学位論文

“Involvement of Stat5 signaling pathway in the regulation of mouse preimplantation development”

(マウス着床前初期胚における Stat5 シグナル伝達系の解析)

新領域創成科学研究科　先端生命科学専攻
資源生物制御学分野
学籍番号 17519
中里　款

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Abbreviations

BSA : Bovine serum albumin

cDNA : complementary deoxyribonucleic acid

DEPC : diethyl pyrocarbonate

DNA : deoxyribonucleic acid

FITC : fluorescein isothiocyanate

G1 phase : gap 1 phase

ICM : inner cell mass

IgG : immunoglobulin gamma

Jak : Janus kinase

MII stage : meiosis II stage

mRNA : messenger ribonucleic acid

PBS : phosphate-buffered saline

PCR : polymerase chain reaction

PMSF : phenylmethylsulfonyl fluoride

PVDF : polyvinylidene difluoride

RNA : ribonucleic acid

RT : reverse-transcription

SDS : sodium dodecyl sulfate

SFKs : Src family kinases

Stat5 : signal transducer and activator of transcription 5

TBS : Tris buffered saline

TE : trophectoderm

UV : ultra-violet light

Abstract

The signal transducer and activator of transcription 5 (Stat5) is an essential factor in signal transduction pathways for a number of cytokines to regulate growth and differentiation in mammalian cells. Mammalian *Stat5* has two closely related genes, *Stat5A* and *Stat5B*. However, the expression and function of Stat5 signaling pathway was unknown during the preimplantation development.

In chapter 1, I investigated Stat5A / 5B expression and localization in the embryos during preimplantation development. The results of RT-PCR and immunoblotting revealed that the both of Stat5s are expressed from the MII stage oocytes to the blastocyst stage embryos. The results of immunocytochemistry showed that Stat5A and Stat5B proteins were localized in the nucleus from the early 1-cell to blastocyst stage. Since only the activated Stat5s are localized in the nucleus, these results suggest that both of Stat5s are activated throughout the preimplantation development.

In chapter 2, to investigate the mechanism regulating Stat5s activation in the preimplantation embryos, I used the inhibitors for two groups of tyrosine kinases, Jak family and Src family kinases (SFKs), which activate Stat5s. Ag490, a Jak kinase family inhibitor, decreased the nuclear localization of Stat5A in the 2-cell, morula and blastocyst stage but not in the 1-cell or 4-cell stages. It also decreased the nuclear localization of Stat5B in the 1-cell, 2-cell and blastocyst stage but not in the 4-cell or morula stages. SU6656, a SFKs inhibitor, did not affect the localization of Stat5s until morula stages. However, in the blastocyst stage, SU6656 decreased the nuclear localization of both Stat5s. These results show that the mechanisms for activation of Stat5s are changed during preimplantation development, and are different between Stat5A and Stat5B. In addition, the inhibitors also affected the development of preimplantation embryos. The developmental stages in which the inhibitors exerted their effects on the development and the nuclear localization of Stat5s were well coincident with each other.

In chapter 3, the results of the in vitro transcription assay and the oligo DNA microarray analysis for 31,000 genes revealed that only a small number of the genes were changed in their expression level by the treatment with Ag490 in the 2-cell stage. However, the real-time PCR analysis following the reverse-transcription revealed that the expressions of several target genes of Jak-Stat5 signaling pathway, *i.e.*, ß-casein and tumor necrosis factor-beta (TNF-ß) were selectively inhibited in the 2-cell embryos by the treatment with Ag490. These results suggest that Jak-Stat5 signaling pathway is functional in the gene expression in the 2-cell stage embryos.

In chapter 4, I investigated the expression of the cytokine receptors that activate Jak2-Stat5 signaling pathway, *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 high level before the 4-cell stage, and that the expressions of GHR, TNFR, IL-3R, IL-5R, and GM-CSFR were increased after the morula stage. The treatment with GM-CSF, a ligand protein of GM-CSFR, revealed that GM-CSFR is functional in the signaling pathway activating Stat5A, but not Stat5B in the morula stage. Since it is known that prolactin and growth hormone phosphorylate Stat5s not only on the tyrosine-residues, but also on the serine-residue, phosphorylation of serine residue was examined. The immunocytochemistry showed that the Stat5s phosphorylated serine-residue (PS-Stat5) was localized in the nucleus from the late 1-cell to morula stage during the preimplantation development.

Taken together with the results in this thesis, it is suggested that complicated signaling pathways activating Stat5s are functional. Stat5s seems to be involved in the preimplantation development *via* regulating gene expression.

General introduction

Preimplantation development, which is the first process of mammalian ontogenesis, have many unique properties in various aspects of cellular function which are dynamically changed during this stage (Figure X-1). After fertilization, mouse MII stage oocytes grow up to the 1-cell embryos that contain male pronuclei and female pronuclei, followed by pronuclei fusion during the first cleavage. Before the 2-cell stage, maternal transcripts regulate the growth of embryo. Although zygotic transcription starts as early as at the late 1-cell stage (Ram and Schultz, 1993; Bouniol et al., 1995; Aoki et al., 1997), the first cleavage between the 1- and 2-cell stages occurs in the presence of alpha-amanitin, which inhibit ribonucleotide synthesis activity (Schultz, 1993). The embryonic control of development starts during the 2-cell stage (Bolton et al., 1984; Schultz, 1993; Schultz et al., 1999; Henery et al., 1995). During the 2-cell stage, maternal RNA is drastically degraded (Bachvarova and Moy, 1985), while gene expression level increase. After the second cleavage, the embryos are quickly divided every 12 h with the cell cycle containing very short G1 phase (Smith and Johnson, 1985; Smith and Johnson, 1986). In the 8-cell embryos, blastomeres connect each other with Gap junction and show compacted state (Lo and Gilula, 1979; Levy et al.,1986; Lee et al., 1987). Differentiation starts at morula stage (Terada et al., 1997; Nishikimi et al., 2001). Blastocyst stage embryos exhibit differentiation into two different cell lineages, *i.e.* inner cell mass and trophectoderm (Handyside and Hunter, 1984; Dyce et al., 1987). During the preimplantation development, various complicated changes thus occur in cellular function. However, the mechanism regulating these changes remains to be elucidated.

In somatic cells, signaling pathways stimulated by cytokines control the gene expression and cell growth. However, it is unclear what signaling pathway is activated during preimplantation development. In this research, I analyzed signal transducers and activators of transcription 5 (Stat5) signaling pathway for understanding the mechanism regulating preimplantation development. Stat5 mediates many cytokine signaling (Barahmand-Pour et al., 1998; Liu et al.,

1998). Upon signaling activation following the binding of ligands to their receptors, Stat5 protein is phosphorylated on tyrosine residues, dimerizes, translocates to the nucleus (Figure X-2) and binds to the promoters of specific target gene at the site named "IFN-gamma activation site (GAS)-like elements" (James and Darnell, 1997; Akira, 1999; Grimley et al., 1999; Imada et al., 2000). Mammalian Stat5 consists of two highly-related homologues, Stat5A and Stat5B (Liu et al., 1995; Mui et al., 1995). Although Stat5A and Stat5B have peptide sequence similarities of >90%, the numbers and positions of tyrosine residues that are phosphorylated by the upstream kinases is different between Stat5A and Stat5B (Liu et al., 1996; Liu et al., 1997; Darnell, 1997; Akira, 1999; Grimley et al., 1999). This difference may be the reason why targeted gene disruptions of Stat5A and Stat5B yielded different phenotypes in mice (Grimley et al., 1999). Stat5A gene targeted mice showed mammaly epitherial defect (Liu et al., 1997; Akira, 1999; Nevalainen et al., 2000), decrease of fat accumulation in adipocyte (Takeda and Akira, 2000) and defect in the development of CD4(+)CD25(+) immunoregulatory T cells that modulate T helper cell differentiation toward Th2 cells (Kagami et al., 2001). On the other hand, Stat5B-null mice showed the reduction of body growth rate in male (Udy et al., 1997) and NK cell development (Imada et al, 1998). Stat5A(-/-)5B(-/-) mice are severely anemic and show higher frequency of erythroid cells' apoptosis (Socolovsky et al., 1999, Snow et al., 2003), shortened life span (Snow et al., 2003), defect in liver growth (Matsumoto et al., 1999) and female infertility (Teglund et al., 1998). In vertebrate, the expression of Stat5 during embryogenesis has been reported only in *Xenopus laevis*'s neurula stage embryos (Pascal et al., 2001). However, the expression of Stat5s is not known in mammalian embryos.

In this thesis, to clarify the regulatory mechanism for preimplantation development, I investigated the whole figure and function in Stat5 signaling pathway. Stat5 signaling pathway consists of the cytokine receptors, upstream kinases, Stat5s and the downstream targeted genes. For the comprehensive understanding of this pathway, the analysis for all of these factors is

indispensable. In chapter 1, I examined the expression and localization of Stat5s in the mouse preimplantation embryos. In chapter 2, to analyze the upstream kinases that activate Stat5s, the effects of the inhibitors for the candidate tyrosine kinases that can activate Stat5s, *i.e.* Ag490 for Jak kinase and SU6656 for Src family kinases, were examined in Stat5 localization and development. In chapter 3, to investigate the functions of Stat5 signaling pathway, the effects of Ag490 on total transcriptional activity and gene expression pattern were examined. In chapter 4, to clarify the regulators of Stat5 signaling pathway, I examined mRNA expression of cytokine receptors those can activate Stat5 signaling pathway. The information obtained from this thesis will contribute to the comprehensive understanding of the mechanisms regulating preimplantation development.

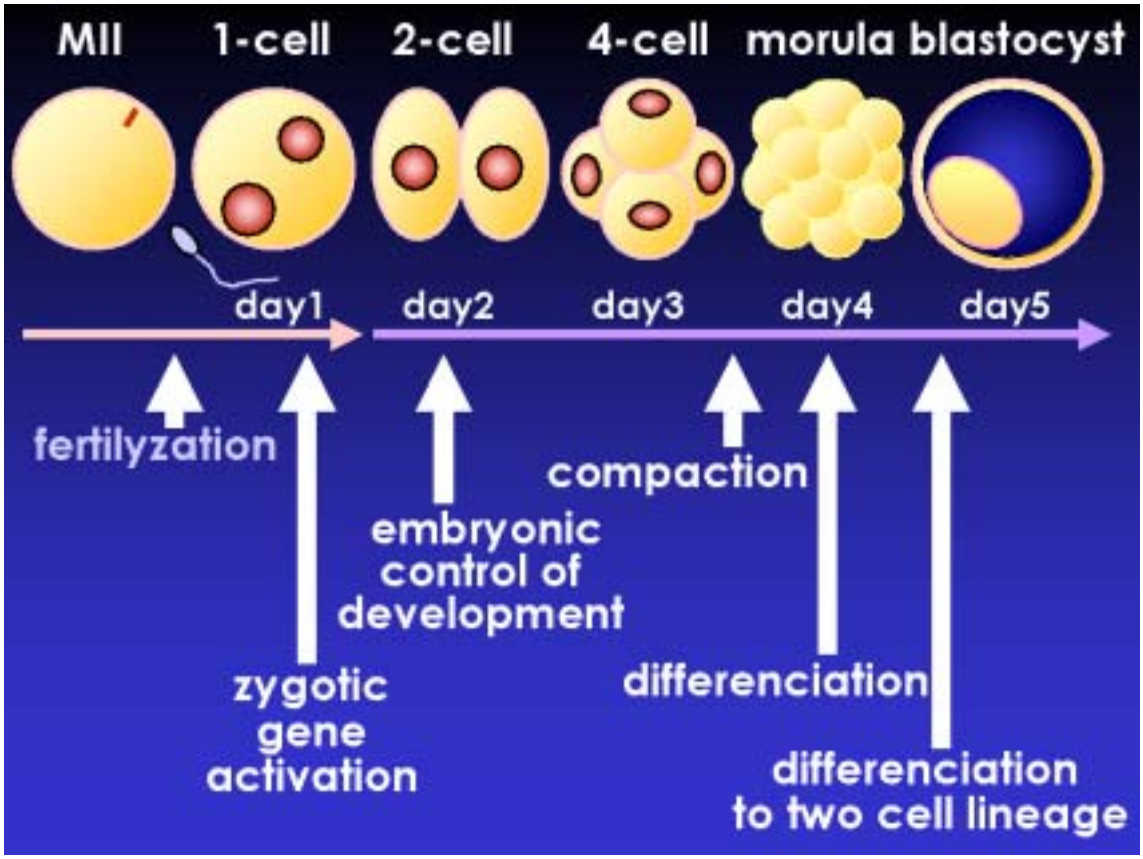

Figure X-1. Dynamics of cell functions during preimplantation development

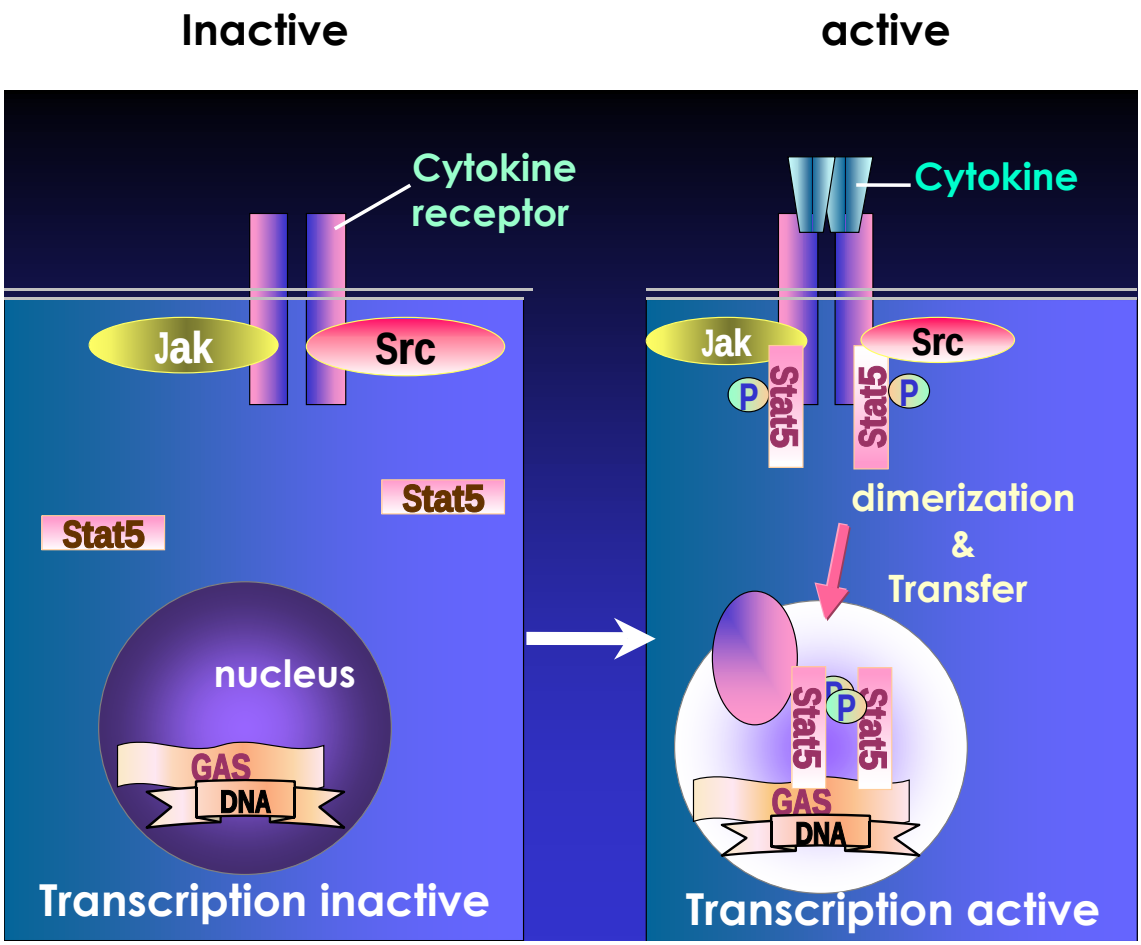

Figure X-2. Diagram of Stat5 signaling pathway

Chapter 1

Stat5s expression during the preimplantation development

Abstract

The signal transducer and activator of transcription 5 (Stat5) is an essential factor in signal transduction pathways for a number of cytokines to regulate growth and differentiation in mammalian cells. Mammalian Stat5 has two closely related genes, Stat5A and Stat5B. In this chapter, I investigated Stat5A/5B expression and localization in the embryos during the preimplantation development. The results of RT-PCR and immunoblotting analysis revealed that although Stat5A and Stat5B are always expressed in the oocytes and embryos during preimplantation development. The amounts were changed during these stages. The expression pattern of Stat5A and Stat5B mRNAs was similar to each other. The results of immunocytochemistry showed that Stat5A and Stat5B proteins were localized in nucleus in the 1-cell stage embryos and that their amounts increased until the 2-cell stage. After the 2-cell stage, Stat5s were always localized in the nucleus and the amounts were kept in a constant level until blastocyst stage. These results suggest that Stat5s continually activated during preimplantation development, and that Stat5s play some roles in mouse preimplantation embryogenesis.

