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Original article

Global decrease in the expression of signalling pathways' genes in murine uterus during preimplantation pregnancy

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ABSTRACT

Early preimplantation embryo-maternal communication is crucial for the establishment and development of pregnancy. Though the involvement of several candidate genes and proteins in this complex event has been described, the hierarchy of molecular networks governing this communication remains unknown. The primary objective of this study was to determine whether the presence of embryos in the uterine lumen stimulated or inhibited gene expression in the uterine tissue on day 3.5 *post coitum*. To answer this question, we investigated the gene expression of dedicated signal transduction pathways in the uterus of CD-1 mice during the preimplantation stage of pregnancy and compared this expression to mice with induced pseudopregnancy. The expression levels of 84 genes assigned to nine intracellular signalling pathways were investigated by real-time PCR. The results demonstrated down-regulation of the uterine gene expression in the majority of pathways. Among target genes, 27 were significantly ($p < 0.05$) down-regulated, and only three were significantly up-regulated. A majority of the down-regulated genes were found to be regulated by the TGFB and NFKB pathways, which suggests that the presence of the embryo selectively regulates signalling within signal transduction pathways. One of the up-regulated genes crucial for early pregnancy was *Ptgs2* ($p < 0.05$). The increased amount of both *Ptgs2* gene and protein products indicates that *Ptgs2* expression may be the earliest positive embryo signal for implantation and pregnancy recognition in mice. In conclusion, our results not only underline which signalling pathways are regulated in embryo-maternal communication before implantation but also support "the quiet state hypothesis" of silencing gene expression.

1. Introduction

Early embryonic death during the preimplantation period is one of the most common reasons for the recent decline in fertility rate observed in both animals and humans. In humans, the incidence of embryo death and pregnancy loss prior to implantation is estimated to be 30%. Similarly, in farm animals, such as cattle, embryonic mortality is estimated to be 35%, whereas 70%–80% of total embryonic loss occurs during the first 3 weeks after insemination [1,2]. In fact, disrupted embryo-maternal communication in the period directly preceding implantation may be one of the most important factors influencing reproductive efficiency. Moreover, the high preimplantation embryo death rate indicates the presence of an intensive selection process and suggests that failure in pregnancy establishment results from disturbances in the interaction between mother and embryo. However, successful embryo development under *in vitro* conditions with subsequent normal pregnancy after an embryo transfer suggests that these interactions are plastic.

In mice, there is a short period during which the uterus prepares for implantation, which leads to both the remodelling of uterine epithelial and stromal cells and metabolic changes in those cells. This reorganization culminates in the opening of the implantation window, which is the period of the highest receptivity of an endometrium. Although the uterus is not permissive to implantation during the prereceptive phase, it is believed that it is important for progression of embryonic development and maintenance of the pregnancy [3]. It is possible that the embryo-maternal cross talk exists during this period, as it was shown that during pregnancy, transcriptional activity of oviducts and uterine cells is altered [4]. Additionally, our previous

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studies have shown that the presence of an embryo during the preimplantation period modulates the phenotype of immune cells at the periphery [5,6]. This finding indicates that even before implantation embryos are able to make their presence known in the maternal organism. However, several publications have shown that the presence of the embryo changes the global transcription activity in different groups of genes involved in particular functions, though none of these papers was dedicated solely to analysing changes in signal transduction pathways. Therefore, we assume that regulation of signal transduction pathways activity may play a role in early interaction between mother and embryo. During the implantation process, the influence of the embryo on signal transduction pathways in uterine tissues is a considerably well-known phenomenon. For instance, the induction and increased activity of WNT (wingless-type MMTV integration site family member) and MAP (mitogen activated protein) kinase pathways are positive signals of the initiation of the trophoblast invasion [7]. However, the signal transduction processes in the uterus directly before implantation are still unknown.

The aim of this study was to determine how the presence of the embryo influences the uterine transcriptional activity of signal transduction pathways. We have investigated nine signal transduction pathways involved in a majority of physiological processes taking place in the preimplantation uterus, including inflammation and cytokine stimulation (NFKB and JAK/STAT pathways), decidualization and tolerance (TGFB pathway), proliferation and apoptosis (P53 and MITOGENIC pathways), cell adhesion and migration (LDL pathway) and implantation (NOTCH, WNT and HEDGEHOG pathways).

We demonstrated that 3.5 days after mating (during the prereceptive phase of the uterine epithelium), the presence of embryos down-regulated the transcription of genes involved in all investigated signal transduction pathways, especially the NFKB (nuclear factor kappa-light-chain-enhancer of activated B cells) and TGFB (transforming growth factor beta) pathways. We also have shown that at that time, the prostaglandin-endoperoxide synthase 2 (*Ptgs2*) gene and its protein expression were up-regulated in uterine tissues, suggesting that these compounds could be early pregnancy signals. Our data support the hypothesis presented by Lesse and colleagues that diminished embryo and maternal metabolic activity is characteristic and favourable for the pregnancy period directly proceeding implantation [8].

