Expression of Cytokine Receptors in Pre-implantation Mouse Embryos

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Abstract: *The proliferation and differentiation of most cells are regulated by cytokine signaling, but the mechanism that regulates pre-implantation development remains unclear. Recently, it has been shown that Jak2, which mediates various cytokine signaling pathways, is expressed in pre-implantation mouse embryos. In this study, we investigated the expression of the cytokine receptors that activate Jak2, i.e., the receptors for prolactin (PrlR), growth hormone (GHR), tumor necrosis factor (TNFR), interleukin-3 (IL-3R), interleukin-5 (IL-5R), and granulocyte-macrophage colony stimulating factor (GM-CSFR). RT-PCR analysis revealed that PrlR was expressed in MII stage oocytes at a relatively high level, and that the level of expression decreased between the 2-cell and 4-cell stages. The expression levels of GHR, TNFR, IL-3R, IL-5R and GM-CSFR were relatively low before the morula stage, but they increased thereafter until the hatched blastocyst stage. These results suggest that various cytokine signaling pathways mediated by Jak2 activation are involved in the regulation of pre-implantation development.*

Key words: *Cytokine receptor, Jak2, Gene expression, Mouse, Embryo*

Introduction

The proliferation and differentiation of most cells are regulated by cytokine signaling. Recent work has shown that Jak2 is expressed in mouse pre-implantation embryos [1]. Jak2 is a non-receptor tyrosine kinase that mediates various signaling pathways via cytokine receptors [2]. It associates with the cytoplasmic domain of cytokine receptors, and is activated upon association of the receptors with their cognate ligands. Activated Jak2 undergoes autophosphorylation [3]. In somatic cells, activated Jak2 phosphorylates the tyrosine residues of Stats, which leads to the activation of Stats as transcription factors [4]. It has been reported that Stat3 is expressed at the blastocyst stage in mouse embryos, and that it functions to maintain the pluripotency of the cells in the inner cell mass [5]. Furthermore, a recent report has demonstrated that Stat1 and Stat3 are expressed in 2-cell stage embryos, and that these transcription factors are phosphorylated [6], which suggests that the Jak2-Stat signaling pathway is activated in pre-implantation mouse embryos.

In this study, to clarify the involvement of the Jak2-Stat signaling pathway in the regulation of pre-implantation development, we investigated the expression of cytokine receptors that activate Jak2. RT-PCR analysis revealed that various cytokine receptors are expressed in pre-implantation embryos, and that their expression patterns vary during pre-implantation development. These results suggest that various Jak2-Stat signaling pathways are involved in the regulation of pre-implantation development.

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Materials and Methods

Animals

Three-week-old female ddY mice and mature male ICR mice were purchased from SLC Japan (Shizuoka, Japan). They were maintained on a constant dark/light cycle (14-h/10-h) with standard mouse food and water being freely available. The housing of the animals was in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

Sperm preparation

Mature male ICR mice were euthanized by cervical dislocation and the cauda epididymis was removed. It

was punctured with a 22-gauge needle, and the sperm mass was squeezed out into a 5% CO_2-saturated 200-μl drop of Whitten's Medium (WM) [7], and covered with paraffin oil. The sperm sample was incubated in 5% CO_2-95% air with 100% humidity at 38°C for 2 h before being used in the experiment.

Oocyte collection and in vitro fertilization

Female ddY mice were superovulated by injection with 5 IU pregnant mares serum gonadotropin (Teikoku Zouki, Tokyo, Japan), followed 48 h later by injection of 5 IU human chorionic gonadotropin (hCG) (Sankyo, Tokyo, Japan). Sixteen hours after the hCG injection, the female mice were euthanized by cervical dislocation and their oviducts were removed. The oocyte-cumulus complexes were isolated in a 5% CO_2-saturated 200-μl drop of WM, and covered with paraffin oil. For *in vitro* fertilization, 5 μl of the sperm suspension was added to medium that contained the oocytes. Inseminated oocytes were incubated in 5% CO_2-95% air at 38°C. Six hours after insemination, the embryos were peeled off from the surrounding cumulus cells, washed three times in a 100-μl drop of CZB medium [8], and incubated in a 200-μl drop of CZB medium.

RNA isolation

Total RNA was isolated from 40 MII stage oocytes and embryos at various developmental stages. The cells were collected in ISOGEN (Wako, Osaka, Japan) and kept at –80°C until use. The samples were thawed at room temperature and mixed with 100 pg of rabbit α-globin mRNA (as the external control) immediately before RNA isolation. The procedure for RNA isolation with ISOGEN followed the manufacturer's protocol (Wako). Briefly, the sample solution was mixed with 100 μl chloroform, incubated at 4°C for 5 min, and then centrifuged at 15,000 rpm for 15 min at 4°C. Supernatant aliquots were mixed with 2 μl of glycogen and 400 μl of isopropanol, incubated at 4°C for 30 min, and then centrifuged at 15,000 rpm for 15 min at 4°C. The precipitated pellet was rinsed in 500 ml of 80% EtOH and centrifuged at 15,000 rpm for 15 min at 4°C. The RNA pellet thus obtained was dried in air.