Introduction

The signal transducer and activator of transcription 5 (Stat5) has been known as an essential factor in signal transduction pathways for a number of cytokines and growth factors (Gouilleux et al., 1994; Groner and Gouilleux, 1995; Groner, 2002). Stat5 was initially identified as a mammaly gland factor (MGF) implicated in the response of cells to prolactin (Wakao et al., 1994). At present, Stat5 is known as regulator of growth and differentiation in mammaly epitherial cells (Kazansky et al., 1995; Streuli et al., 1995; Miyoshi et al., 2001), mammaly gland (Cui et al., 2004), neuron (Cattaneo et al., 1996; De-Fraja et al., 1998), myeloid cells (Takatsu, 1998; Coffer et al., 2000; Kieslinger et al., 2000; Piazza et al., 2000; Smithgall et al., 2000; Goetz et al., 2004), hematocytes (Snow et al., 2002; Yoon and Watowich, 2003; Debierre-Grockiego, 2004; Schuringa et al., 2004), mast cell (Shelburne et al., 2002; Shelburne et al., 2003), T cell (Kelly et al., 2003) and adipocytes (Nanbu et al., 2002; Floyd and Stephens, 2003; Richter et al., 2003).

Stat5 activation is regulated by phosphorylation of tyrosine residues in C-terminal region. Activated Stat5s are dimerized by interaction of their phosphorylated tyrosine residue with Src-homology (SH2) domain of their counterparts and *vice versa*. Stat5 dimer translocates to the nucleus and activates the transcription of downstream gene (Darnell, 1997; James and Darnell, 1997; Liu et al., 1998; Akira, 1999; Grimley et al., 1999; Imada et al., 2000; Groner, 2002).

Mammalian Stat5 has two closely related gene, Stat5A and Stat5B (Liu et al., 1995; Mui et al., 1995). Although Stat5A and Stat5B have peptide sequence similarities of > 90% and mRNA sequence similarities of > 96%, the numbers and positions of tyrosine residues in C-terminus that are phosphorylated by the upstream kinases are different between each other (Liu et al., 1996; Liu et al., 1997; Darnell, 1997; Akira, 1999; Grimley et al., 1999). These two Stat5 homologues make homodimer or heterodimer (Cella et al, 1998; Grimley et al., 1999). Stat5A and Stat5B co-regulate cell growth and differentiation in various types of cells (Beisenherz-Huss et al., 2001; Friedrichsen et al., 2003; Shelburne et al., 2003; Baskiewicz-Masiuk and Machalinski, 2004), but it is also

reported that there are many differences in mechanism for their activation (Frasor et al., 2001; Ridderstrale and Groop, 2001), preferency of their targets DNA sequences (Verdier et al., 1998), and function in cell growth (Leong et al., 2002) between Stat5A and Stat5B.

In this chapter, I investigate Stat5A/5B expression and localization in the embryos during preimplantation development. RT-PCR analysis and immunoblotting were carried out to examine Stat5s expression. Immunocytochemistry was carried out to investigate the localization of Stat5s, by which the activation states of Stat5s are deduced; active Stat5s are localized in the nucleus. These results show that Stat5A and Stat5B are expressed and activated during the preimplantation development.

Materials and Methods

Animals

Three weeks old female ddY mice and mature male ICR mice were purchased from SLC Japan (Shizuoka, Japan). They were maintained on a constant light/dark cycle (14h / 10h) with standard mouse food and water available. Their housing 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 epididymidis* was removed. It was punctured with a 22-gauge needle, and a mass of sperm was squeezed put into a 5 % CO_2-satured 200 µl drop of Whitten's Medium (WM) (Whitten, 1971) covered with paraffin oil. The sperm was incubated in 5 % CO_2-95% air with 100% humidity at 38 °C for 2 h before used.

Oocyte collection and in vitro fertilization

Female ddY mice were superovulated by the injection with 5 IU pregnant mare's serum gonadotrophin (Teikoku Zouki, Tokyo, Japan) followed by 5 IU human chorionic gonadotropin (hCG) (Sankyo, Tokyo, Japan) 48 h later. Sixteen hours after the hCG injection, the female mice were euthanized by cervical dislocation and their oviducts were removed. The oocyte-cumulus cell complexes were isolated in a 5 % CO_2-satured 200 µl drop of WM covered with paraffin oil. For in vitro fertilization, 5 µl of the sperm suspension was added to medium containing the oocytes. Inseminated oocytes were incubated in 5 % CO_2-95% air at 38 °C. Six h after insemination, the embryos were peeled off from surrounding cumulus cells. The embryos which had two pronuclei were collected, washed in a 100 µl drop of CZB medium (Chatot et al, 1989) 3 times 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 into ISOGEN (Wako, Osaka, Japan) and kept at -80 °C until use. The samples were melted at room temperature and added with 100 pg of rabbit alpha-globin mRNA as an external control immediately before RNA isolation. The procedure for RNA isolation followed the manufacture's protocol of ISOGEN. Briefly, sample solution was added with 100 µl chloroform, stayed at 4 °C for 5 min and then centrifuged by 15,000 rpm for 15 min at 4 °C. Supernatant aliquot of each tube was added with 2 µl of glycogen and 400 µl of isopropanol, kept at 4 °C for 30 min and then centrifuged by 15,000 rpm for 15 min at 4 °C. The precipitated pellet was rinsed in 500 ml of 80 % EtOH and centrifuged 15,000 rpm for 15 min at 4 °C. RNA pellet thus obtained was dried in air.

RT-PCR assay

RNA pellets were resolved in 27 µl of DEPC treated water and added with 2 µl of Oligo(dT)$_{12\text{-}18}$ primer (Invitrogen Corp., Carlsbad, CA) and 2 µl of 10 mM dNTP mix (Takara Bio Inc., Kyoto, Japan). After incubation at 70 °C for 5 min, the sample mixture was added with 4 µl of 10x 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), and incubated at 42 °C for 90 min followed by the incubation at 75 °C for 15 min. The sample solution was cooled at 4 °C and used as the cDNA template for PCR.

Each of PCR mixture (25 µl) was made of 15.3 µl of double distilled water, 2.5 µl of 10x PCR buffer, 0.5 µl of 10mM dNTP mix, 0.3 µl of 250 mM $MgCl_2$, 0.25 µl of EX Taq HS (Takara Bio Inc.), 0.5 µl each of 10 µM gene-specific sense and antisense primers and 2 µl of cDNA template. PCR was carried out in the cycles consisting of denaturation at 94 °C for 30 s, annealing 30 s and extention at

72 °C for 1 min, and a final extention at 72 °C for 10 min. For Stat5A and Stat5B, 40 cycles of PCR were conducted in the protocol of annealing temperature at the 58.4 °C and 62 °C, respectively. For rabbit alpha-globin, PCR was carried out through 27 cycles in the protocol of annealing temperature at 58 °C. Sequences of each gene specific primers are shown in Table 1-1.

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

Immunoblotting

After washing three times in PBS that containing 3 mg/ml of polyvinylpiyrrolidone, 150 MII stage oocytes and embryos at various developmental stages were collected into 12 µl of modified RIPA buffer (100 mM NaCl, 1 % Nonidet P-40, 0.1 % SDS, 0.5 % deoxycholic acid, and 50 mM Tris-HCl, pH8.0 at 12 °C, 15 mM EGTA, 1mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 µg/ml pepstatin A). The samples were kept at -80 °C until use. The samples were melted at room temperature, added with 12 µl of x2 SDS sample buffer (12 % ß-mercaptethanol, 4 % SDS, 20 % glycerol, and 0.1 M Tris-HCl, pH6.8 at 12 °C), and incubated at 95 °C for 3 min. The samples were separated by electrophoresis in a 10 % polyacrylamide gel, followed by transferring to PVDF membrane. The membrane was incubated in the primary antibody solution at 4 °C for overnight. The primary antibody solutions were prepared by diluting the antibodies with Tris bufferd saline (TBS; 137mM NaCl, 2.68mM KCl, 25mM Tris) containing 0.1 % (v/v) Tween-20 and 0.2 % ECL advance blocking agent (Amersham Biosciences Corp., Piscataway, NJ, USA) to the concentration of 0.4 µg/ml. Anti-Stat5A rabbit polyclonal antibody (L-20; Santa Cruz Biotechnology, Charlottesville, VA) or anti-Stat5B mouse monoclonal antibody

(G-2; Santa Cruz Biotechnology) was used as the primary antibody. After washed with TBS containing 0.1 % (v/v) Tween-20 (TBS-T) three times, the membranes were incubated in the secondary antibody solution at room temperature for 1 h. The secondary antibody solutions were prepared by diluting the 1:1000 (v/v) antibodies with TBS-T containing 0.2 % ECL advance blocking agent (Amersham Biosciences Corp., Piscataway, NJ, USA). Anti-rabbit IgG, peroxidase-linked spiecies-specific donkey whole antibody (Amersham Biosciences Corp.) or anti-mouse IgG horseradish peroxidase peroxidase-linked species-specific sheep whole antibody (Amersham Biosciences Corp.) was used. The adsorbed antibodies were reacted with ECL advance Western Blotting Detection Kit (Amersham Biosciences Corp.) and detected by FUJIFILM LAS-1000plus luminoimage analyzer (FUJI PHOTO FILM Corp., Kanagawa, Japan).

To examine the specificity of antibodies, for 50 embryos at 2-cell stage were subjected to immunoblotting in which primary antibodies were preincubated with their antigen peptides for 1 h before use.

Immunocytochemistry

Oocytes and embryos were fixed in a 3.7 % paraformaldehyde in PBS for overnight at 4 °C after removal of the *zona pellucida* with acid MEMCO (Evans et al., 1995). After washing three times in PBS containing 1 mg/ml of BSA (PBS/BSA), the fixed cells were permeabilized for 15 min with PBS containing 0.5 % (v/v) Triton X-100. After washing four times in PBS/BSA, the cells were incubated for 60 min in PBS/BSA that contained 2 μg/ml primary antibody (anti-STAT5A rabbit polyclonal antibody; L-20 Santa Cruz Biotechnology) or anti-STAT5B mouse monoclonal antibody (Zymed Laboratories Inc., San Francisco, CA, USA). After washing four times in PBS/BSA, the cells were incubated with 1:100-diluted secondary antibody. For the detection of Stat5A and Stat5B, anti-rabbit IgG FITC-conjugated donkey antibody (Jackson ImmunoResearch, West Grove, PA, USA) and anti-mouse IgG FITC-conjugated goat antibody (Jackson ImmunoResearch) were used as

the secondary antibody, respectively. After washing four times in PBS/BSA, the cells were mounted on a glass slide in Vectashield anti-bleaching solution (Vector Laboratories, Burlingame, CA, USA). Fluorescence was detected using a Leica TCS SP2 laser-scanning confocal microscope.

Statistical analysis

Statistical analysis was performed by student's *t*-test.

Results

Expression of Stat5s mRNA

The temporal changes in the expression levels of Stat5A and Stat5B mRNA were examined during preimplantation development (Figure 1-1A). The relative expression levels of Stat5s are shown in Figure 1-1B. Stat5A mRNA decreased by 80 % between the MII and 4-cell stages. During the 2-cell to 4-cell stage, Stat5A mRNA was significantly decreased ($P < 0.05$) by 40 %, and then significantly increased ($P < 0.01$) five-times during the 4-cell to blastocyst stage eight-times. The expression pattern of Stat5B mRNA was similar to that of Stat5A. The relative expression level of Stat5B mRNA prominently decreased from the MII to 4-cell stage about 70 %, and then significantly increased ($P < 0.01$) eighty-times during the 4-cell to blastocyst stage twenty-times.

Expression of Stat5s protein

The amounts of Stat5A and Stat5B proteins were changed in a similar manner during preimplantation development (Figure 1-2). The amounts slightly decreased between the MII and 1-cell stage. In the 1-cell stage, the expression level of Stat5s proteins did not change between 6 h and 12 h after insemination. After the 2-cell stage, abruptly increased and were kept in high level until blastocyst stage.

Preincubation of the primary antibodies with antigen peptide resulted in the disappearance of the bands corresponding to Stat5A of Stat5B on immunoblot (Figure 1-3), indicating that the antibodies were specific.

Localization of Stat5 proteins in the preimplantation embryos

The localization of Stat5A and Stat5B was examined during preimplantation development by imminocytochemistry (Figure 1-4). No localization of the Stat5A and Stat5B were observed in the MII stage oocytes. However, the proteins were localized in nucleus in the 1-cell stage embryos. They

were detected 6 h after insemination. Between the 1- and 2-cell stages, the concentrations of Stat5s in the nucleus increased. After the 2-cell stage, Stat5s were always localized in the nucleus until blastocyst stage.

Discussion

In this chapter, I examined the expression and localization of Stat5s during mouse preimplantation development. Since in some type of cells, Stat5s were very important transcriptional regulator (Barahmand-Pour et al., 1998; Liu et al., 1998), the investigation of Stat5s expression is important in understanding the mechanisms of transcriptional regulation during preimplantation development. In the vertebrate embryos, the expression of Stat5 during embryogenesis has been reported only in neurula stage embryos of *Xenopus laevis* (Pascal et al., 2001). My finding is the first one demonstrating the expression of Stat5 in the mammalian preimplantation embryos.

RT-PCR analysis revealed that the expression of Stat5A and Stat5B were changed during preimplantation development (Figure 1-1). The relative expression levels of Stat5s mRNAs decreased between the MII and 4-cell stages, and then prominently increased from the morula stage, which suggests that maternally derived mRNAs were gradually degraded until the 4-cell stage, and the expression of Stat5s from embryonic genomes starts from morula stage. In goat mammary gland, it was reported that the expression of Stat5 was induced by the treatment with growth hormone (GH) (Boutinaud and Jammes, 2004). In mouse preimplantation embryos, it was known that GH mRNA is expressed after morula stage (Pantaleon et al., 1997). Therefore, it is likely that the expression of Stat5s were induced by GH autocrine after morula stage.

The expression patterns of Stat5A and Stat5B mRNAs were similar to each other. This similarity suggests that the expressions of Stat5A and Stat5B are regulated by a common mechanism during mouse preimplantation development. In human, their genes are present in the adjacent locus on chromosome 17. There is no difference in the function of human Stat5A and Stat5B promoters (Crispi et al., 2004). Mouse Stat5s genes are also present in the adjacent locus on chromosome 11 (Copeland et al., 1995; Cui et al., 2001). Therefore, it is likely that the mechanisms of transcriptional regulation for mice Stat5A and Stat5B are also comparable. This is consistent

with the results that the expression patterns of Stat5A and Stat5B mRNAs were similar. However, the rate of increase in the expression level of mRNA after the 4-cell stage was different between Stat5A and Stat5B. During the 4-cell to blastocyst stages, the expression level of Stat5A mRNA increased eight-times, while that of Stat5B increased twenty-times. This difference may be caused by the difference in the stability of these mRNA. Stat5A mRNA may be more labile than Stat5B mRNA. Indeed, the rate of degradation of maternally stored mRNA of Stat5A seemed to be faster than that of Stat5B after fertilization (Figure 1-1B).

Stat5s proteins were expressed in the all stages of preimplantation development. The expression patterns of Stat5s proteins did not reflect those of mRNAs. Although Stat5s protein increased between the 1- and 2-cell stages, their mRNA increased before morula stage. This difference may be caused by the change of the efficiency in translation of mRNA.

The localization of Stat5s proteins in the nucleus were detected even at the early 1-cell stage. It is known in the somatic cells that the Stat5 localization in the nucleus occurs under activation of Stat5, and the activated Stat5 can induce transcription (Darnell, 1997; James and Darnell, 1997; Liu et al., 1998; Akira, 1999). Thus, Stat5s localization in the early 1-cell stage nuclei suggests that Stat5s are activated and regulate the transcription in the early 1-cell stage. In mouse embryos, however, embryonic transcription starts at the late 1-cell stage (Aoki et al., 1997). Therefore, Stat5s seem to be localized in nucleus for preparation of the initiation of transcription during early 1-cell stage, and functional for transcription after the late 1-cell stage. After 1-cell stage, Stat5s were always localized in the nucleus until blastocyst stage, suggesting that Stat5s are continuously activated in the all stages of preimplantation embryo.

The changes in the amounts of Stat5 proteins in the nucleus were not consistent with those in the total amounts of proteins detected by immunoblotting. Although the total amounts prominently increased between the 1- and 2- cell stages, only slight increase in the nuclear concentration of the proteins was observed. This inconsistency would be caused by that Stat5s

proteins were expressed excessively after 2-cell stage, and only a part of the proteins was localized in the nucleus. It is also suggested that this small amount of activated Stat5 is sufficient for transcriptional activation in the preimplantation embryos.

In summary, the expression and activation of Stat5s has been shown in this chapter. It suggests that Stat5s signaling pathways are activated and Stat5-mediated transcription occurs during the preimplantation development. In the next chapter, I will show the mechanism by which Stat5s are activated during preimplantation development.

