THE ROLE OF THE PROGESTERONE-INDUCED BLOCKING FACTOR IN TUMOUR SPREADING AND IN THE EMBRYO-MATERNAL COMMUNICATION

Ph.D. thesis

Tímea Balassa

Basic Sciences Doctoral School

Chair of the doctoral school: Dr. Dóra Reglődi

Program leader: Dr. Éva Mikó

Tutor: **Dr. Júlia Szekeres-Barthó**

Department of Medical Biology and
Central Electron Microscope Laboratory

Medical School

University of Pécs

Pécs

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INTRODUCTION

The thesis deals with two seemingly independent issues: the role of the progesterone-induced blocking factor (PIBF) in tumour invasion, and the harmful effect of light exposure on the preimplantation embryo. In fact, these are two aspects of the same problem. Both the receptive endometrium and the competent embryo are indispensable for successful implantation. The trophoblast of the implanted embryo invades the decidua, creating an interface for embryo-maternal communication. Invasiveness is a common feature of the trophoblast and malignant tumours however, while trophoblast invasion is strictly regulated both in space and time, tumour invasion is uncontrolled.

1. The conditions for successful implantation

The establishment of pregnancy depends on a receptive endometrium and a competent embryo. The absence of either of these requirements results in infertility - a condition that affects millions of couples worldwide.

The implantation capacity of the embryo might be influenced by environmental factors. One of these is light exposure during the laboratory procedures of assisted reproduction, which either directly, or via altered gene expression affects the implantation capacity of the embryos.

The immune system creates a tolerant environment for the foetus, while and at the same time retains its ability to fight infections. The selective inhibition of anti-foetal immune responses is mainly manifested by a Th2 biased cytokine pattern and reduced NK activity. Following recognition of foetal antigens, activated decidual γ/δ T cells express progesterone receptors,

and in the presence of progesterone, produce the progesterone-induced blocking factor, which mediates the immunological effects of progesterone.

2. The progesterone-induced blocking factor (PIBF)

PIBF is present in activated lymphocytes as well as in the placenta, decidua and the embryo.

The mRNA transcribed from the PIBF1 gene contains 18 exons and codes for a 90 kDa protein, which - due to alternative splicing - produces several smaller molecular weight isoforms. The full length PIBF and the small forms exert different functions. The small molecular weight secreted isoforms induce Th2 dominant cytokine production, and down-regulate both decidual and peripheral NK activity. The full length PIBF is associated with the nucleus and plays a role in cell cycle regulation. PIBF produced during the peri-implantation period supports decidual transformation and regulates trophoblast invasion.

3. The role of PIBF in invasion

Earlier studies on trophoblast and tumour cell lines revealed a regulatory effect of PIBF on invasiveness. The present study aims at confirm the previous results in primary tumour cell cultures, e.g., primary lung adenocarcinoma (LC), primary ovarian carcinoma (OC) as well as a choriocarcinoma (JEG-3) cell line, and to investigate the involvement of PIBF in the complex mechanism of tumour growth.

Invasion is a multi-step process, including cell-cell interactions, cell-extracellular matrix (ECM) interactions, degradation of the ECM and migration of cells through the eroded connective tissue.

Invasiveness is a common feature of the trophoblast and of malignant tumours furthermore, physiologic (trophoblast) and pathological (tumour) invasion activate the same signalling pathways (MAPK, FAK, PI3K/Akt, STAT and Wnt). PIBF is strongly expressed in immature, rapidly proliferating cells, e.g. in embryonic cells and tumour cells. In addition to the immunomodulatory small isoforms, tumour cells express the full length PIBF, which - via its effect on cell cycle regulation - might play a role in tumour progression.

PIBF controls the transcription of invasion-related genes in a tissue specific manner, thereby down regulating invasion of the trophoblast while upregulating tumour cell invasion. Both its immunomodulatory effects and those on invasion might contribute to the complex mechanism of tumour formation.

The role of PIBF in cell-cell adhesion is yet to be clarified. Investigating the expression of E-cadherin in PIBF-deficient cells might contribute to our understanding of the role of PIBF in tumorigenesis.

AIMS

I. The effect of light exposure on implantation capacity of cultured murine embryos.

Problem: Does light exposure

- affect the viability, development and implantation capacity of murine embryos?
- induce DNA fragmentation in the nuclei of cultured murine embryos?
- alter the expression of pro- and anti-apoptotic molecules?

II. PIBF regulates invasion of primary tumour cells.

We aim to investigate:

- the expression profile of PIBF in tumours with different metastatic potentials.
- the efficiency of RNA interference on PIBF production and the dynamics of PIBF expression after gene silencing
- the relationship between PIBF and E-cadherin expression in tumour cells.
- the effect of PIBF on the adhesion of primary tumour cells to ECM components, as well as on migration and invasion of the cells.

