,5]. The highly coiled arterioles that supply blood to the endometrium are known as spiral arteries. They transport the maternal blood into the intervillous space. The hemochorial placentation requires an epithelial-to-endothelial conversion and arises primarily through differentiation, proliferation, migration and invasion of the endometrium and its vasculature by the CTB [6,7].

Similarities have been frequently asserted between trophoblastic and cancer cells in terms of invasion, migration and proliferation [8–10]. Malign cells have similarities with CTB and EVT, which show highly invasive characteristics especially during implantation and first trimester of pregnancy. There is involvement of autocrine/paracrine communications, adhesion molecules and proteases. Numerous intracellular signaling pathways controlling these processes have been described, such as JAK/STAT, MAP kinases, Wnt and others [11–13]. Leukemia Inhibitory Factor (LIF) is a major inducer of trophoblast invasiveness via activation of STAT3 [14]. Previous studies have demonstrated that in mouse embryonic stem cells LIF-mediated STAT3 activation leads to upregulation of Proviral Integration site of Moloney murine leukemia virus 1 (PIM1) and PIM3 [15].

* Corresponding author.
E-mail address: markert@med.uni-jena.de (U.R. Markert).
[1] www.placenta-labor.de.

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Table 1
Primers used in qPCR reactions.

Gene Name (PCR Product)	Primer sequence		Gene ID
***PIM1*(109 bp)**	Fwd	5′CATCCTTATCGACCTCAATCG-3′	NM_001243186.1
	Rev	5′TATACACTCGGGTCCCATCG-3′	
***PIM2* (107 bp)**	Fwd	5′TCACAGATCGACTCCAGGTG-3′	NM_006875.3
	Rev	5′CATAGCAGTGCGACTTCGAG-3′	
***PIM3* (128 bp)**	Fwd	5′ATGCTGCTCTCCAAGTTCGGCTCCCTGGCG-3′	NM_001001852.3
	Rev	5′ TCCTGTGCCGGCTCGGGTCGCTCCAGCACC 3′	
***HMBS* (281 bp)**	Fwd	5′ GAAATCATTGCTATGTCCACCAC 3′	NM_008093.1
	Rev	5′ CTTCCCACCACACTCTTCTC 3′	

PIM kinases belong to the serine/threonine kinases family and consist of three different isoforms (PIM1, PIM2 and PIM3) which share several homologies and are highly evolutionary conserved [16]. They were reported first in the 1980s in murine Moloney leukemia virus (MuLv)-induced T-cell lymphomas in a process related to transcriptional activation of *Pim-1* gene [17]. PIM genes were identified as oncogenes [18]. So far, their expression has been reported in almost all kind of human cancer, marking its presence to be important in cell survival, proliferation, migration, and apoptosis [19]. There is evidence that PIM kinases overexpression leads to tumor progression [17]. In fact, aberrant expression of PIM kinases in pancreatic cancer plays a pivotal role in the regulation of cell cycle, apoptosis, stem cell properties, metabolism, autophagy, drug resistance and targeted therapy. In this scenario, blocking the activities of PIM kinases prevents pancreatic cancer development [20]. PIM kinases are also involved in embryonic development [15,21].

One of the primary machineries of survival and apoptosis for PIM kinases is elicited through their pro-survival effect via the Bcl-2 family members, that has both pro-apoptotic (BAD and BAX) and anti-apoptotic (BCL-2 and BCL-XL) effects. PIM kinases phosphorylate BAD at ser112, which disrupts the association with BCL-2, promoting binding and retention in the cytosol [22], hence resulting in anti-apoptotic activity. PIM kinases also inhibit the activation of caspase 3 (CASP3) and CASP9 [23,24]. PIM kinases act synergistically on MYC which is a transcription factor and proto-oncogene with relevant functions in cell survival/apoptosis, proliferation, differentiation and metabolism. Because of similarities of trophoblast and tumor behavior, it may be expected that PIM kinases play a similar role in both. However, the presence and the role of PIM kinases in trophoblast cells have not been examined so far, and therefore, have been analyzed in this study.