Table 1-1. PCR Primers

Target gene		Sequences	Product size
Stat5A	Sense	5' - ATTACACTCCTGTACTTGCGA -3'	212bp
	Antisense	5' - GGTCAAACTCGCCATCTTGG -3'	
Stat5B	Sense	5' - TCCCCTGTGAGCCCGCAAC -3'	321bp
	Antisense	5' - GGTGAGGTCTGGTCATGACT -3'	
rabbit alpha globin	Sense	5' - GCAGCCACGGTGGCGAGTAT -3'	257bp
	Antisense	5' - GTGGGACAGGAGCTTGAAAT -3'	

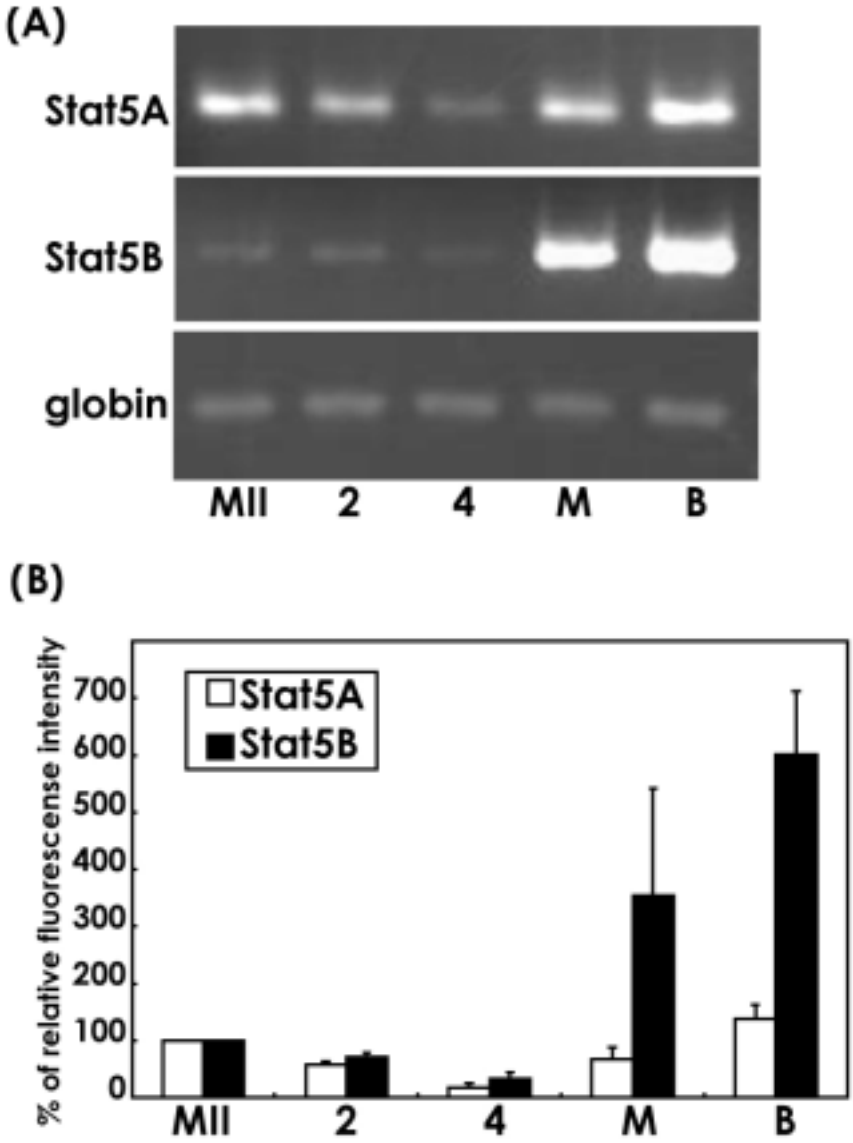

Figure 1-1. Expression patterns of Stat5s during the preimplantation development in mice. Total RNA was isolated from the oocytes at MII stage (MII) and the embryos at the stages of 2-cell (2), 4-cell (4), morula (M) and blastocyst (B) collected 31, 48, 72 and 96 h after insemination, respectively, and subjected to RT-PCR analysis for the expression of Stat5A and Stat5B. Rabbit globin mRNA was added as an external control. The PCR products were subjected to agarose gel electrophoresis followed by staining with ethidium bromide. The images of PCR bands on agarose gels (A) and the results of quantification of PCR products (B) are shown. For quantification, the relative fluorescence intensities of PCR bands were determined. The value in the MII oocyte was set as 100% and the values of other stages were calculated relative to this value. The experiment was performed three times and the results are presented as the mean with SEM.

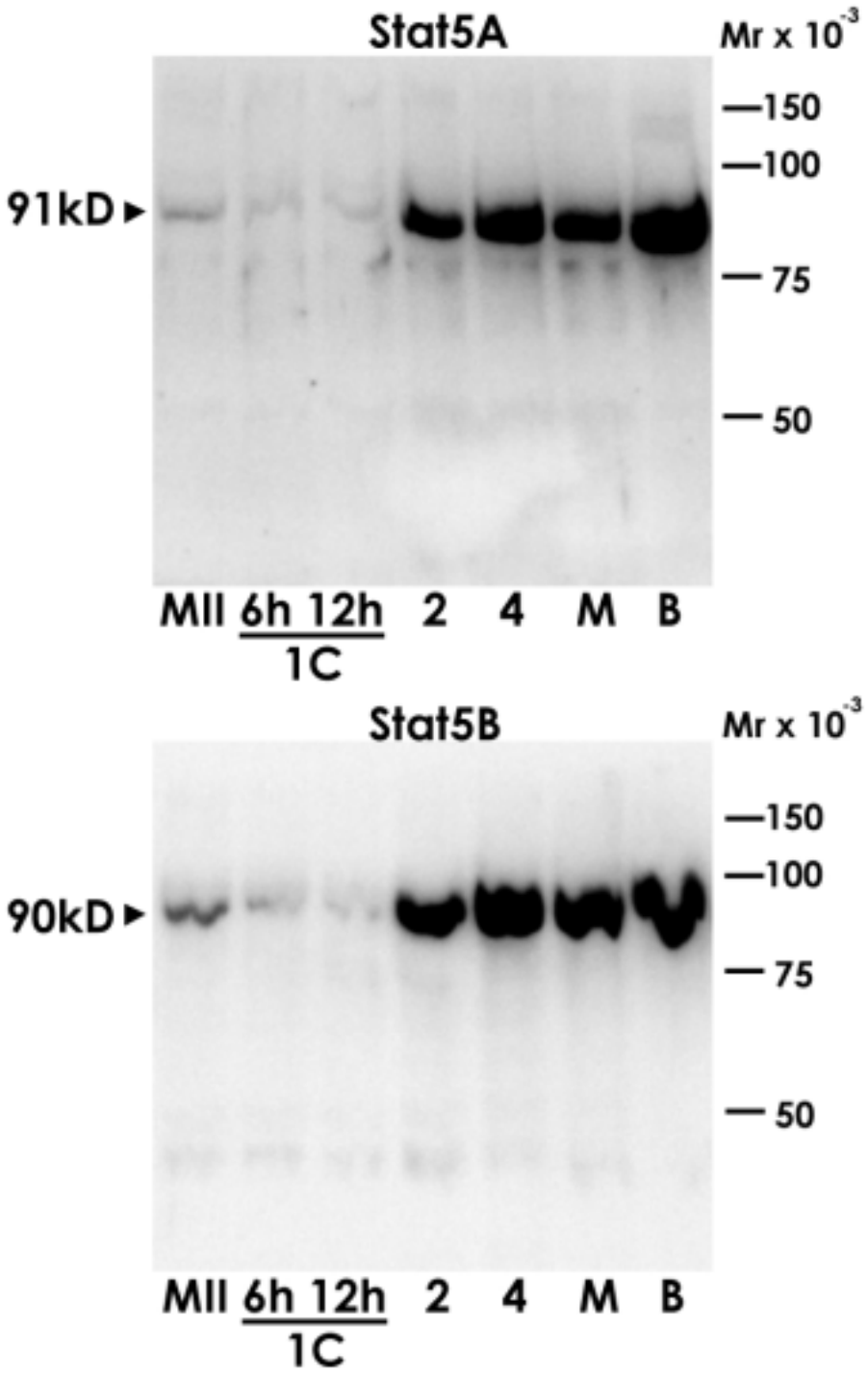

Figure 1-2. Western blot probed for Stat5 proteins.
The extracts of the oocytes at MII stage (MII) and the embryos at the stages of 1-cell (1 C; 6 and 12 h), 2-cell (2), 4-cell (4), morula (M) and blastocyst (B) collected 6, 12, 31, 48, 72 and 96 h after insemination, respectively, were subjected to immunoblotting with the antibody for Stat5A and Stat5B. Arrowheads indicate the molecular weight of Stat5A and Stat5B.

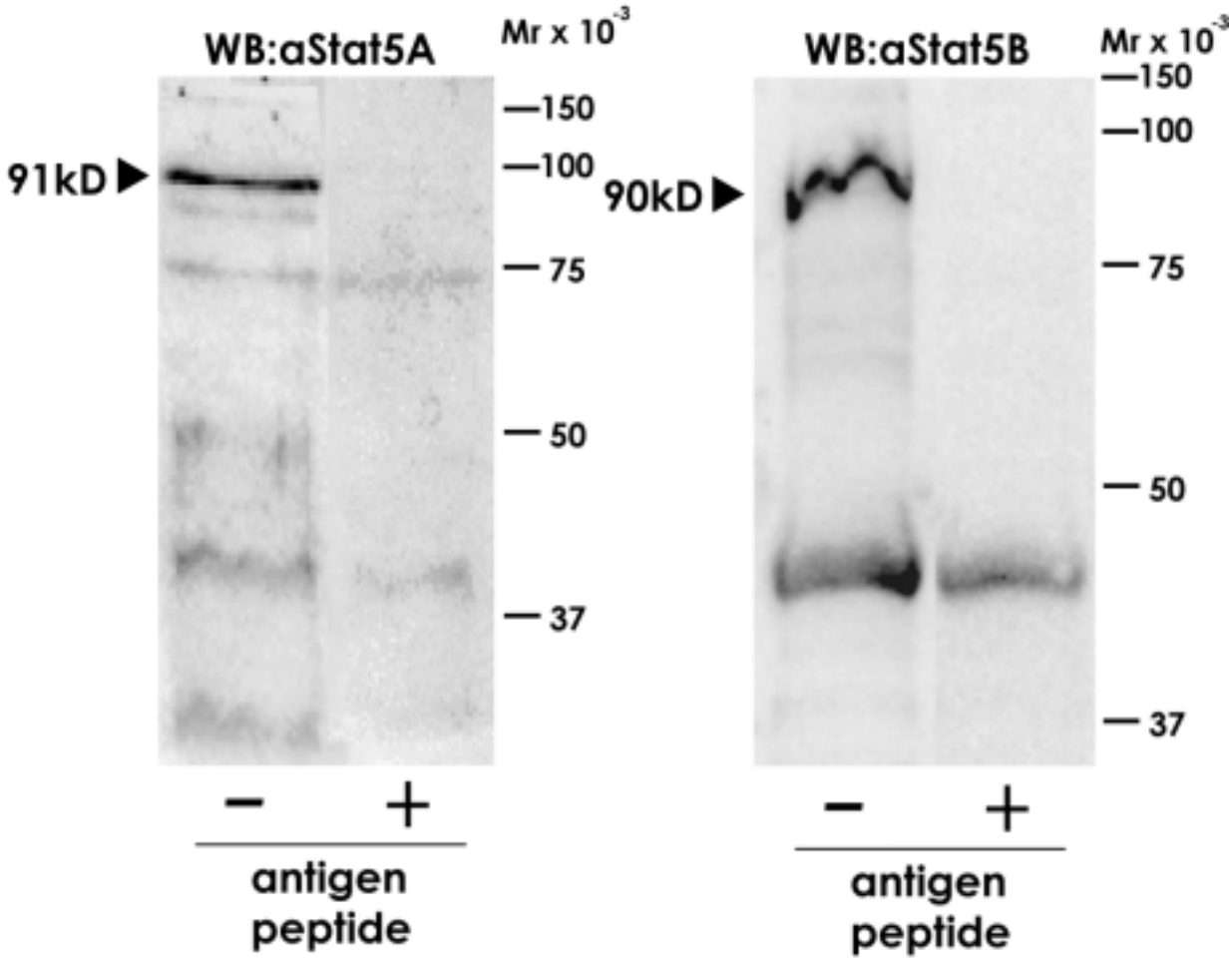

Figure 1-3. The Specificity of the antibody for Stat5A and Stat5B.
The extracts of 2-cell stage embryos were subjected to immunoblotting with the antibody for Stat5A / Stat5B. The antibody was preincubated with (+) or without (-)antigen peptides of each antibody. Arrowheads indicate the molecular weight of Stat5A and Stat5B.

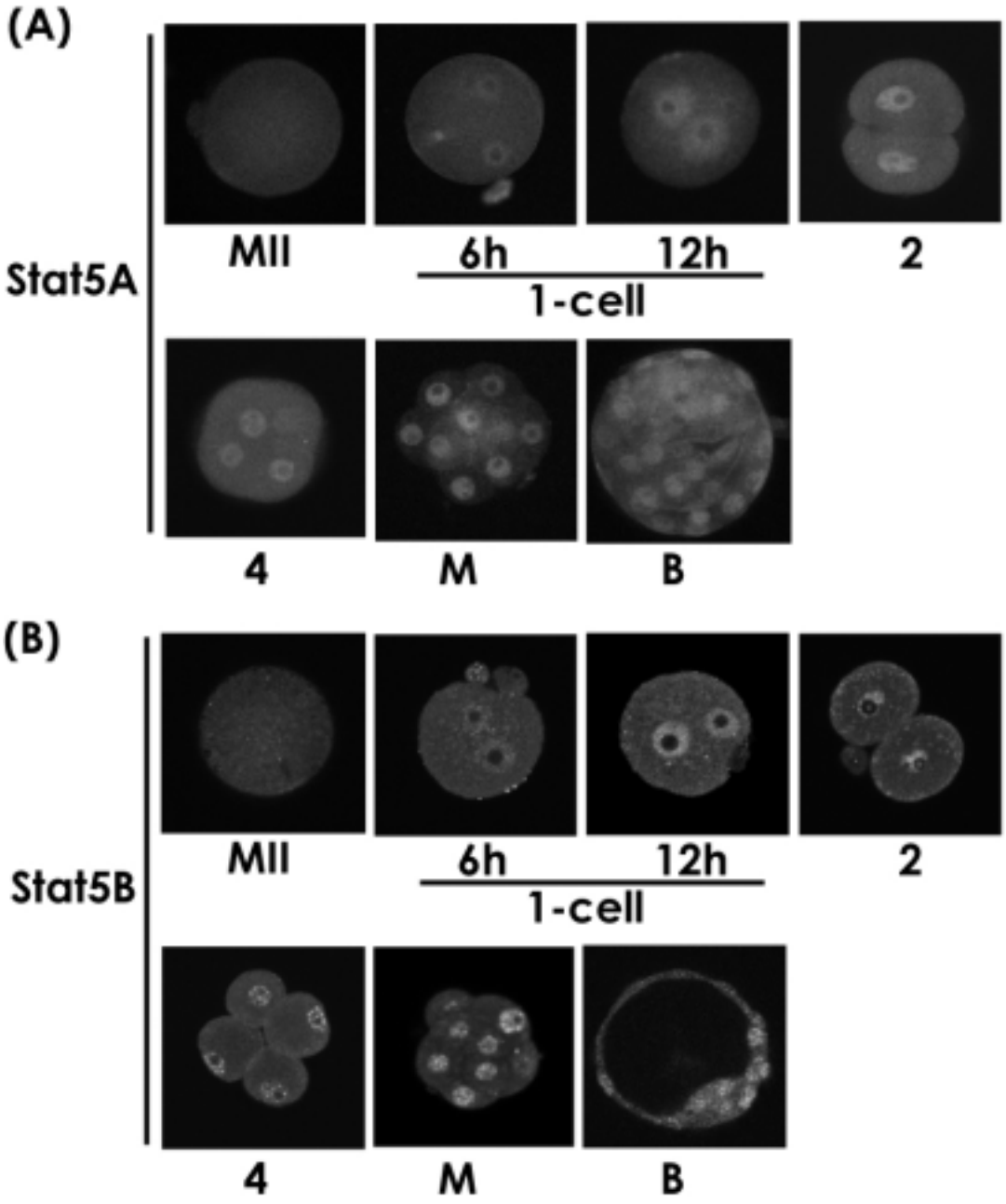

Figure 1-4. Localization of Stat5s proteins in the preimplantation embryos. The immunofluorescent confocal microscopy of mice oocytes and preimplantation embryos stained with anti-Stat5A / Stat5B antibody. The oocytes at MII stage (MII) and the embryos at the stages of 1-cell (6 and 12 h), 2-cell (2), 4-cell (4), morula (M) and blastocyst (B) collected 6, 12, 31, 48, 72 and 96 h after insemination,respectively, were stained with specific antibody for Stat5A (A) and Stat5B (B).

Chapter 2

Roles of tyrosine kinases in the activation of Stat5s during the preimplantation development

Abstract

In the previous chapter, I showed that Stat5s are expressed and activated during preimplantation development. However, the mechanisms for the activation of Stat5s remain to be elucidated. In this chapter, to investigate the mechanism regulating Stat5s activation in the preimplantation embryos, I used the inhibitors for two groups of tyrosine kinases, Jak family and Src family kinases (SFKs), which activate Stat5s. After the embryos were cultured with Ag490, a Jak kinase family inhibitor, and SU6656, a SFKs inhibitor, they were examined for the nuclear localization of Stat5s in the various stages during preimplantation development. In the 1-cell stage, Ag490 decreased the nuclear localization of Stat5B, but not Stat5A. On the contrary, in the morula stage embryos and the trophectoderm (TE) of blastocysts, Ag490 decreased the localization of Stat5A but not of Stat5B. SU6656 did not affect the localization of Stat5s until the morula stages. However, in the blastocyst stage, the localizations of Stat5s were decreased by the treatment with SU6656. The patterns of the Stat5s localization in the blastocyst stage embryos were similar to the ones with Ag490. These results show that the mechanisms for activation of Stat5s are changed during preimplantation development, and different between Stat5A and Stat5B. In addition, the inhibitors also affected the development of preimplantation embryos. The stages in which the inhibitors exerted their effects on the development and the nuclear localization of Stat5s were well coincident with each other. Their results suggested that Stat5s play roles in the regulation of preimplantation development.