RT-PCR assay

The RNA pellets were resolved in 27 μl DEPC-treated water and then mixed with 2 μl of the Oligo(dT)$_{12-18}$ primer (Invitrogen Corp., Carlsbad, CA) and 2 μl of 10 mM dNTP mix (TaKaRa, Kyoto, Japan). After incubation at 70°C for 5 min, the samples were combined with 4 μl of 10 × RT reaction buffer, 4 μl of dithiothreitol, 0.5 μl of Reverscript II (Wako), and 0.5 μl of RNasin ribonuclease inhibitor (Promega, Madison, WI). This mixture was incubated at 42°C for 90 min, followed by incubation at 75°C for 15 min. The sample solution was cooled to 4°C and used as the cDNA template for PCR.

Each PCR mixture (25 μl) comprised 15.3 μl double-distilled water, 2.5 μl 10 × PCR buffer, 2.5 μl 2 mM dNTP mix, 1.5 μl 25 mM $MgCl_2$, 0.2 μl of Ampli Taq Gold (Applied Biosystems, Foster City, CA), 0.5 μl each of the 10 μM stocks of the gene-specific sense and antisense primers, and 2 μl of cDNA template. For all the cytokine receptors examined, with the exception of the prolactin receptor (PrlR) and granulocyte-macrophage colony stimulating factor receptor (GM-CSFR), PCR was carried out for 40 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. For PrlR, PCR was conducted with the same protocol but only for 35 cycles. For GM-CSFR, the annealing temperature was changed to 60°C. For rabbit α-globin, PCR was carried out for 27 cycles with the protocol described above for the other cytokine receptors.

The sequences of the gene-specific primers used for PCR are shown in Table 1. The interleukin-3 receptor (IL-3R), interleukin-5 receptor (IL-5R), and GM-CSFR consist of an α-chain, which is specific for each cytokine, and a β-chain, which is common to all these cytokines [9]. The gene-specific primers for these cytokines were designed with the sequences of their α-chains.

The PCR products were separated by electrophoresis in a 2% agarose gel and stained with ethidium bromide. The image of the gel was obtained with the DT-20MP UV illuminator (ATTO, Tokyo, Japan), and the relative amounts of the PCR products were determined by measuring the densities of the bands with a NIH Image (National Institute of Health, Bethesda, MD). The values for the cytokine receptors were normalized with that for rabbit α-globin.

Statistical analysis

Statistical analysis was performed with Student's *t*-test. All statements of significance are based on a probability level of $p<0.05$.

Results

The changes in expression levels of the cytokine receptors that activate Jak2, e.g., PrlR, GHR, TNFR, IL-

Table 1. PCR primers

Target gene (primer)	Sequence (5'–3')	Product size (bp)
PrlR (sense)	CATCACAGTAAATGCCACGAACGAA	586
PrlR (antisense)	GGCACTCAGCAGTTCTTCAGACTTG	
GHR (sense)	ACCCCAGGATCTATTCAGCT	205
GHR (antisense)	ATGTCTCCACGAATCCCGGT	
TNFR (sense)	GTACTGCGCCTTGAAAACCC	348
TNFR (antisense)	GATGCTTGGAGTTTGGCTGG	
IL-3R (sense)	AGGAAGGGCAGGGACATCTT	245
IL-3R (antisense)	TCACGCCAGAACATCCGGTA	
IL-5R (sense)	CTAGCGTGAGGACCATTCTG	210
IL-5R (antisense)	TCCTTCCCAACAAGCCAGGT	
GM-CSFR (sense)	CTGCTCTTCTCCACGCTACT	230
GM-CSFR (antisense)	TCCTGAACCAGCAGCGGCAA	
α-globin (sense)	GCAGCCACGGTGGCGAGTAT	257
α-globin (antisense)	GTGGGACAGGAGCTTGAAAT	

3R, IL-5R, and GM-CSFR, were examined during pre-implantation development (Fig. 1). The results for each receptor are documented below.

PrlR

PrlR was expressed at relatively high levels in MII stage oocytes and 2-cell stage embryos. The expression level decreased by 80% between the MII stage oocytes and 4-cell stage embryos. After the 4-cell stage, PrlR expression was maintained at a low level until the blastocyst stage, whereas at the hatched blastocyst stage, the expression level increased slightly.