2. Materials and methods

2.1. Cell culture

The immortalized human primary trophoblast cell line HTR8/SVneo was a kind gift from Dr. Charles Graham, Ontario, Canada [25], the choriocarcinoma cell line JEG-3 was obtained from ATCC® HTB-36™. Both cell lines were regularly verified free of mycoplasma by PCR. HTR8/SVneo were grown in RPMI medium and JEG-3 in Ham's F12 medium (Life Technologies, Grand Island, New York, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, Steinheim, Germany), penicillin and streptomycin (Life Technologies), under standard conditions (37 °C, 5% CO_2).

2.2. Isolation of primary trophoblast cells

The primary trophoblast cells were isolated from term placenta tissue not more than 1 h following delivery. The protocol for isolation was adapted in our Placenta Laboratory [26,27]. In brief, placenta tissue was cut into small pieces, washed in sterile PBS to remove blood and enzymatically digested with a mixture of collagenase type IV, proteases type IV and DNase type IV for 30 min at 37 °C. After a washing step, cell suspension was centrifuged at 100×*g* for 10 min and separated by a Percoll gradient (Percoll, Pharmacia, Sweden). The layer within 25% Percoll was carefully collected and washed. To avoid erythrocyte contamination, red blood cell lysis buffer was added to the isolated cells. Subsequently, trophoblast origin was confirmed by flow cytometry using anti-cytokeratin-7 and anti-HLA-G antibodies.

2.3. RNA isolation and PCR

Total cellular RNA was extracted using TRIzol reagent (Invitrogen, Darmstadt, Germany) and quantified in a Nano Drop ND-1000 spectrophotometer (PeqLab Biotechnologies GmbH, Erlangen, Germany). Samples with purity ratio higher than 1.8 at A260/A280 were selected and stored at − 80 °C. cDNA was generated from 150 to 300 ng of DNase-free RNA using Maxima Reverse Transcriptase (Thermo Fisher Scientific Biosciences GmbH, Waltham, MA, USA) and Mastercycler ep gradient S (Eppendorf, Hamburg, Germany).

Quantitative real time PCR was performed using SYBR green master mix with ROX reference dye (Thermo Fisher) in a thermocycler Stratagene Mx3005 P (Agilent Technologies Inc., Santa Clara, CA, USA). qPCR primers for PIM1 and PIM2 were designed using software tools NCBI, genome browser, and Primer3. PIM3 primer sequence was used as published previously (Table 1) [28]. Specificity was confirmed using Keinefold and InsilicoPCR UCSC genome browser. Amplification efficiency for the different primers ranged between 96.4% and 114.9%.

Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method and Hydroxymethylbilane Synthase (HMBS) as reference gene [29].

2.4. Western blotting

Protein expression of PIM kinases (PIM1, PIM2 and PIM3) was analyzed by Western blot. Briefly, cells were seeded in 6-well plates at a density of 4.5×10^5 cells/well, and each plate was incubated for 24 h to reach adherence and confluence. Subsequently, after serum starvation for 4 h, cells were stimulated with or without LIF (10 ng/ml) at different intervals from 2 to 60 min. Then, cells were rinsed with cold PBS, harvested and lysed using cell lysis buffer (Cell Signaling Technologies Inc., Beverly, Massachusetts). Lysates were centrifuged at 10,000×*g* for 10 min at 4 °C. Protein concentrations of the resulting supernatant were determined using a Pierce® BCA Protein Assay (Thermo Fisher). Protein samples (20–30 μg) were resolved in 10–12% SDS PAGE gels and transferred to a PVDF membrane (Thermo Fisher). Membranes were blocked with 5% bovine serum albumin (BSA) 1 h before overnight incubation at 4 °C with the following primary antibodies (1:1000 dilution): anti-PIM1, anti-PIM2, anti-PIM3, anti-BCLXL, anti-phospho-BAD, anti-BAD, anti-c-MYC, anti-phospho-c-MYC, anti-CASP 3, anti-cleaved CASP 3 and anti-cleaved PARP (all purchased from Cell Signaling). Blots were rinsed with TBST and incubated with a secondary antibody anti-rabbit IgG, HRP-linked (Cell Signaling) at a dilution of 1:10,000 for 90 min at RT. Reactive bands were visualized by exposure to Luminata Forte Western HRP substrate (Merck Millipore, Billerica, Massachusetts). Documentation