Introduction

In the previous chapter, I showed that Stat5s are expressed and activated during preimplantation development. However, the mechanisms for the activation of Stat5s remain to be elucidated. In this chapter, I address the mechanism regulating Stat5s activation in the preimplantation embryos.

In the somatic cells, there are many protein tyrosine kinases that phosphorylate the tyrosine residues of Stat5s to activate them, and almost all of these kinases are classified into two groups. The first group is Jak kinase family, *i.e.* Jak1, Jak2, Jak3 and Tyk2. These activate Stat5 in the signaling pathways of a variety of cytokines, *e.g.* prolactin, growth hormone (GH), granulocyte-macrophage colony stimulating factor (GM-CSF), and etc. (Finbloom and Larner, 1995; Ihle and Kerr, 1995; Sattler et al., 1995). The second group is Src family kinases (SFKs) which consist of c-Src, c-Yes, Fyn and Lyn. SFKs activate Stat5 in the signaling pathways of a variety of cytokines, *e.g.* epidermal growth factor (EGF), transforming growth factor-alpha (TGF-α), transforming growth factor-beta (TGF-β) and etc. (Okutani et al., 2001; Buettner et al., 2002; Xi et al., 2003).

In this chapter, I use the two inhibitors for these two groups of tyrosine kinases to clarify the signalling pathways activating Stat5s during preimplantation development. The used inhibitors are Ag490, a Jak kinase family inhibitor which selectively suppresses the kinase activity of Jak2 and Jak3 (Meydan et al., 1996; Behbod et al., 2001) and SU6656, a SFKs inhibitor which selectively suppresses the kinase activity of c-Src, c-Yes, Fyn and Lyn (Blake et al., 2000). I examined the effects of these inhibitors on the development as well as the activation of Stat5s, which will reveal the role of Stat5s in the preimplantation development.

Materials and methods

Treatment with tyrosine kinase inhibitors

Sixty embryos of each developmental stages were collected into 200 µl of CZB medium and incubated with 0.2 % (v/v) of tyrosine kinase inhibitor solutions which had been prepared by dissolving Ag490 (Merck Biosciences, Darmstadt, Germany) or SU6656 (Merck Biosciences) with dimethylsulfoxide (DMSO) to make the final concentrations of 20 µM Ag490 or 5 µM SU6656. As a control, 0.2 % DMSO instead of tyrosine kinase inhibitor solution was added to the medium. The embryos were observed and the percentages of the embryos that had developed to 2-cell, 4-cell, morula, blastocyst stages at 31 h (day 2), 48 h (day 3), 72 h (day 4) and 96 h (day 5) after insemination, respectively, were determined.

Immunocytochemistry and quantification of fluorescence intensity

The procedure for immunocytochemistry was described in Chapter1. Semi-quantification analysis of the fluorescence intensity on the images of laser-scanning confocal microscopy was conducted using the NIH Image program (National Institute of Health, Bethesda, MD, USA). The average pixel value/unit area of the nucleus was subtracted by the average cytoplasmic pixel value/unit area and multiplied by nuclear dimensions to yield the relative values of fluorescence intensity. In every quantification procedure, the averaged value of the control embryo was set as 100 % and the fluorescence intensity observed in each sample was expressed relative to this value.

Results

Ag490 and SU6656 inhibit the localization of Stat5s in the nucleus

The effects of inhibiting Jak and Src kinases in the nuclear localization of Stat5s were examined by treating the embryos with their specific inhibitors, Ag490 and SU6656, respectively. The embryos were incubated with Ag490 or SU6656 starting from 6, 31, 48 and 72 h after insemination, and then examined for the nuclear localization of Stat5s at 31, 48, 72 and 96 h, respectively. The embryos that were incubated with inhibitors from 6 h after insemination were also examined at 12 h.

In the embryos that were incubated with Ag490, the different localization patterns were observed between Stat5A and Stat5B (Figure 2-1 and 2-2). By the treatment with Ag490, Stat5A localization significantly decreased in the 2-cell ($P < 0.001$; Figure 2-4A), morula ($P < 0.05$; Figure 2-6A.) and blastocyst stage ($P < 0.001$; TE and ICM; Figure 2-7A) but not in the 1-cell (Figure 2-3A) or 4-cell stages (Figure 2-5A) when compared to the control embryos without the treatment. On the other hand, Stat5B localization significantly decreased in the 1-cell ($P < 0.001$; male and female pronucleus; Figure 2-3B), 2-cell ($P < 0.001$; Figure 2-4B) and blastocyst stage ($P < 0.001$; ICM only; Figure 2-7B) but not in the 4-cell (Figure 2-5B) or morula stages (Figure 2-6B). Thus, Ag490 inhibited the localization only of Stat5B but not Stat5A in the 1-cell embryos. On the contrary, the inhibition was observed only for Stat5A but not Stat5B in the morula stage embryos. Interestingly, although the localization of Stat5A was inhibited both in ICM and TE in blastocysts, the inhibition for Stat5B was observed only in the ICM but not TE (Figure 2-2, 2-7B).

SU6656 did not affect the nuclear localization of Stat5A and Stat5B between the 1-cell and morula stages (Figure 2-3-2-6). In the blastocyst stage embryos, however the patterns of the inhibition were similar to the ones with Ag490. Although SU6656 significantly inhibited the nuclear localization of Stat5A both in ICM and TE ($P < 0.001$; Figure 2-7A), the decrease in the localization of Stat5B was observed only in ICM ($P < 0.001$) but not TE (Figure 2-7B).

Ag490 and SU6656 inhibited the development in the specific stages during preimplantation development.

The embryos were incubated with Ag490 or SU6656 starting from 6 h after insemination and examined for their development until 96 h. Although Ag490 did not affect the first cleavage, it significantly inhibited the second one ($P < 0.001$): only 30 % of embryos treated with the inhibitor developed to the 4-cell stage on day 3. After that, no embryo developed to the blastocyst stage on day 5 on which more than 50 % of control embryos developed. The treatment with SU6656 did not affect the development before blastocyst stage. However, the percentage of the embryos that had developed to the blastocyst stage on day 5 was significantly lower ($P < 0.05$) in the inhibitor-treated group than the control.

Since the effects of inhibitors on the nuclear localization of Stat5s were dependent on the developmental stage (Figure 2-3-2-7), the embryos were incubated with Ag490 or SU6656 starting from 6, 31, 48 and 72 h after insemination, and then examined for the development at 31, 48, 72 and 96 h. At first, the embryos were incubated with Ag490 or SU6656 starting from 31 h (day 2) after insemination and examined for their development until morula stage. The treatment with neither of Ag490 or SU6656 affected the cleavage of embryos between the 2-cell and morula stage (Figure 2-8B). Next, the embryos were incubated with the inhibitors starting from 48 h (day 3), and examined for their development until blastocyst stage. Although the treatment with neither of Ag490 or SU6656 affected the development until morula stage on day 4, the treatment with any of inhibitors significantly inhibited the development to the blastocyst on day 5 ($P < 0.05$). The percentage of the embryos that had developed to the blastocyst stage was lower in the Ag490-treated group than the control one by 25 %, and in the SU6656-treated group than the control by 20 % (Figure 2-8C). Finally, the embryos were incubated with inhibitors starting from 72 h (day 4) after insemination, and examined for their development until blastocyst stage. Both of

Ag490 and SU6656 significantly inhibited the cleavage between day 4 and 5 ($P < 0.05$) (Figure 2-8D).

Discussion

By the experiments using immunocytochemistry, it was revealed that tyrosine kinase inhibitors, Ag490 and SU6656, inhibited the translocation of Stat5s into the nucleus during the preimplantation development (Figure 2-1, 2-2). However, the patterns of the inhibition by these two inhibitors were different among the stages of preimplantation development, between Stat5A and Stat5B, and between TE and ICM in the blastocysts.

In terms of the stage of development, Ag490 inhibited the nuclear localization of Stat5A in the 2-cell, morula and blastocyst stages but not in the 1-cell or 4-cell stage, and that of Stat5B in the 1-cell, 2-cell and blastocyst stages but not 4-cell or morula stage (Figure 2-1 and 2-2). These results suggested that Jak kinase regulates the activation of Stat5A or Stat5B in specific stages during preimplantation development. Moreover, the treatment with SU6656 inhibited the Stat5s activation only in the blastocyst stage, but not other stages (Figure 2-1, 2-2). These results suggested that the mechanisms of activation of Stat5s changed during the preimplantation development. The activities of the upstream kinases involved in the activation of Stat5s are regulated by cytokine and cytokine receptor in the signaling pathways. Thus, it was suggested that the expressions of cytokines and cytokine receptors that involve Stat5s in their signaling pathways are changed during the preimplantation development.

On the other hand, in terms of the difference between Stat5A and Stat5B, the mechanisms for their activation seem to be different between Stat5A and Stat5B during the preimplantation development. In the 1-cell, morula and blastocyst stages, the patterns of the inhibition for the nuclear localization by the treatment with inhibitors were different between Stat5A and Stat5B (Figure 2-3, 2-6 and 2-7). In the 1-cell stage, Ag490 inhibited the localization of Stat5B in the nucleus, but not Stat5A (Figure 2-3). In contrast, in the morula and TE of blastocyst, Ag490 decreased the localization of Stat5A, but not Stat5B (Figure2-6 and 2-7). SU6656 also inhibited the nuclear localization of Stat5A, not but Stat5B, in the blastocysts (Figure 2-7). Although in somatic cells, Stat5A and Stat5B are activated by common mechanisms in many cytokines signaling

pathways, it was also known that some cytokine receptors activate only one of these two species. The receptors for transforming growth factor-alpha (TGF-αR) and epidermal growth factor (EGFR) selectively activate Stat5B, but not Stat5A in epithelial cells (Leong et al., 2002). Insulin receptor (IR) also activates Stat5B, but not Stat5A in rhabdomyosarcoma cells (Storz et al., 1999). On the contrary, granulocyte-macrophage colony stimulating factor receptor (GM-CSFR) preferentially activates Stat5A, but not Stat5B in human blood monocytes (Rosen et al., 1996). It seems that the signaling pathways via cytokine receptors that selectively activate the one of Stat5s, *e.g.* EGFR and GM-CSFR as described above, are functional in addition to the pathways activating both of Stat5s during the preimplantation development.

In terms of the cell lineage, the difference in the inhibition of Stat5s nuclear localization between TE and ICM suggested that the mechanisms for the activation of Stat5s are different between in these two lineages in the blastocysts, In the Ag490-treated blastocysts, the localization of Stat5B in the nucleus was decreased in ICM, but not TE (Figure 2-7). This difference may be caused by the mechanisms regulating the differentiation of the cell lineages in the blastocyst stage.

Ag490 and SU6656 did not affect the translocation of Stat5A into the nucleus in the 1- and 4-cell stage embryos, and that of Stat5B in the 4-cell, morula and TE of blastocyst stage embryos (Figure 2-3 - 2-7). Probably, in these stages, Stat5s are activated by both of Jak and Src family kinases, redundantly, or other upstream kinases that are not inhibited by Ag490 and SU6656, *e.g.* Jak1 and Tyk2 (Liu et al., 1997; Darnell, 1997; Akira, 1999). There are a number of reports demonstrating the signaling pathways that involve Jak1 and Tyk2 to regulate cell growth. In Ba/F3 cell, a murine lymphoid cell lineage, Jak1 activates Stat5 to regulate the cell growth in interleukin-4 mediating signaling pathway (Friedrich et al., 1999) and in interleukin-9 mediating signaling pathway (Demoulin et al., 2000). Tyk2 is involved in the Stat5 activating mechanism via interleukin-12 mediating signaling in myeloid leukemia cell (Morita et al., 1996). Finally, it have been reported that growth hormone (GH), granulocyte-colony stimulating factor (G-CSF), interleukin-2, 5, 7, 9, 10, 13 and etc. activate Stat5s via Jak1, and that thrombopoietin (TPO),

interferon-α/ß, and interleukin-10 activate Stat5s via Tyk2 (reviewed in Grimley et al., 1999).

The stages in which the inhibitors exerted their effects on the development and the nuclear localization of Stat5s were well coincident with each other. Ag490 affected the development between the 2- and 4-cell stages, and between the morula and blastocyst stages (Figure 2-8), and also affected the nuclear localization of Stat5s in the similar stages (Figure 2-1, 2-2 and 2-8). Although SU6656 did not prevent neither the development nor the nuclear localization of Stat5s before blastocyst stage, it affected both of the nuclear localization of Stat5s and the development in the blastocyst stage (Figure 2-7). The correlation between the inhibition of the localization of Stat5s in the nucleus and the development during the preimplantation development suggests that the Stat5s are involved in the mechanism for development in the preimplantation embryos.

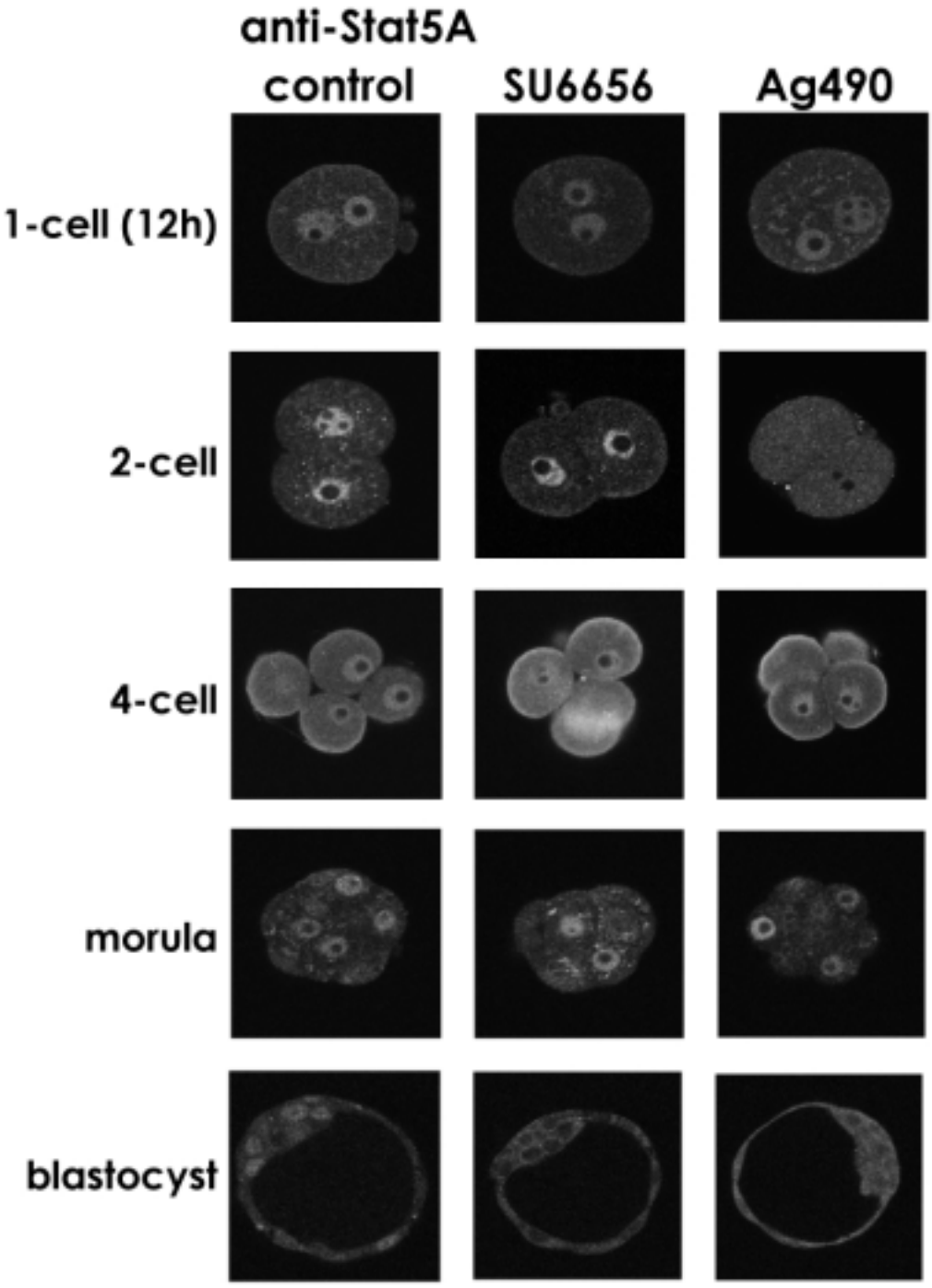

Figure 2-1. The changes of Stat5A localization in the presense of tyrosine kinase inhibitors, Ag490 or SU6656, during the preimplantation development in mice.
The embryos were incubated with Ag490 or SU6656 starting from 6, 31, 48 and 72 h after insemination, and then examined for the localization of Stat5A at 31 (2-cell), 48 (4-cell), 72 (morula) and 96 h (blastocyst), respectively. The embryos that were incubated with inhibitors from 6 h after insemination were also observed at 12 h (1-cell (12 h)). The embryos were subjected to immunocytochemistry with anti-Stat5A antibody and observed by using a laser-scanning confocal microscope.