GHR

GHR expression peaked at the hatched blastocyst stage. From the blastocyst to the hatched blastocyst stage, the expression level increased significantly. During the other stages, the GHR expression level showed relatively little fluctuation.

TNFR

TNFR expression increased after fertilization, although only marginal expression was detectable until the 4-cell stage. The level of TNFR expression increased significantly between the 4-cell stage and the hatched blastocyst stage. The relative expression level did not change between the blastocyst and hatched blastocyst stages.

IL-3R

The expression pattern of IL-3R was similar to that of GHR. Peak expression was observed at the hatched blastocyst stage. The expression level was relatively low before the hatched blastocyst stage, and increased significantly at this stage. The difference in IL-3R expression levels between the 4-cell and hatched blastocyst stages was significant.

IL-5R

Peak expression of IL-5R was observed at the morula stage. The expression level was relatively low before the morula stage, but increased abruptly once the cells reached this stage. The difference in IL-5R expression levels between the 4-cell and morula stage was significant. Thereafter, the expression level decreased gradually until the hatched blastocyst stage.

GM-CSFR

The expression pattern of GM-CSFR was similar to that of IL-5R. Peak expression was detected at the morula stage. The expression level increased abruptly, but not significantly, at the morula stage, and decreased subsequently at the blastocyst stage.

Discussion

We examined the expression of cytokine receptors that activate Jak2, and found that their expression patterns could be classified into two groups. The first group contains PrlR, which was expressed at relatively high levels before the 2-cell stage. The second group comprises the other receptors (GHR, TNFR, IL-3R, IL-5R, and GM-CSFR), which were expressed at high levels after the morula stage.

PrlR was expressed at relatively high levels in the MII stage oocytes and 2-cell embryos. Expression decreased abruptly to the marginal level at the 4-cell stage. In somatic cells, PrlR is expressed in many