METHODS, RESULTS AND DISCUSSION

I. The effect of light treatment on the development and implantation capacity of murine embryos

Among physiological conditions in mammals, the preimplantation embryo develops to blastocyst in the dark conditions of the fallopian tubes, whereas during assisted reproduction, the embryo is removed from its natural environment, and is exposed to light during the laboratory procedures.

In order to investigate the effect of light exposure on the physiology of developing embryos, in vitro cultured 4 cell stage murine embryos were exposed to 450 or 1130 Lux white light or red filtered light for 2x25 minutes. Control embryos were kept in dark. The embryos were further cultured to the morula stage, and then transferred to the uteri of pseudo-pregnant females. The mice were sacrificed on day 8.5 and implantation sites were counted. DNA fragmentation in control and light exposed embryos was detected with a TUNEL assay. The effect of light on the expression of apoptosis-related molecules was assessed in an apoptosis protein array.

Light exposure had no effect on the viability or the development of the embryos, but significantly reduced their implantation capacity. The harmful effect was related to the wavelength, rather than to the brightness of the light. The number of nuclei with fragmented DNA significantly increased in embryos treated with white light. The lysates of untreated and light exposed embryos were subjected to an apoptosis array. Compared to the controls, the expression of two anti-apoptotic molecules Bcl-2 and Bcl-x expression increased by 47% and 33 % respectively, in white light exposed embryos, suggesting that the DNA fragmentation was not connected with apoptotic damage.

Though there is no information on the light sensitivity of human embryos, these data suggest that embryo manipulation during IVF and ICSI should be performed with caution.

II. The role of PIBF in tumour progression

Earlier information concerning the expression and the role of PIBF in regulation of invasiveness stems from studies on trophoblast and tumour cell lines. The present study was aimed at confirming these results on primary tumour cells as well as performing a more complex investigation on the involvement of PIBF in the development of malignant tumours.

II.1. The expression of PIBF and E-cadherin in tumours

PIBF and E-cadherin expression was detected in paraffin sections from malignant tumours by immunohistology.

The cells in the tumour-infiltrated tissue showed a strong cytoplasmic and perinuclear anti-PIBF reactivity, while the tumour-free parts of the section expressed no PIBF.

A negative relationship was observed between PIBF and E-cadherin expression. PIBF positive cells failed to express E-cadherin, while E-cadherin positive cells did not react with the anti-PIBF antibody.

II.2. PIBF and E-cadherin expression in JEG-3, LC and OC cells

All of these invasive cell types showed strong cytoplasmic and nuclear PIBF reactivity. Western blot revealed the 90 kDa form, together with a 67 kDa isoform. As shown earlier, the full length PIBF is involved in cell cycle regulation, therefore we focused on this form. In order to investigate the role of PIBF, we transiently knocked down PIBF production by siRNA treatment.

The efficiency of knock down was 70% in JEG-3 and in LC cells respectively, and 65% in OC cells.

E-cadherin expression increased in PIBF-deficient cells compared to the controls, suggesting that PIBF down regulates E-cadherin expression, thereby loosening cell-cell interactions and increasing the mobility of tumour cells.

II.3. The effect of PIBF on cell-ECM adhesion

We investigated the adhesion capacity of the cells to ECM components, including collagen I, II, IV, laminin, fibronectin, tenascin and vitronectin, using a colorimetric ECM-cell adhesion test. The cells bound the ECM components were stained with crystal violet and the absorbance of the dye extracted from the cells (proportional with the number of adhered cells) was detected by spectrophotometry.

Though all PIBF-deficient cells showed a lower adhesion to ECM than intact cells, only PIBF-deficient JEG-3 cells adhered at a significantly reduced extent to collagen I than the controls.

Structural collagen proteins not only provide a passive background for the metastatic process, but also actively regulate metastasis formation and collagen I often accumulates in malignant tumours. Our data suggest that PIBF may affect the cell-ECM connection, however, to confirm its role, further studies are required.

II.4. The effect of PIBF on tumour cell migration

The scratching assay was used for assessing the migration of JEG-3, LC and OC cells. The rate of migration (monitored under the microscope) was defined by the closure of the scratched surfaces.

We found no differences between migration of intact and PIBF-deficient cells.

Considering that PIBF down regulated E-cadherin expression, further studies are required to explain this negative result. Migration and invasion are however different issues. While migration is a two dimensional process, not affected by obstacles inhibiting the movement of the cells, invasion takes place through a three dimensional matrix and implies secreting proteases to digest the matrix that would otherwise block the entry of cancer cells.

II.5. The effect of PIBF on tumour cell invasion

Invasion of PIBF-knock down and control tumour cells, was detected by an Oris Cell Invasion and Detection Assay following the manufacturer's instructions. PIBF silencing resulted in 80%, 65%, and 50% reduction of invasion in JEG-3 cells LC cells and OC cells respectively.