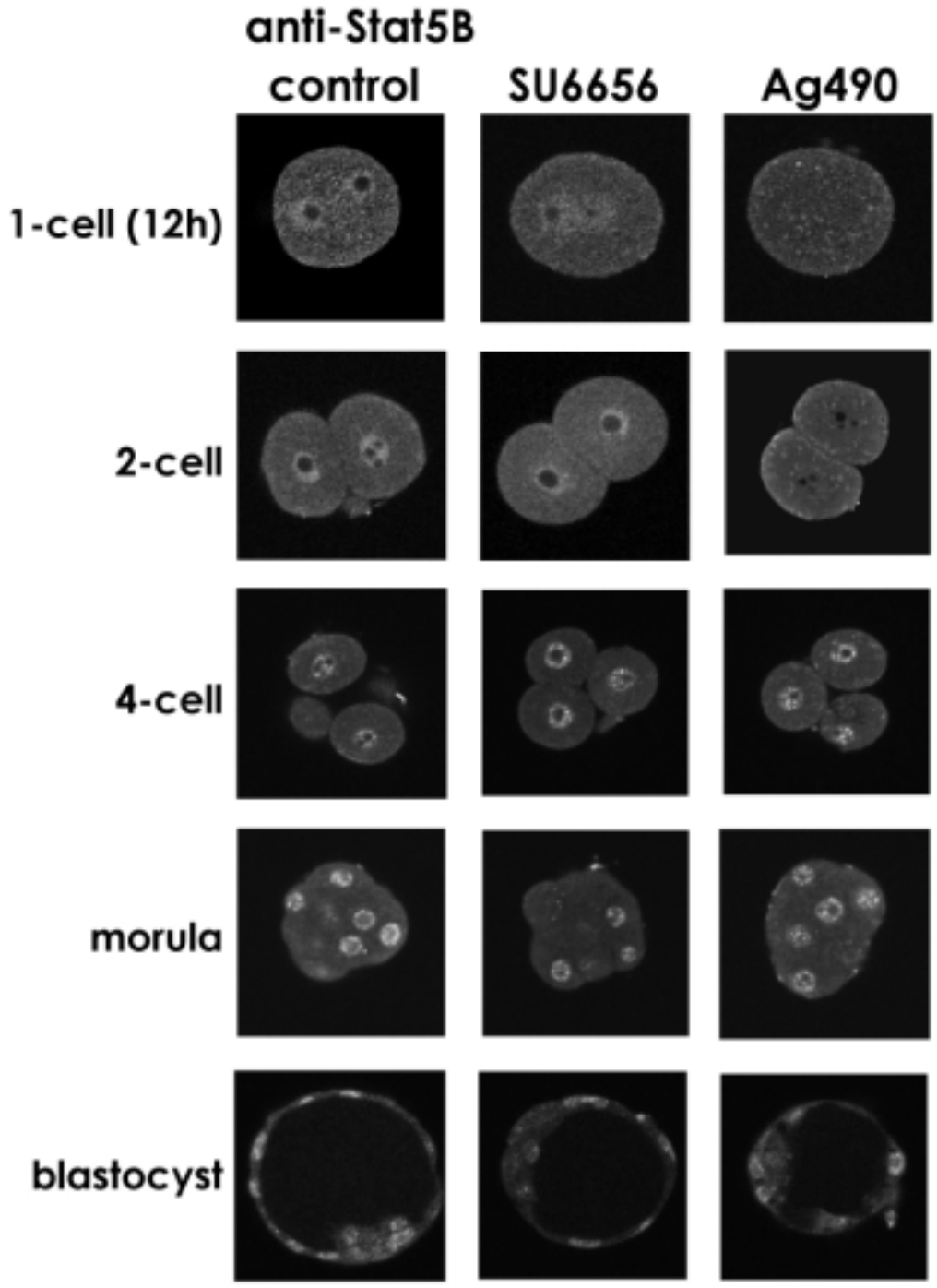

Figure 2-2. The changes of Stat5B localization in the presense of tyrosine kinase inhibitors, Ag490 or SU6656, during the preimplantation development in mice.
The embryos were incubated with Ag490 or SU6656 starting from 6, 31, 48 and 72 h after insemination, and then examined for the localization of Stat5B at 31 (2-cell), 48 (4-cell), 72 (morula) and 96 h (blastocyst), respectively. The embryos that were incubated with inhibitors from 6 h after insemination were also observed at 12 h (1-cell (12 h)). The embryos were subjected to immunocytochemistry with anti-Stat5B antibody and observed by using a laser-scanning confocal microscope.

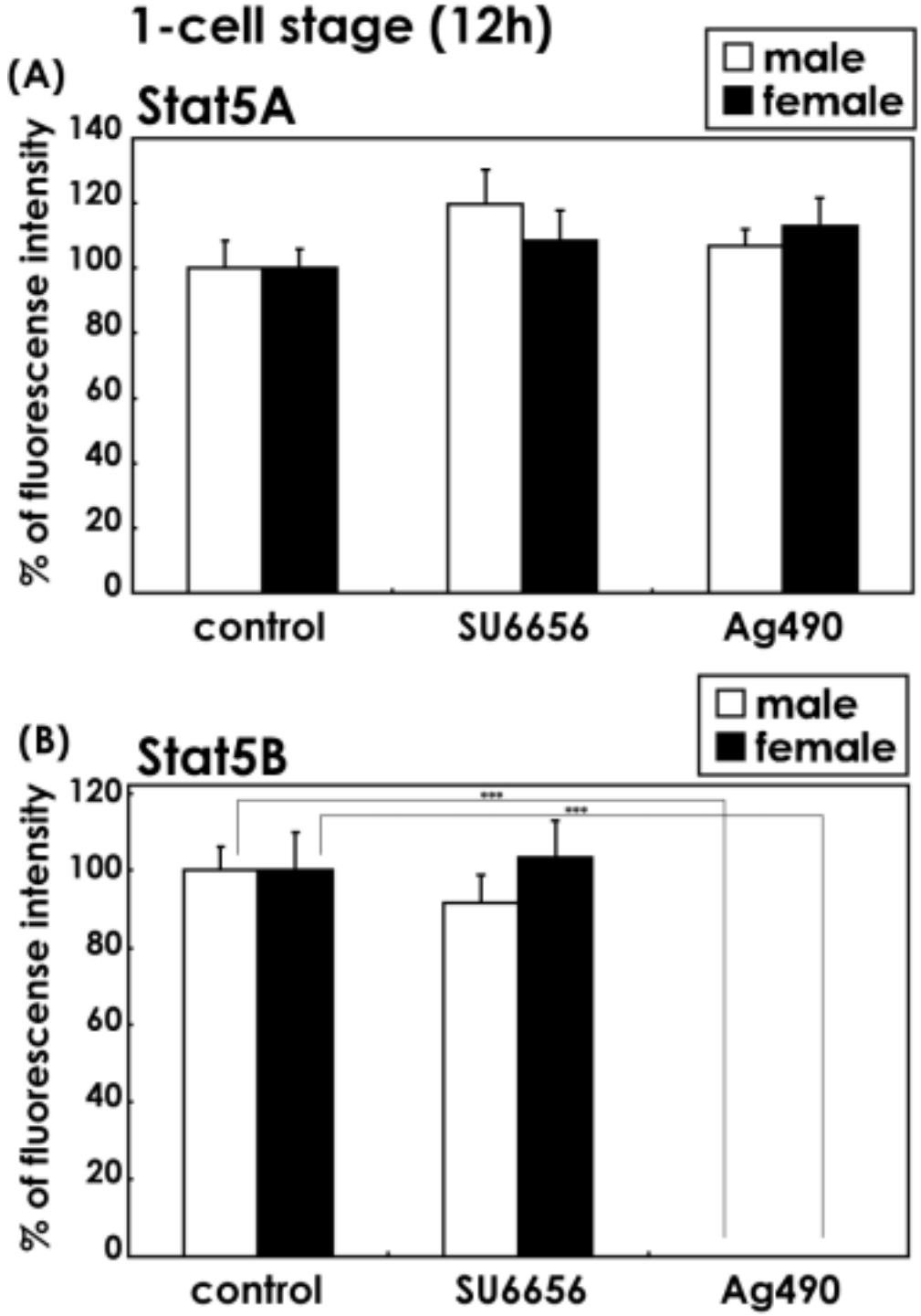

Figure 2-3. Quantification of nuclear Stat5s in the 1-cell stage embryos treated with Ag490 or SU6656.
The embryos were cultured with Ag490 or SU6656 from 6 to 12 h after insemination, followed by immunocytochemistry with anti-Stat5A antibody (A) or anti-Stat5B antibody (B). For quantification, the relative fluorescence intensities of nucleus were determined. The averaged values of controls were set as 100% and the values of others were calculated relative to this value. The results are presented as the mean with SEM. n=15. The open and closed columns represent the male and female pronucleus. ***;$P < 0.001$

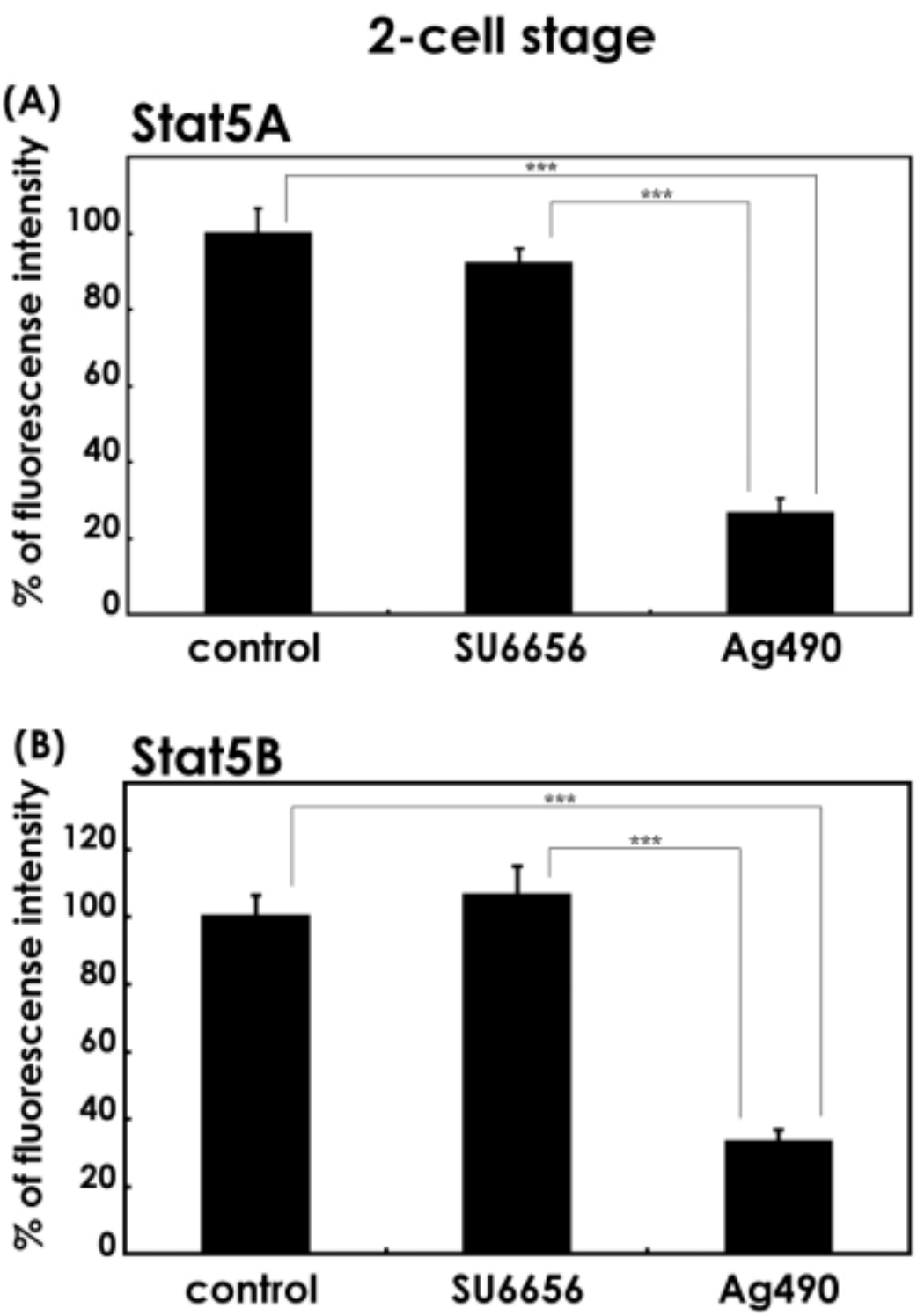

Figure 2-4. Quantification of nuclear Stat5s in the 2-cell stage embryos treated with Ag490 or SU6656.
The embryos were cultured with Ag490 or SU6656 from 6 to 31 h after insemination, followed by immunocytochemistry with anti-Stat5A antibody (A) or anti-Stat5B antibody (B). For quantification, the relative fluorescence intensities of nucleus were determined. The averaged values of controls were set as 100% and the values of others were calculated relative to this value. The results are presented as the mean with SEM. n=20. ***;$P < 0.001$

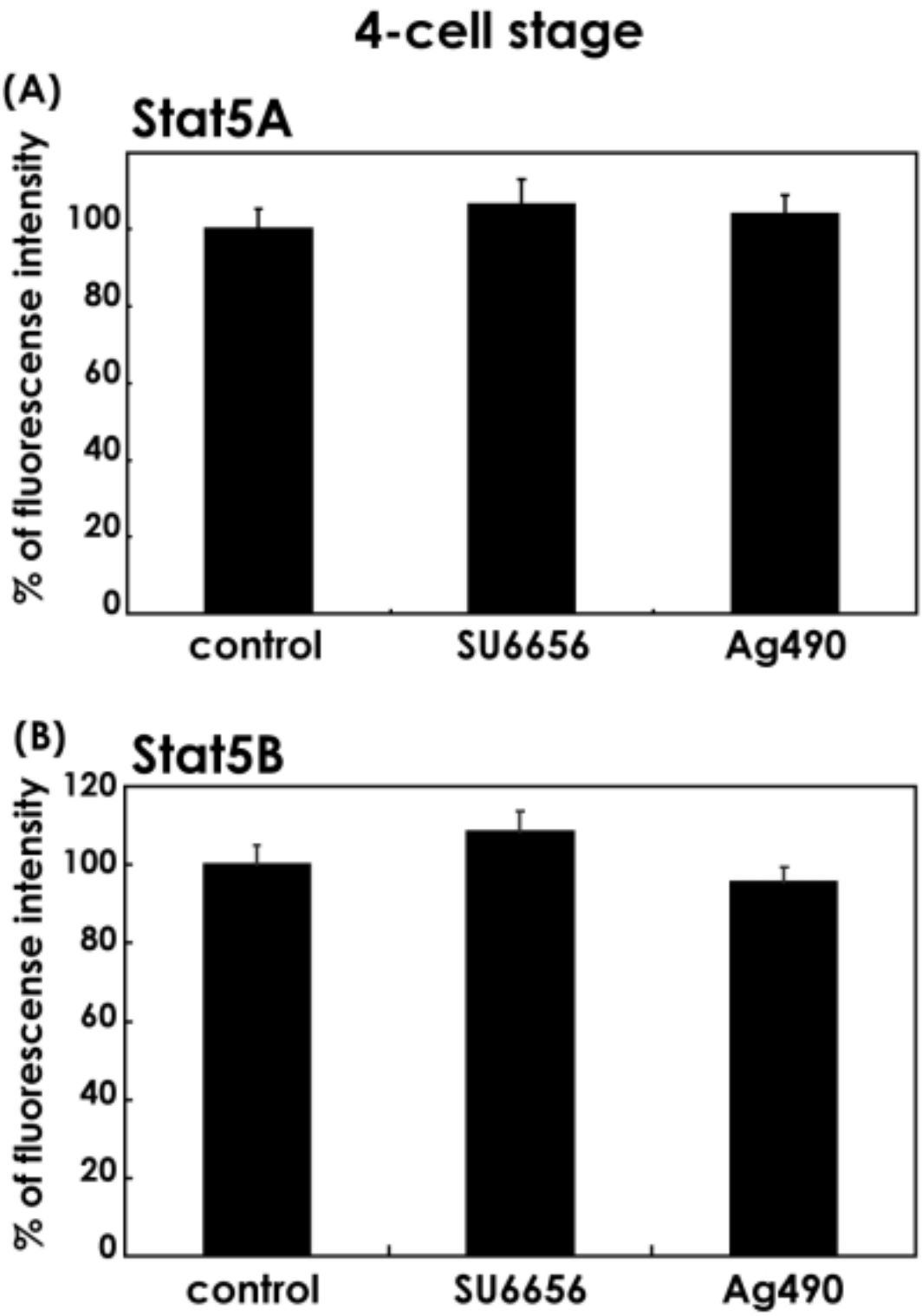

Figure 2-5. Quantification of nuclear Stat5s in the 4-cell stage embryos treated with Ag490 or SU6656.
The embryos were cultured with Ag490 or SU6656 from 31 to 48 h after insemination, followed by immunocytochemistry with anti-Stat5A antibody (A) or anti-Stat5B antibody (B). For quantification, the relative fluorescence intensities of nucleus were determined. The averaged values of controls were set as 100% and the values of others were calculated relative to this value. The results are presented as the mean with SEM. n=60.