2. Materials and methods

2.1. Ethics statement

Outbred CD-1 (Crl:CD1(Icr)) mice were purchased from Charles Rivers Laboratories (Suzfeld, Germany) and housed in a dark-light cycle (12:12) under specific pathogen-free conditions in the Animal Breeding Centre of the Institute of Immunology and Experimental Therapy. All animal experiments were approved by the I Local Ethics Committee for Experiments on Animals at the Institute of Immunology and Experimental Therapy in Wroclaw No 44/2010. All surgery was performed under isoflurane anaesthesia and meloxicam analgesia, and all efforts were made to minimize suffering.

2.2. Animal preparation and material collection

Two groups of 6–8-week-old females, 10 animals per group, were investigated: a control group of pseudopregnant female mice and an experimental group of pregnant mice. All females were treated with 5 IU of pregnant mare serum gonadotropin (Folligon, Intervet, Poland), to compensate for the hormonal background followed by 5 IU human chorionic gonadotropin (Chorulon, Intervet, Poland) 46 h later. Female mice from the control group were mated with vasectomized males, while mice from the experimental group were mated with males of proven fertility. Male vasectomy was performed by surgical excision of 1 cm segments of each *vas deferens* under general anaesthesia. After surgery and recovery, males were kept with fertile females to completely empty the male reproductive tract of the residual sperm. Mating of experimental animals was confirmed by the presence of the copulatory plug. The day of the vaginal plug formation was designated 0.5 dpc (days *post coitum)*. Mice were sacrificed at 3.5 days of pregnancy and pseudopregnancy and uteri were dissected. Whole uteri from control and experimental group (with confirmed presence of embryos/oocytes) were carefully flushed with cold phosphate-buffered saline (PBS) and stripped of any adjacent tissues. Subsequently, uterine corpus and oviducts were separated and then both uterine horns were flushed separately with cold PBS. The developmental status and quality of the collected embryos were confirmed by visual observation under microscope. The average frequency of blastocysts and morulae flushed from uterine horns of mated mice was equal to 59% and 41%, respectively. The horns with confirmed presence of embryos (pregnant mice) or oocytes (pseudopregnant mice) were cut into pieces and randomly sampled for further RNA isolation and Western blot analysis. Samples were preserved in −80 °C.

2.3. RNA isolation, cDNA synthesis

Total RNA was isolated from homogenized (OmniTips™ Homogenizer Kit, OmniInternational, USA) uterine horns using Trizol Reagent (Invitrogen, Paisley, U.K.) by the phenol-chloroform method. Total RNA samples were further purified using an RNeasy Mini Kit (Qiagen, USA) with additional on-column DNase digestion (DNase I, Qiagen, USA) to remove genomic DNA contamination from the samples. The RNA purity (A_{260}/A_{280}) was measured (Nanodrop, ThermoScientific, USA) according to the Bustin et al. protocol [9]. The resulting RNA was combined into three pools in the pregnant group of mice (consisting of RNA samples from 3, 3 and 4 uteri, respectively) and three RNA pools in the control pseudopregnant mice (consisting of RNA samples from 3, 3 and 4 uteri, respectively). During optimization of the quantitative real-time polymerase chain reaction (real-time PCR) protocol, RNA quality was evaluated by RNA gel electrophoresis (1% agarose gel). The 18S and 28S fragments were visualized using the GBOX imaging system (Amersham Pharmacia, Piscataway, New Jersey, USA) versus a 1-kb DNA ladder (Invitrogen) and were observed in the expected 1:2 ratio in all isolated samples. For global screening of the uterine preimplantation transcriptome, cDNA was prepared with the use of the RT^2 First Strand Kit (Qiagen, USA) from total RNA (in an amount of approximately 1 μg from each uterus).

2.4. Real-Time PCR

Before experimentation, preliminary/pilot studies on gene expression in pregnant and pseudopregnant mouse uteri were performed by real-time PCR with the use of the Signal Transduction Pathway Finder Array (SABioscience, USA), which is an array allowing for the investigation of 18 different signalling pathways. Based on the results of changes in gene expression (statistical significance) in those 18 signalling pathways, nine pathways were selected for further experiments (data not shown). The chosen target genes were selected according to the suggested list of genes for single pathways available from the manufacturer website (for details see http://www.sabiosciences.com/ArrayList.php?pline=PCRArray). Moreover, the authors extended the sets of genes for analysis in some pathways due to gene function analysis based on available scientific data (example of references for the NFKB