The matrix metalloproteinases (MMPs) cleave type IV collagen, thus play a crucial role in the process of invasion to the extracellular matrix. Therefore, cell-conditioned media from the invasion assay was used to examine the matrix metalloproteinase activity by gelatine substrate zymography.

In the media from PIBF-silenced JEG-3, LC and OC cells, MMP-2 activity was reduced to 36% 35%, and 65% respectively, while MMP-9 activity of PIBF-deficient JEG-3 and LC cells was reduced to 32% and 36% of those produced by the control cells. MMP-9 was not detectable in the medium of OC cells.

Though PIBF knock down did not affect migration, it resulted significant decrease of invasion of JEG-3 cells, LC and OC primary tumour cells respectively. PIBF silencing resulted in increased E-cadherin expression,

suggesting that by down regulating E-cadherin expression, PIBF might interfere with the cell-cell adhesion mechanisms and by increasing MMP activity induced extracellular matrix degradation, definitely facilitates the invasion of tumour cells.

These data suggest that PIBF increases MMP activity, thereby the invasive capacity of tumour cells. During active invasion, proteases secreted by mobile cells cleave the ECM components, and organize the collagen filaments in a pattern that enables tumour cells to invade.

SUMMARY

I. The effect of light exposure on cultured murine embryos

- Light exposed embryos have an impaired implantation capacity; using a red filter mitigates the harmful effect of white light.
- Exposure of embryos to white, but not to red filtered light results in DNA fragmentation in the nuclei.
- White light exposure of mouse embryos does not induce the production of pro-apoptotic molecules.

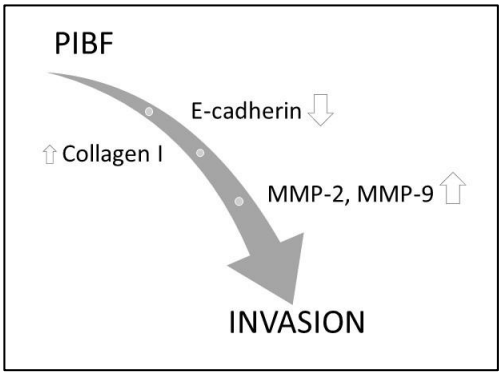
Characteristic		
Development (morphology)	∅	
Viability	∅	
Implantation capacity	↓	↓
DNA fragmentation	∅	↑
Anti-apoptotic proteins	—	↑

Light stress induced alterations in murine embryos.

Light exposure of the embryos is unavoidable during the laboratory procedures of IVF. Since there is no information on the light sensitivity of human embryos, embryo manipulation during IVF and ICSI should be performed with caution.

II. The role of PIBF in invasion of tumour cells

- PIBF is strongly expressed in the cytoplasm and perinuclear region of tumour cells.
- SiRNA treatment of JEG-3, primary lung adenocarcinoma and primary ovarian carcinoma cells results in a transient, but significant reduction of PIBF expression.
- PIBF and E-cadherin expression are inversely related in invasive tumours.
- E-cadherin expression is increased in PIBF-deficient cells.
- Adhesion of PIBF-deficient choriocarcinoma cells to type I collagen is reduced.
- There is no difference between the migration of PIBF-deficient and control tumour cells.
- The invasiveness of PIBF-deficient primary tumour cells is significantly decreased, together with a decreased MMP activity.



Effects of PIBF promoting distribution of tumour cells.

Considering that, most tumours express PIBF and that treatment of tumour patients with progesterone receptor blockers has a beneficial effect by inhibiting the production of progesterone-induced PIBF (Check et al., 2014), further characterization of PIBF functions in early steps of tumour invasion may provide novel therapeutic opportunities.

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PUBLICATIONS

The thesis is based on the following publications:

Bognár Z, Csabai TJ, Pállinger É, **Balassa T**, Farkas N, Schmidt J, Görgey É, Berta G, Szekeres-Barthó J, Bódis J. **2019** The effect of light exposure on the cleavage rate and implantation capacity of preimplantation murine embryos. J Reprod Immunol. 132:21-28. *IF:4,018*

Balassa T, Berta G, Jakab L, Bohonyi N, Szekeres-Barthó J. **2017** The effect of the progesterone-induced blocking factor (PIBF) on E-cadherin expression, cell motility and invasion of primary tumour cell lines. J Reprod Immunol. 125:8-15. *IF: 2,654*

Other publications:

Meggyes M, Nagy DU, **Balassa T**, Gödöny K, Péterfalvi Á, Szereday L, Polgár B. **2021** Influence of Galectin-9 treatment on the phenotype and function of NK-92MI cells in the presence of different serum supplements. Biomolecules. 11(8):1066. *IF: 4,879*

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