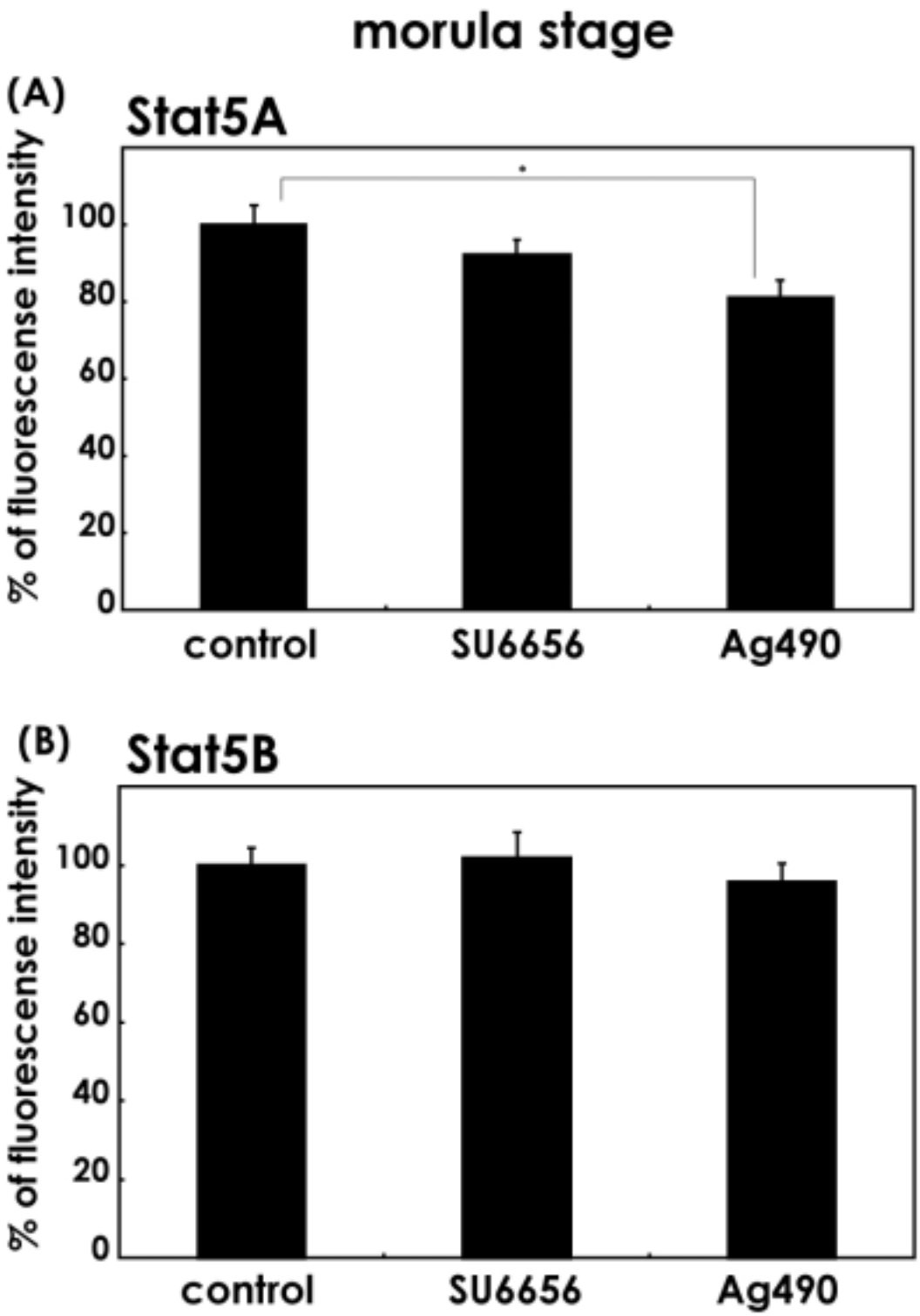

Figure 2-6. Quantification of nuclear Stat5s in the morula stage embryos treated with Ag490 or SU6656.
The embryos were cultured with Ag490 or SU6656 from 48 to 72 h after insemination, followed by immunocytochemistry with anti-Stat5A antibody (A) or anti-Stat5B antibody (B). For quantification, the relative fluorescence intensities of nucleus were determined. The averaged values of controls were set as 100% and the values of others were calculated relative to this value. The results are presented as the mean with SEM. n=60. *;$P < 0.05$

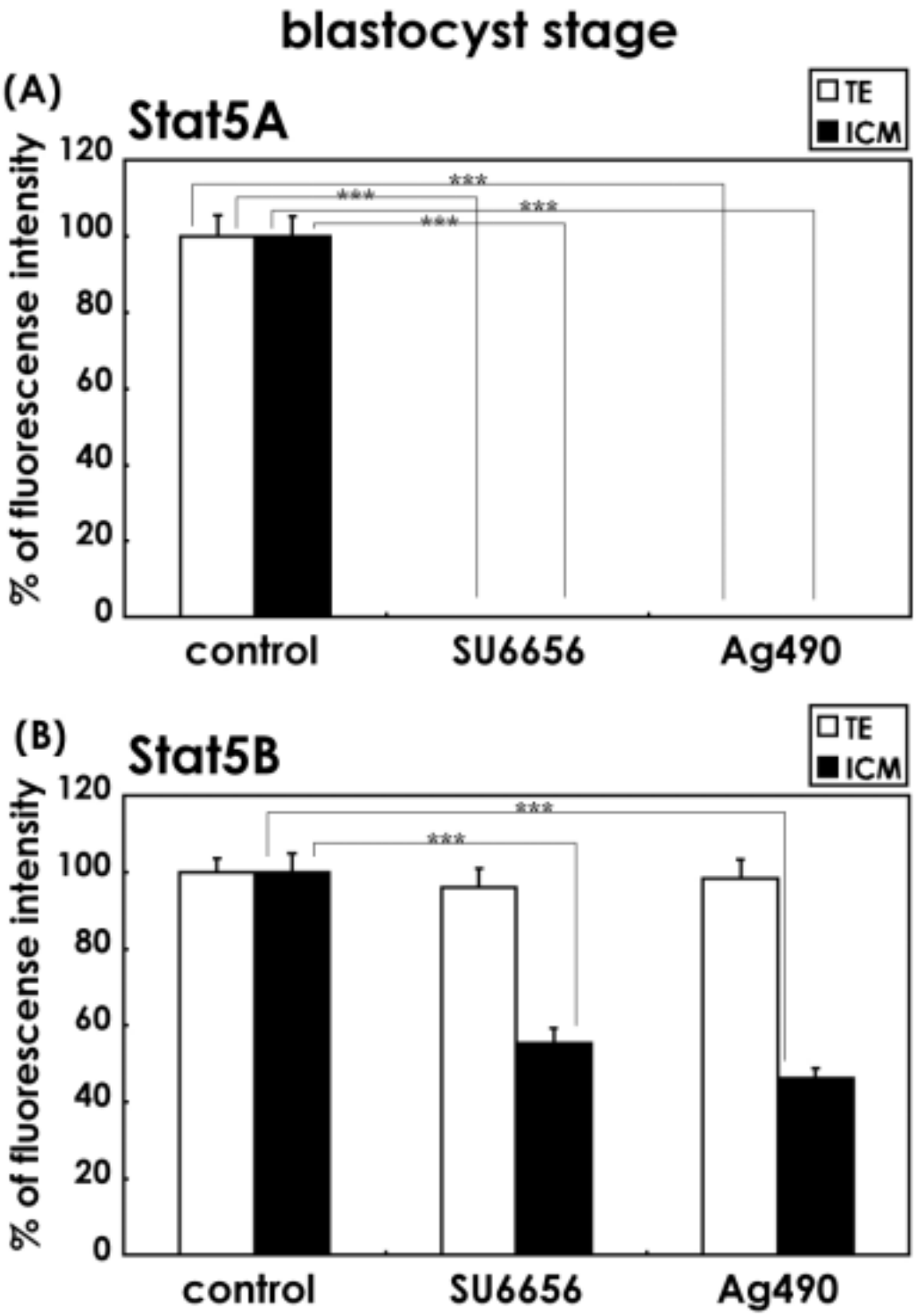

Figure 2-7. Quantification of nuclear Stat5s in the blastocyst stage embryos treated with Ag490 or SU6656.
The embryos were cultured with Ag490 or SU6656 from 72 to 96 h after insemination, followed by immunocytochemistry with anti-Stat5A antibody (A) or anti-Stat5B antibody (B). For quantification, the relative fluorescence intensities of nucleus were determined. The averaged values of controls were set as 100% and the values of others were calculated relative to this value. The results are presented as the mean with SEM. n=60. The open and closed columns represent the TE and ICM nucleus.
***;$P < 0.001$

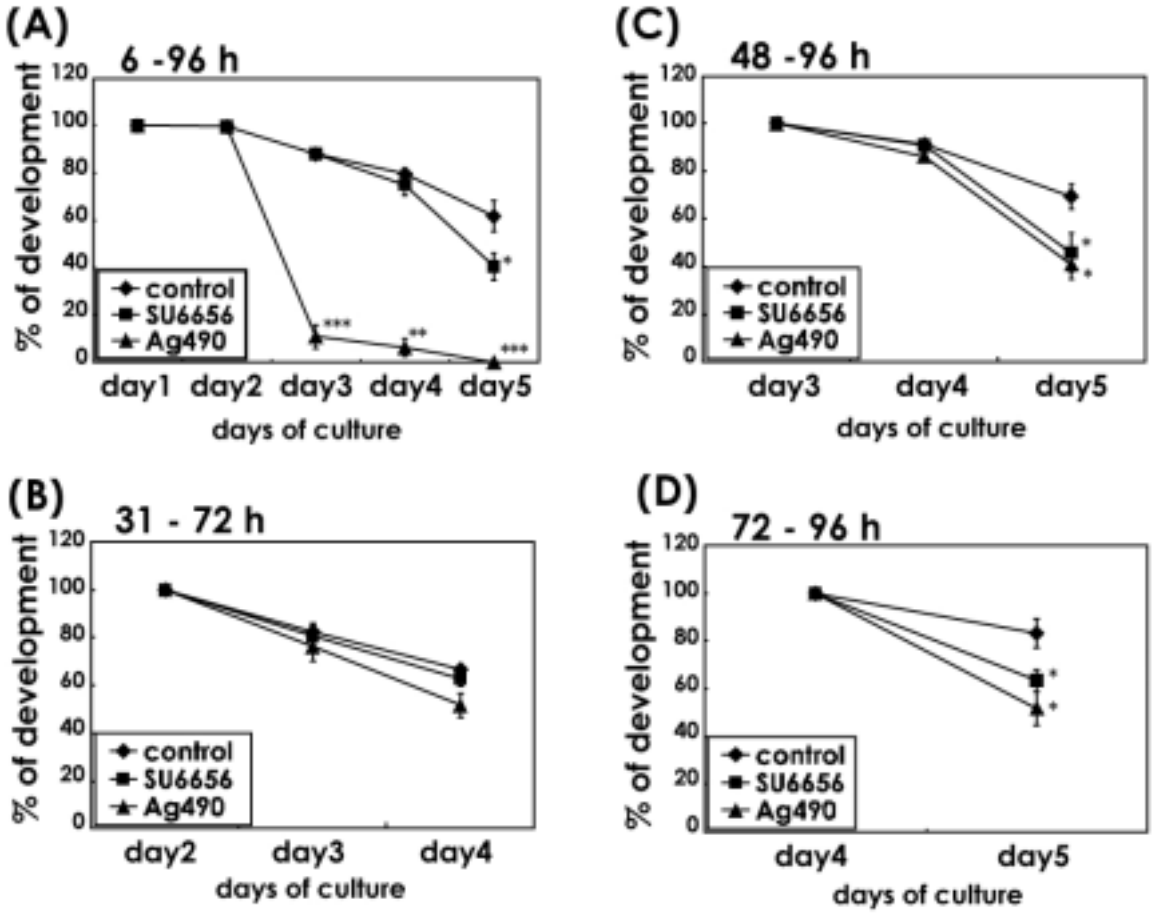

Figure 2-8. Effects of the treatment with Ag490 and SU6656 on the development of mouse preimplantation embryos.
The embryos were cultured with Ag490 or SU6656 from 6 to 96 h (A), 31 to 72 h (B), 48 to 96 h (C) and 72 to 96 h (D). The percentages of the embryos that had developed to 2-cell, 4-cell, morula, blastocyst stages at 31 h (day2), 48 h (day3), 72 h (day4) and 96 h (day5) after insemination, respectively, were determined. The experiment was performed four times. The results are presented as the mean with SEM. *, **, *** indicate the significant differences from the corresponding controls at $P < 0.05$, 0.01, 0.0001, respectively.

Chapter 3

Role of Jak-Stat5 signaling pathway in the gene expression of the 2-cell embryos

Abstract

In the previous chapter, I showed that Ag490, a Jak kinase selective inhibitor, affected the localizations of both Stat5A and Stat5B in the nucleus of the mouse 2-cell embryos. In this chapter, I examined whether Jak-Stat5 signaling pathway is functional in the regulation of gene expression at the 2-cell stage. The results of the *in vitro* transcription assay showed that the treatment with Ag490 did not affect the total transcriptional activity. Moreover, the oligo DNA microarray analysis for 31,000 genes revealed that only a small number of the genes were changed in their expression level by the treatment with Ag490. However, the real-time PCR analysis following the reverse-transcription revealed that the expressions of several target genes of Jak-Stat5 signaling pathway, *i.e.*, ß-casein and tumor necrosis factor-beta (TNF-ß) were selectively inhibited in the 2-cell embryos by the treatment with Ag490. These results suggest that Jak-Stat5 signaling pathway is functional in the gene expression in the 2-cell stage embryos. Taken together with the results in the chapter 2 that Ag490 inhibited the proliferation between the 2- and 4-cell stages, Jak-Stat5 signaling pathway would be involved in the mechanism regulating the preimplantation development, *via* the expression of their downstream genes.

Introduction

In the previous chapters, the activation of Stat5 signaling pathway *via* Jak- or Src family kinases was shown in the mouse preimplantation embryos. In the 2-cell stage embryos, Ag490, a Jak kinases selective inhibitor affected the localizations of both Stat5A and Stat5B in the nucleus (Figure 2-1 and 2-2 in chapter 2). During the 2-cell stage, maternal RNA is drastically degraded (Bachvarova and Moy, 1985), while the amounts of mRNA transcribed from zygotic genome increases. In mouse, the transition from maternal to embryonic control of development occurs in the 2-cell stage embryo (Flach et al., 1982; Bolton et al., 1984; Schultz, 1993; Schultz et al., 1999; Henery et al., 1995). Since the treatment with Ag490 inhibited the development of the embryos between 2- and 4-cell stages (Figure 2-8 in chapter 2), the transcripts of which expressions are regulated by Jak-Stat5 signaling pathway is likely to play roles in the regulations of the development during these stages. In the Jak-Stat5 signaling pathway, Stat5s proteins are phosphorylated on the tyrosine residues in the C-terminal domain by Jak kinases. Phosphorylated tyrosine residue in Stat5s binds to the Src-homology 2 (SH2) domain of the counterpart Stat5, to dimerize. Stat5s dimer translocates to the nucleus, binds to the target sequences in the promoter of specific gene named "IFN-gamma activation site (GAS)-like elements" that was composed by the sequences of "TTCNNNGAA" (James and Darnell, 1997; Akira, 1999; Grimley et al., 1999; Imada et al., 2000), and induces the transcription of the target genes. Thus, it is likely that the inhibition of Jak-Stat5 signaling pathway by Ag490 affects the expression of the specific genes those have GAS-like elements in their promoter. Therefore, to investigate the involvement of Jak-Stat5 in the regulation of gene expression, I examined the change in the profile of gene expression in the 2-cell stage embryos by the treatment with Ag490.

Materials and Methods

In vitro transcription assay

All treatments were performed at room temperature unless otherwise specified. The 2-cell stage embryos were washed three times with physiological buffer (PB; 1 mM $MgCl_2$, 100 mM $KC_2H_3O_2$, 30 mM KCl, 1 mM DTT, 0.2 mM PMSF, 1 mM Na_2ATP, 10 mM Na_2HPO_4 and 50 units/ml of RNasin Inhibitor (Promega)) (Jackson et al., 1993; Ferreria and Carmo-Fonseca, 1995) and the plasma membrane was permeabilized by treating the embryos for 1-2 min with 0.05 % Triton X-100 in PB. Following this treatment, the embryos were briefly washed three times with PB and then transferred to PB containing 1 mM ATP, 0.4 mM each of GTP, CTP, and BrUTP and 1 mM $MgCl_2$. After a 15 min incubation at 33 °C, the embryos were washed briefly three times with PB and the nuclear membrane was permeabilized by the treatment with PB containing 0.2 % Triton X-100 for 3 min. The embryos were then washed with PB three times and fixed in a 3.7 % paraformaldehyde in PB for 1 h.

The incorporated BrUTP was detected by immunostaining with anti-BrdU monoclonal antibody. The embryos were washed five times in 15 µl drops of PBS containing 3 mg/ml of BSA (PBS/BSA) over a period of 15 min and then incubated for 45 min with 2 µg/ml of the anti-BrdU monoclonal antibody (Boehringer-Mannheim, Mannheim, Germany). The embryos were then washed four times with PBS/BSA over the course of 10 min and subsequently incubated in PBS/BSA containing 0.5 µg/ml anti-mouse IgG antibody conjugated with Cy5 (Jackson ImmunoResearch) for 45 min.