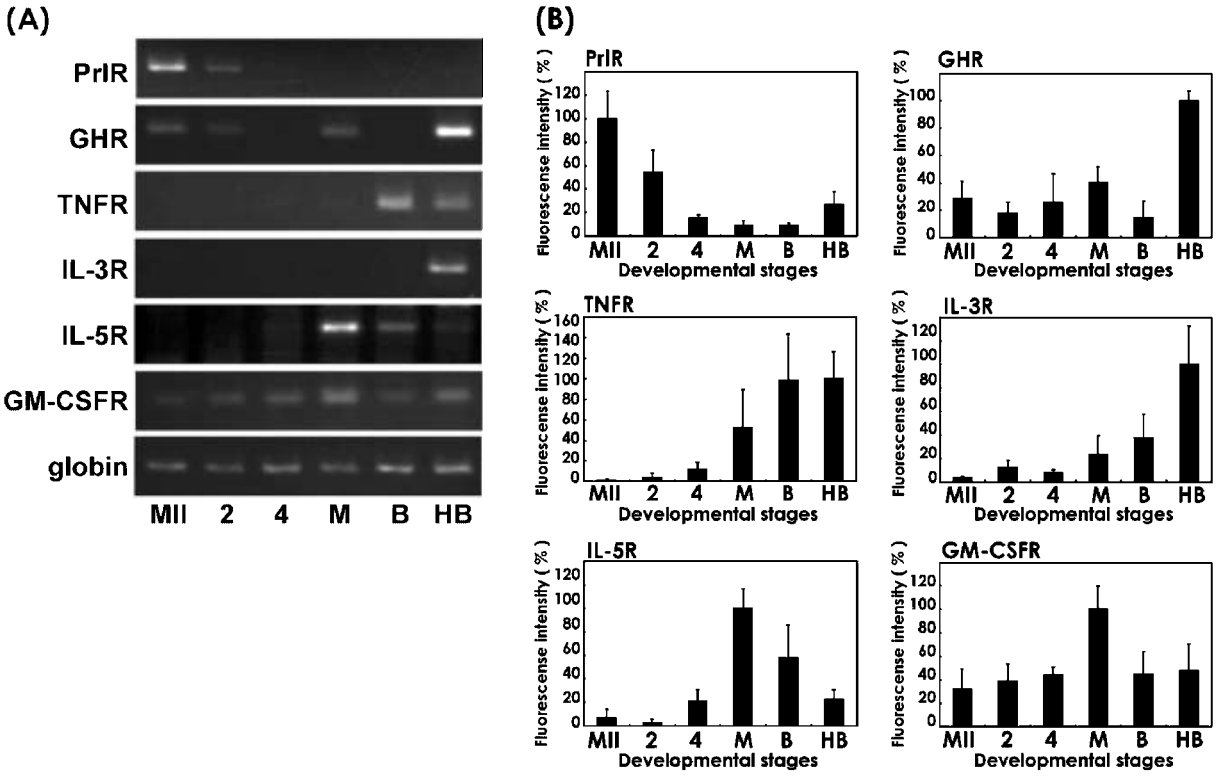

Fig. 1. Expression patterns of cytokine receptors during pre-implantation development in the mouse. Total RNA samples were isolated from oocytes at the MII stage (MII) and from embryos at the 2-cell (2), 4-cell (4), morula (M), blastocyst (B), and hatched blastocyst (HB) stages, which corresponds to collection at 31, 48, 72, 96 and 120 h after insemination, respectively. The samples were subjected to RT-PCR analysis for the expression of prolactin receptor (PrlR), growth hormone receptor (GHR), tumor necrosis factor receptor (TNFR), interleukin-3 receptor (IL-3R), interleukin-5 receptor (IL-5R), and granulocyte-macrophage colony stimulating factor receptor (GM-CSFR). Rabbit globin mRNA (globin) was included as an external control. The PCR products were subjected to agarose gel electrophoresis, followed by staining with ethidium bromide. The images of the PCR-amplified bands on agarose gels (A) and the results of the quantification of the PCR products (B) are shown. For quantification, the relative fluorescence intensities of the PCR bands were determined. The highest value in the developmental stages was set as 100% for each cytokine receptor, and the values for the other stages were calculated relative to this value. The experiment was performed three times and the results are presented as the mean ± SEM.

types of cells, such as mammary epithelial cells [10, 11], breast cancer cells [12], and lymphocytes [13]. PrlR mediates the signaling pathway that regulates lactation, reproduction, cell growth, brain behavior, immunomodulation, and electrolyte balance [14], by activating Stats 1, 3, 5a, and 5b [15, 16]. PrlR knockout mice exhibited reduced rates of fertilization and pre-implantation development, but did not reveal any disorder of male reproductive function [17], but the mechanism by which PrlR regulates pre-implantation development remains to be elucidated. Recently, it has been reported that tyrosine-phosphorylated Stat1 and Stat3 are expressed in mouse 2-cell stage embryos [6]. Thus, PrlR may be involved in the regulation of development until the 2-cell stage by activating Stat1 and Stat3.

All of the cytokine receptors examined in this study, except for PrlR, showed peak expression at the morula stage or thereafter. Since the differentiation of cells starts at the morula or blastocyst stage [18, 19], these cytokine receptors appear to regulate cellular differentiation and proliferation at these developmental stages by activation of the Jak2-Stat signaling pathway. In somatic cells, IL-5R mediates the differentiation of mature B-1 and B-2 cells from splenic B cells [20]. In TNFR-null mice, early hematopoietic progenitor cells fail to differentiate into dendritic cells [21].

The expression levels of IL-5R and GM-CSFR were

high at the morula stage. Both of these receptors mediate the signal that stimulates hematopoiesis [22]. They consist of a cytokine-specific α-chain, which binds the ligand with low affinity, and a common beta-chain [9]. In β-chain-null mice, the bone marrow cells did not respond to either IL-5 or GM-CSF, and the numbers of peripheral eosinophils were reduced [23]. GM-CSF knockout mice showed reduced rates of pre-implantation development [24]. These facts suggest that GM-CSFR is functional in morula stage embryos, but IL-5 knockout mice did not show any defect in reproduction [22]. GM-CSFR and IL-5R may work redundantly during pre-implantation development, and GM-CSFR may complement the function of IL-5R in the embryos of IL-5 knockout mice.

The expression levels of GHR and IL-3R were high at the hatched blastocyst stage. Although the expression patterns of GHR transcripts during pre-implantation development in mice have been described [25], that report examined the embryos up to the blastocyst stage and not at the hatched blastocyst stage. Our results show that the expression of GHR increases abruptly at the hatched blastocyst stage. Since growth hormone increases the number of cells in blastocyst stage embryos [26], GHR may function by mediating the proliferation of cells in pre-implantation embryos. On the other hand, the function of IL-3R during pre-implantation development is not clear. In somatic cells, IL-3R is known to regulate the proliferation of hemocytes [27]. IL-3R as well as GHR may regulate the proliferation of cells at the hatched blastocyst stage during pre-implantation development.

The expression of TNFR increased between the 4-cell stage and hatched blastocyst stage. It has been shown that TNF-α selectively regulates the proliferation of the cells of the inner cell mass at the blastocyst stage [28]. These results suggest that TNFR is involved in cell differentiation after the morula stage.

In conclusion, our results suggest that cytokine receptors that activate the Jak2-Stat signaling pathway are involved in the regulation of development at various stages during pre-implantation development.

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