Fluorescence was detected by using a Leica TCS SP2 laser-scanning confocal microscope and quantified in the protocols that are described in chapter2.

Preparation of cDNA samples for microarray analysis

Total RNA was isolated from 250 MII stage oocytes, 2-cell stage embryos that had been incubated with Ag490 starting from 6 h after insemination, and control 2-cell stage embryos. The 2-cell stage embryos were collected at 31 h. The control 2-cell embryos were prepared by incubating them with 0.2 % DMSO instead of Ag490 from 6 to 31 h. The protocol of total RNA isolation was shown in chapter 1. The RNA samples were purified using Nucleospin RNA II Kit (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) according to manufactured protocols. Purified RNA samples were subjected to reverse-transcription using Super SMART PCR cDNA Synthesis Kit (Becton, Dickinson and Company) and SUPERase in Rnase inhibitor (Ambion, Inc., Austin, TX, USA).

Synthesized cDNA samples were amplified by PCR in the 25 cycles. PCR was carried out under the protocols of Super SMART PCR cDNA Synthesis Kit (Becton, Dickinson and Company). Amplified cDNA samples were purified by using NucleoSpin Extraction Kit (Becton, Dickinson and Company) and then used as the cDNA template for oligo microarray hybridization.

Oligo microarray hybridization

I used the microarray that was spotted the oligo DNAs for the 31,000 genes. In this experiment, I examined the three groups of cDNA samples prepared from the MII oocytes, the 2-cell embryos that were treated with Ag490 and the control 2-cell embryos, comparing two of those three groups in any combinations. The cDNA sample was divided into 2 portions and each of them was labeled with Cyanine 3 (Cy3) or Cyanine 5 (Cy5). To control the difference in the sensitivities for the fluorescence detection between these two dyes, I performed double microarray assays in which the dyes were swapped in the single comparison. The averaged values in these double microarray assays were used for analysis. These experiments were performed twice using independent cDNA samples in each comparison. All treatments were performed at room temperature unless otherwise

specified. In pre-hybridization, oligo microarray was incubated in pre hybridization buffer that containing 5 × SSC, 0.1 % SDS and 0.1 % BSA, at 42 ° C for 1 h. Hybridization was performed at 42 °C for 17 h in the Hybridization solution (Cy3-labelled and Cy5-labelled cDNA, 15 % formamide, 5 × SSC, 0.5 % SDS and 20 μg/μl Acetylated BSA (Invitrogen Corp.)). Following hybridization, the oligo microarray was washed once in 2 × SSC / 0.1% SDS solution at 42 °C for 5 min, and washed once in 0.1 × SSC / 0.1% SDS solution for 10 min. Then, oligo microarray was washed twice in 0.1 × SSC solution for 2 min, by using a Wash Station (TeleChem International, Sunnyvale, CA). After washing, oligo microarray was dried by centrifugation. Hybridization images were detected using a ScanArray Express (Perkin Elmer) fluorescence laser scanner.

Microarray analysis

Fluorescence intensities on the oligo microarray were quantified using QuantArray 3.0 (Perkin Elmer). Of all genes in each array, the genes whose expression levels were lower than those of negative control spots were removed. The ratios of the signal intensities between two templates were calculated from Lowess normalization.

Quantification of the amounts of mRNA by real-time, fluorescence-monitored RT-PCR

The relative amounts of mRNA were determined by real-time PCR by using Smart Cycler System (TaKaRa Bio Inc.) following reverse-transcription. Except for ß-casein, the reaction mixture was made as described in chapter 1. Sequences of each gene specific primers are shown in Table 3-1. For the detection of the double-strand DNA, 1/30000 (v/v) of SYBR Green I (BioWhittaker Molecular Applications, Maine, USA) was added to the PCR mixtures. For ß-casein detection, the reaction mixture (25 μl) was made of 2.5 μl of 10x PCR buffer, 2 μl of 2.5 mM dNTP mix, 1.5 μl of 25 mM $MgCl_2$, 0.25 μl of EX Taq HS (Takara Bio Inc.), 2.5 μl each of 3 pM gene-specific sense and antisense primers, 2.5 μl of 2 pM gene-specific TaqMan probe and 2 μl of cDNA template. The

sequences of the PCR primers and TaqMan probe used are shown in Table 3-1. PCR was performed using 35 cycles containing the steps of denaturation at 95°C for 15 s, annealing for 15 s, extension at 72 °C for 20 s, and measurement of fluorescence for 6 s. The annealing and measuring temperatures were shown in Table 3-1. The relative amounts of each cDNA in the samples were determined by using a cDNA standard curve obtained by the amplification of a threefold dilution series of a cDNA.

Results

The treatment with Ag490 did not show significant effect on the total transcriptional activity in the 2-cell embryos.

In chapter 2, I showed that Ag490, a selective inhibitor of Jak family kinases, affected the localization of Stat5s in the nucleus in the 2-cell stage embryos. Since Stat5s function as transcription factors, it is likely that Ag490 inhibits the transcription of the genes targeted by the Jak-regulating signaling pathway in the 2-cell stage embryos. Thus, I examined the total transcriptional activity in the embryos that were incubated with or without Ag490, using the BrUTP incorporation assay.

Since Jak-mediating signaling pathways stimulate the transcription of particular target genes, it was expected that the inhibition of Jak kinase by Ag490 would affect the expression of some specific genes, but not global ones. The results of in vitro transcriptional activity assay was consistent with this hypothesis, showing that the embryos that had been treated with Ag490 from 6 to 29 h after insemination did not exhibit any differences from the control embryos (Figure 3-1). Thus, Ag490 did not affect the total transcriptional activity in the 2-cell stage embryos.

The treatment with Ag490 affected the expression levels in a small number of the genes in the 2-cell embryos.

To search the genes whose expressions were changed by the treatment with Ag490, the analysis by using oligo DNA microarray for 31,000 genes were performed. In the comparison between the MII stage oocytes and 2-cell stage embryos, the transcripts of 248 and 853 genes showed over twofold and 1.5fold increase, respectively, while 306 and 761 genes showed the decrease into below half and two-thirds levels, respectively (SD < 0.4) (Figure 3-2A). When compared to these numbers of the genes, the comparison between the 2-cell stage embryos that

were treated with and without Ag490 showed only a small numbers of the genes whose expression levels were different: no genes changed their expression levels over twofold or below half by treating the 2-cell embryos with Ag490 (SD < 0.4). Only 69 and 75 genes showed over 1.5 fold increase and below half level decrease, respectively (SD < 0.4) (Figure 3-2B).

The treatment with Ag490 selectively prevented the expressions of the genes whose expression were regulated by Jak-Stat5s signaling pathway in the 2-cell embryos.

Analyzing the results of microarray, in the list of the genes whose expression level decreased by Ag490, I found several genes whose expressions are known to be regulated by Jak-Stat5s signaling pathway in somatic cells, *i.e.* Socs-3 (Davey et al., 1999; Morales et al., 2002), bcl-2L (Lord et al., 2000), beta-casein (Chida et al., 1998; Grimley et al., 1999; Inuzuka et al., 1999; Yamashita et al., 2001), gamma-casein (Kolb, 2002) and limphotoxin alpha (TNF-beta) (Lu et al., 1998). These microarray results showed that these Jak-Stat5 regulating gene decreased by the treatment with Ag490.

To confirm these results, quantification of the transcripts by using real-time PCR were performed. In this experiment, I selected the four monitoring genes, *i.e.* ß-casein, TNF-ß, p21 and eIF-1A. The expressions of ß-casein, and TNF-ß genes are regulated by Jak-Stat5 signaling pathway in somatic cells (Liu et al., 1996; Chida et al., 1998; Nosaka et al., 1999; Lu et al., 1998), and were inhibited by the treatment with Ag490 in the results of microarray analysis. The p21 gene was a sample of the gene whose expression is regulated by Jak-Stat5 signaling pathway (Grimley et al., 1999; Takahashi et al., 2004), but the result of microarray analysis did not show any changes by the treatment with Ag490. The eIF-1A gene is a control gene which was not regulated by Jak-Stat5 signaling pathway.

The results of real-time PCR showed that the expression levels in the all of four genes were significantly increased between the MII and 2-cell stages ($P < 0.05$) (Figure 3-3). Comparing the

expression levels between the 2-cell embryos treated with and without Ag490, the expression levels of the ß-casein and TNF-ß were significantly inhibited by the treatment (Figure 3-3A and B). The expression levels of p21 were slightly lower in the embryos treated with Ag490 (Figure 3-3C), but this difference was not significant. The amounts of mRNAs for ß-casein, TNF-ß and p21 were decreased 70 %, 60 % and 15 %, respectively, by Ag490. On the contrary, the expression level of eIF-1A was not affected by the treatment (Figure 3-3D).

Discussion

In previous chapters, I revealed that Stat5s was localized in the nucleus during the preimplantation development (Figure 1-4 in chapter 1), and this localization was regulated by Jak family kinases at the 2-cell stage (Figure 2-1 and 2-2 in chapter 2). Since Stat5s work as the transcription factor in the nucleus (Darnell, 1997), in this chapter, I investigated the function of Jak-Stat5 signaling pathway for the gene expression in the 2-cell embryos. In the Jak-Stat5 signaling pathway, Stat5s that are phosphorylated on their tyrosine-residue by Jak, form their homo- or hetero-dimer, and are transferred into the nucleus. The Stat5s dimer binds to the promoter region of the target genes, and activates the transcription (Darnell, 1997). The downstream genes of Stat5 signaling pathway has common sequence which is named as "GAS like region" (TTCNNNGAA; N means indefinite nucleotide) in their promoter region (Grimley et al., 1999). Since the appearance for these sequences in genomes is not frequently, it was expected that the number of the genes whose expression are regulated by Jak-Stat5 signaling pathway is small in the 2-cell embryos. The results of the *in vitro* transcription assay and the microarray analysis for the Ag490 treated 2-cell embryos supported this idea as described below. The result of the *in vitro* transcription assay showed the treatment with Ag490 did not affect the total transcriptional activity (Figure 3-1). It means that the quantity of the transcripts from the downstream genes of Jak-mediated signaling pathway is relatively small in the 2-cell embryos. Additionally, the results of microarray analysis also revealed that the only small number of the genes was affected in their expression by the treatment with Ag490 (figure 3-2). Moreover, the result of the real-time PCR showed that the expressions of several downstream genes of Jak-Stat5 signaling pathway, *i.e.* ß-casein and TNF-ß, were selectively suppressed by the treatment with the inhibitor (Figure 3-3). These results confirmed that Jak-Stat5 signaling pathway is functional in the expression of their downstream genes at the 2-cell stage.

Among the genes whose promoter sequences contain GAS-like region, although the

expression of ß-casein and TNF-ß were inhibited by the treatment with Ag490, the expression of p21 was not changed significantly (Figure 3-3A, B and C). This difference could be explained by the facts that the expression of p21 gene is stimulated by not only Jak-Stat5 signaling pathway, but also another signaling pathway. In somatic cells, the expression of p21 is also stimulated by EGFR-Src (Sato et al., 2003) and PDTC-p38MAPK signaling pathway (Moon et al., 2004). These pathways also may function in the 2-cell embryo.

In chapter 2, I showed that the treatment with Ag490 inhibited the second cleavage of the embryos (Figure 2-8 in chapter 2), suggesting that Jak kinase activity is required for the cleavage between the 2- and 4-cell stages. In the 2-cell stage, a burst of zygotic gene activation occurs (Kidder, 1992; Thompson et al., 1998), and the transcripts in this stage are essential for the development, because the inhibition of transcription by the treatment with α-amanitin cause the arrest at the 2-cell stage (Bolton et al., 1984, Schultz, 1993; Rambhatla and Latham, 1995). These results suggest that Jak-Stat5 signaling pathway is involved in the development after the 2-cell stage *via* the expression of their downstream genes.

In somatic cells, Jak-Stat5 signaling pathway regulates the expression of the genes that is required for the cell growth, *e.g.* cyclins (Miyatake et al., 1997; Grimley et al., 1999; Brockman et al., 2002; Shelburne et al., 2003), and insulin-like growth factor-I (Davey et al., 2001; Frost et al., 2002). Although the result of microarray analysis did not show appreciable differences in the expression levels of these transcripts, I found a candidate gene that is likely to be involved in the development between the 2- and 4-cell stages, which was RGS14. In the result of the microarray analysis, the treatment with Ag490 reduced its expression level in the 2-cell stage. RGS14 is a mitotic spindle protein, and the RGS14 null mouse embryo is not able to develop after 2-cell stage (Martin-McCaffrey et al., 2004). Therefore, RGS14 expression may be involved in the regulation of the preimplantation development *via* Jak-Stat5 signaling pathway.

Table 3-1. PCR primers				Temparature
Target gene		**Sequences**	**Product size**	**annealing/measurement**
TNF-β	Sense	5' - CCCTTCCATGTGCCTCTCCT -3'	442bp	60.0/89.0
	Antisense	5' - CCCATGTCTGTCCCTCCTTC -3'		
p21 (WAF1/CIP1)	Sense	5' - AAGTGTGCCGTTGTCTCTTC -3'	391bp	63.5/86.0
	Antisense	5' - TCTGCGCTTGGAGTGATAGA -3'		
eIF-1A	Sense	5' - AAGAAGTCTGAAGGCCTATG -3'	169bp	57.0/82.8
	Antisense	5' - CAGAGAACTTGGAAGGTAGC -3'		
α-globin	Sense	5' - CAGAGAACTTGGAAGGTAGC -3'	171bp	58.0/91.0
	Antisense	5' - CAGAGAACTTGGAAGGTAGC -3'		
β-casein	Sense	5' - GATGCCCCTCCTTAACTCTGAA -3'	72bp	60.0/95.0
	Antisense	5' - TTAGCAAGACTGGCAAGGCTG -3'		
Taqman probe for β-casein		5' - CTGTGCTCCGCCTCATAAACTCTCAAATCC -3'		

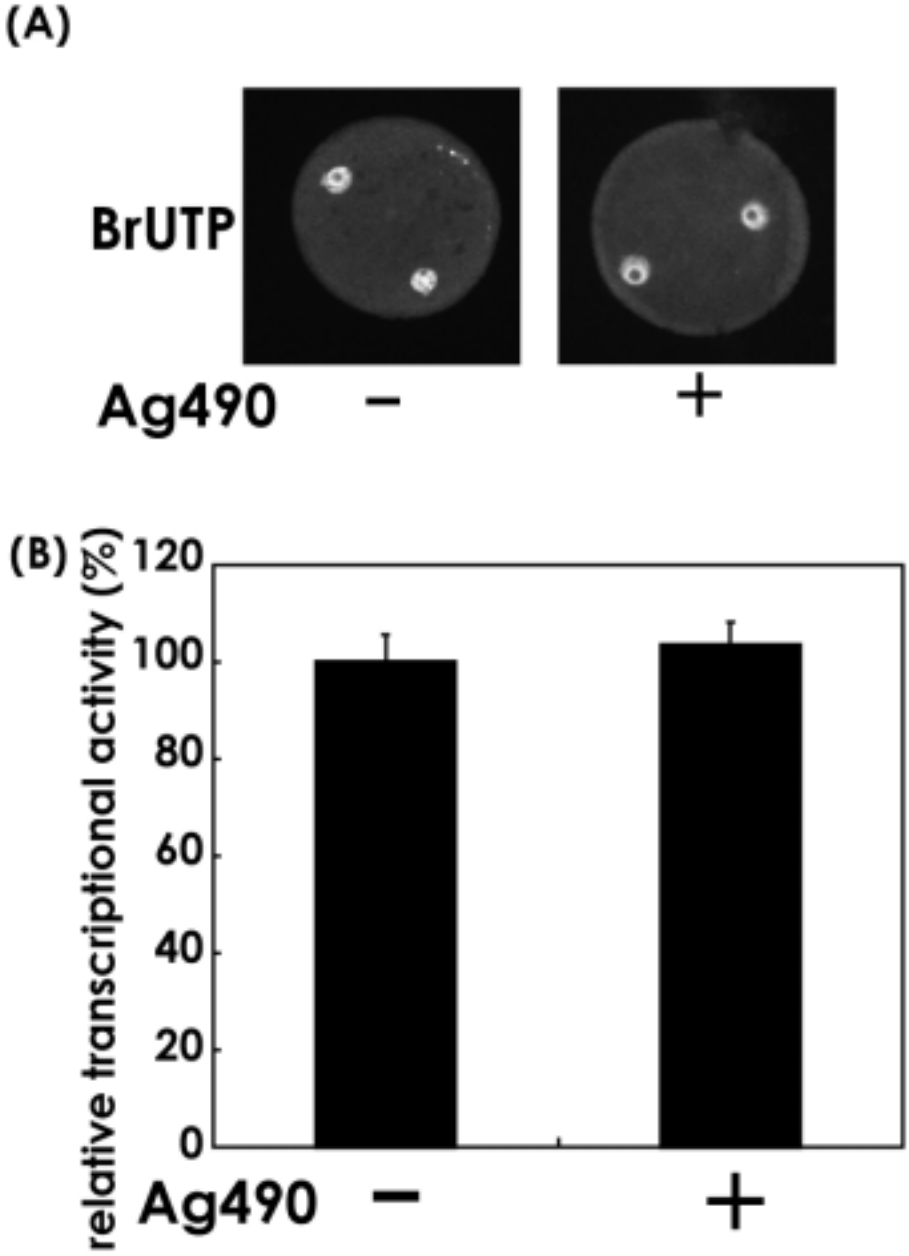

Figure 3-1. Total transcriptional activity in the mice 2-cell stage embryos treated with Ag490.
The embryos that had been incubated with (Ag490 +) or without Ag490 (Ag490 -) from 6 h after insemination were collected at 29 h, and then subjected to in vitro transcription assay. (A) Confocal microscopic images of the embryos in which the incorporation of BrUTP was detected by immunocytochemistry. (B) Quantification of incorporated BrU. For quantification, the relative fluorescence intensities of nucleus were determined. The averaged values of control embryos were set as 100%. The experiment was performed three times and the data were accumulated. The results are presented as the mean with SEM. n=36 (Ag490 -) and 44 (Ag490 +).

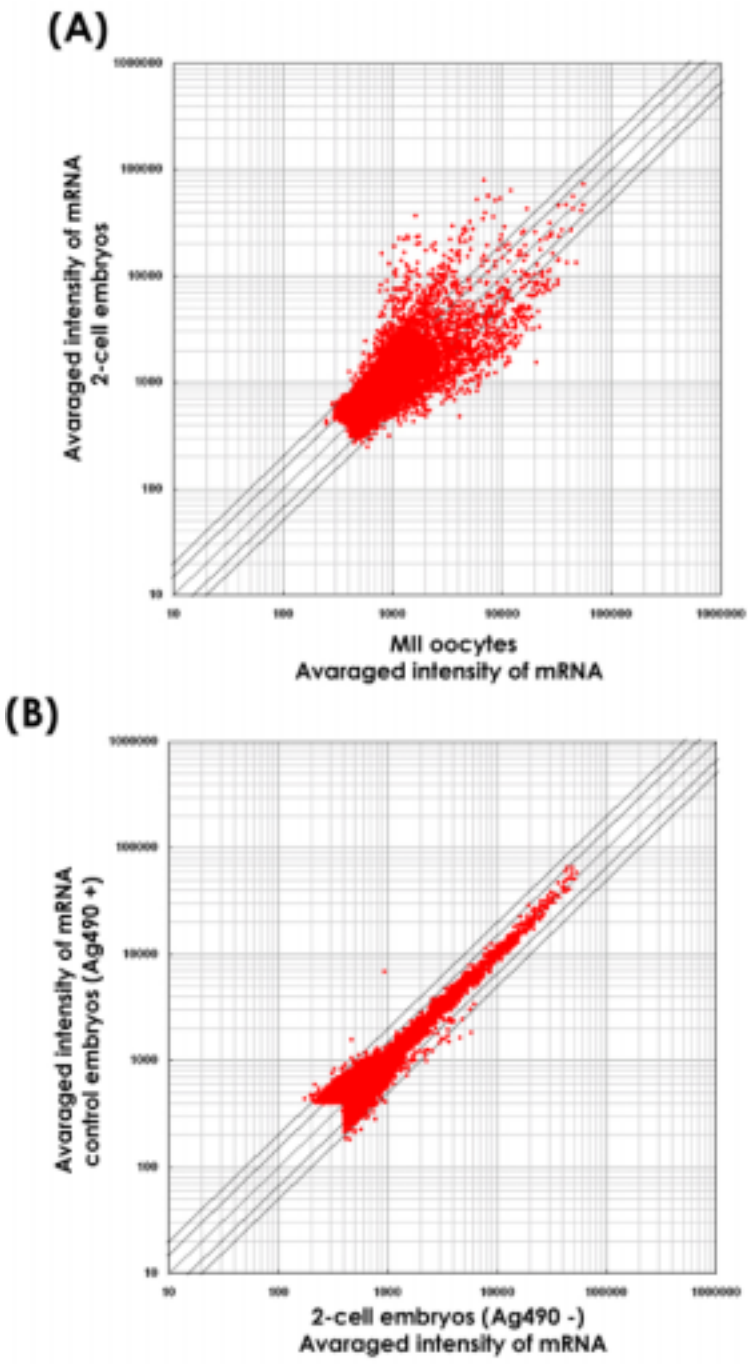

Figure 3-2. Scatter plot of QIAGEN 31000 oligo DNA microarray for mice embryos.
cDNAs samples were amplified by Super-SMART cDNA synthesis kit, followed by hybridization and detection. (A) Comparison of the gene expression levels between the MII stage oocytes and 2-cell stage embryos that were incubated with 0.2 % (v/v) DMSO, a solvent for Ag490, from 6 to 31 h after insemination. (B) Comparison of the gene expression levels between the 2-cell stage embryos that were treated with and without Ag490 from 6 to 31 h. The embryos without Ag490 were incubated with 0.2 % DMSO as a control.

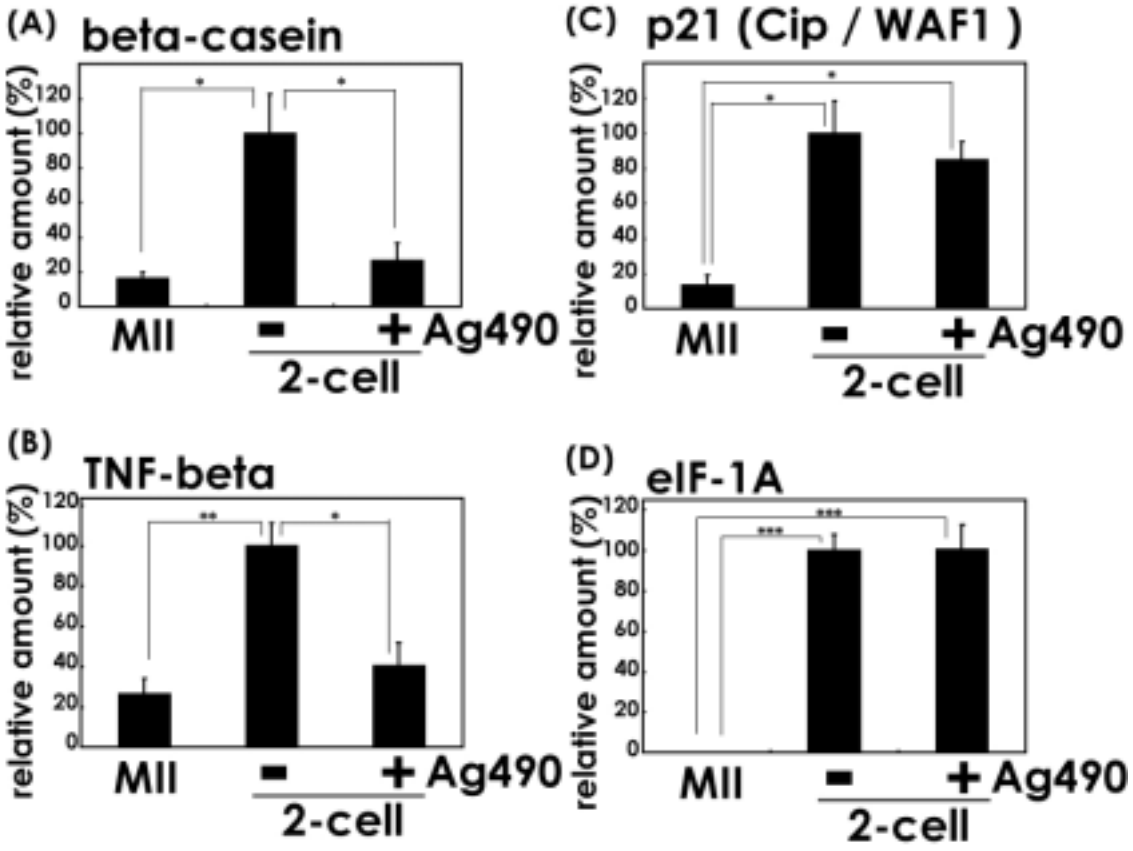

Figure 3-3. Quantification of mRNA in the MII stage oocytes and 2-cell stage mice embryos treated with or without Ag490 by using real-time, florescence-monitored RT-PCR. Total RNA was isolated from the oocytes at MII stage and the 2-cell embryos collected 31 h after insemination, and subjected to real-time RT-PCR analysis for the transcripts of beta-casein, TNF-beta, p21 and eIF-1A genes. For quantification, the value of the 2-cell embryos treated without Ag490 embryos (Ag490 -) was set as 100% and the values of samples were calculated relative to this value. The experiment was performed four times for beta-casein (A) or three times for others (B-D). The results are presented as the mean with SEM. *, **, *** indicate the significant differences from the corresponding controls at $P < 0.05$, 0.01, 0.001, respectively.

Chapter 4

Expression and function of the cytokine receptors activating Jak2-Stat5 signaling pathway in the preimplantation embryos

Abstract

In the previous chapters, I showed that the mechanisms regulating the activation of Stat5 changed during the preimplantation development, and that Jak-Stat5 signaling pathway is involved in the regulation of gene expression in the 2-cell embryos. In many types of somatic cells, various cytokine receptors activate Jak2-Stat5 signaling pathway, and regulates the proliferation and/or differentiation. Therefore, in this chapter, I firstly investigated the expression of the cytokine receptors that activate Jak2-Stat5 signaling pathway, *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. Secondly, I investigated the role of GM-CSF, the ligand of GM-CSFR, on the signaling pathway involving Stat5s. The treatment with GM-CSF increased the nuclear localization of Stat5A, but not Stat5B, in morulae, which suggests that GM-CSFR is involved in the activation of Stat5A in the preimplantation embryos. Finally, I examined for the phosphorylation on serine residues of Stat5s. In somatic cells, prolactin and growth hormone induce the phosphorylation of Stat5s not only on the tyrosine-residues, but also on the serine-residue. The immunocytochemistry showed that the Stat5s phosphorylated serine-residue (PS-Stat5) was localized in the nucleus from the late 1-cell to morula stage during the preimplantation development. These results suggest that various cytokine receptors are involved in the regulation of preimplantation development at various stages *via* the gene expression regulated by Jak2-Stat5 signaling pathway and PS-Stat5.

Introduction

In the previous chapters, I showed that the mechanisms regulating the activation of Stat5 altered during the preimplantation development (chapter 2) and that Jak-Stat5 signaling pathway is involved in the regulation of gene expression in the 2-cell embryos (chapter 3). Recent work reported that Jak2, a Jak family protein, is expressed in the preimplantation embryos (Ito et al., 2004). In many types of somatic cells, Jak2-Stat5 signaling pathway regulates the proliferation and/or differentiation (Gouilleux et al., 1994; Groner and Gouilleux, 1995; Chida et al., 1999; Ilaria et al., 1999; Ogilvie et al., 2000; Brockman et al., 2002; Xie et al., 2002; Friedrichsen et al., 2003; Zoubiane et al., 2004). Therefore, it is likely that Jak2-Stat5 signaling pathway is activated and regulates the proliferation and/or differentiation during the preimplantation development.

In somatic cells, many cytokine receptors are involved in the activation of Jak2- Stat5 signaling pathway (reviewed in Schindler and Darnell, 1995; Darnell, 1997; Grimley et al., 1999). These receptors may also activate Jak2-Stat5 signaling pathway in the preimplantation embryos. In this chapter, to clarify the involvement of these receptors in the regulation of the preimplantation development *via* the Jak2-Stat5 signaling pathway, I investigate the expression of various cytokine receptors that are known to activate Jak2-Stat5 signaling pathway in somatic cells.

Among the ligands for cytokines activating Jak2-Stat5 signaling pathway, prolactin (Prl) and growth hormone (GH) are involved in the phosphorylation of serine-residues in the C-terminus of Stat5s, mainly serine 726 residue in Stat5A and serine 730 residue in Stat5B (Yamashita et al., 1998; Beuvink et al., 2000; Park et al., 2001; Yamashita et al., 2001), in addition to their tyrosine residue whose phosphorylation is required for the Stat5s translocation in the nucleus (Darnell, 1997). Phosphorylation on the serine residue also plays an important role in the regulation of the activity of Stat5s as transcription factors. In COS-7 cell, it was shown that the phosphorylation on the serine residues of Stat5s affected their binding activity to the promoter regions (Beuvink et al.,

2000). Therefore, I also examine the nuclear localization of serine (726/730) phosphorylated Stat5s protein (PS-Stat5) during the preimplantation development.

Materials and Methods

RT-PCR of the transcripts of the cytokine receptors

The MII stage oocytes and the embryos at 2-cell, 4-cell, morula, blastocyst and hatched blastocyst stage were collected 31, 48, 72 and 96 h after insemination, respectively. The 1-cell embryos were collected both of 6 and 12 h. Total RNA was isolated from the oocytes and embryos and used for RT-PCR. The procedure for total RNA isolation and RT-PCR were described in chapter1.

For all the cytokine receptors examined, with the exceptions for the prolactin receptor (PrlR) and granulocyte-macrophage colony stimulating factor receptor (GM-CSFR), PCR was performed through 40 cycles consisting of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min and a final extension at 72°C for 10 min. For PrlR, PCR was conducted using the same protocol but only for 35 cycles. For GM-CSFR, the annealing temperature was changed to 60°C. For rabbit alpha globin as an external control, the conditions of PCR were described in chapter1. Sequences of each gene specific primers are shown in Table 4-1. 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 ß-chain, which is common to all these cytokines (Geijsen et al., 2001).

Treatment with granulocyte-macrophage colony stimulating factor (GM-CSF)

Forty morula stage embryos were collected at 71 h, and then transferred into the 200 μl drop of CZB medium with or without 20 ng/ml recombinant mouse GM-CSF protein (BIOSOURCE, Camarillo, CA, USA). For control, the drop of CZB medium without GM-CSF protein was added with 0.2 % (v/v) PBS which was a solvent for GM-CSF protein. The embryos were incubated for 1 h in 5 % CO_2-95% air at 38 °C before collected for immunocytochemistry.

Immunocytochemistry for serine-residue phosphorylated Stat5 (PS-Stat5),

All procedure for immunocytochemistry was the same as that of described in Chapter 1. In the detection for the localization of PS-Stat5, the antibody recognizing Stat5 phosphorylated on serine residue (726/730) (Upstate, Lake placid, NY, USA, cat.# 06-867) and anti-rabbit IgG FITC-conjugated donkey antibody (Jackson ImmunoResearch) were used as the first and secondary antibodies.

Results

The expression levels of the cytokine receptors that activate Jak-Stat5 signaling pathway were changed during the preimplantation development.

The changes in expression levels of the cytokine receptors that activate Jak-Stat5 signaling pathway, *i.e.*, 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), were examined during the preimplantation development (Figure 4-1). 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. Before hatched blastocyst stage, it was expressed relatively at a low level and showed small fluctuations. The expression level of GHR slightly decreased between the MII and 2-cell stage, increased between the 2-cell and morula stages, and then decreased again in the blastocyst stage. From the blastocyst to the hatched blastocyst stage, the expression level increased significantly ($P < 0.05$).

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 hatched blastocyst stage ($P < 0.05$). 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 4-cell stage. From the 4-cell to the hatched blastocyst stage, it was abruptly increased. The difference in IL-3R expression levels between the 4-cell and hatched blastocyst stages was significant ($P < 0.05$).

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 ($P < 0.05$). 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.

The treatment with GM-CSF increased the localization of Stat5A, but not Stat5B, in the nucleus of the morulae.

To certify whether or not the cytokine receptors whose expression were detected in the preimplantation embryos are involved in the activation of the Stat5s, I examined the effect of the

treatment with GM-CSF, the ligand protein of GM-CSFR, on the nuclear localization of the Stat5s in the morula stage, since the transcripts of GM-CSFR prominently exhibited peak expression in this stage (Figure 4-1). The results showed that the treatment with GM-CSF significantly increased the nuclear localization of Stat5A ($P < 0.01$). However, Stat5B localization was not changed by this treatment (Figure 4-2).

The nuclear localization of PS-Stat5 was observed between the late 1-cell and morula stages.

The localization of the serine residue phosphorylated-Stat5 (PS-Stat5) was examined by the immunocytochemistry with the specific antibody that recognize both of Stat5A phosphorylated on serine 726 residue (S726) and Stat5B phosphorylated on serine 730 residue (S730). The results of the immunocytochemistry showed that the serine residues of Stat5s were phosphorylated in the preimplantation embryos (Figure 4-3). Although the nuclear localization of the PS-Stat5 protein was not observed until 6 h after insemination (early 1-cell stage), it was observed from 12 h (late 1-cell stage) to 72 h (morula stage). The signal for PS-Stat5 was especially strong in the edge of the nucleolus. This uneven localization in the nucleus was observed until the 4-cell stage, and thereafter a uniform localization was observed at the morula stage. Although the nuclear accumulation of PS-Stat5 gradually increased between the 2-cell and morula stages, it was suddenly diminished at the blastocyst stage (Figure 4-3).

Discussion

In this chapter, I examined the expression of cytokine receptors that activate Jak2-Stat5 signaling pathway (Figure 4-1), 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 types of cells, such as mammary epithelial cells (Sakai et al., 1978; Bole-Feysot et al., 1998), breast cancer cells (Wennbo and Tornell, 2000), and lymphocytes (Clevenger and Medaglia, 1994). PrlR mediates the signaling pathway that regulates lactation, reproduction, cell growth, brain behavior, immunomodulation, and electrolyte balance (Kelly et al., 2001), by activating Stat5A, and Stat5B (Schroeder et al., 2001). It was also reported that Jak2 was expressed in the preimplantation embryos (Ito et al., 2004) and was essential kinase for the activation of Stat5s by PrlR in mammary epitherial cells (Shillingford et al., 2002). Since Jak-Stat5 signaling pathway is involved in the gene expression in the 2-cell stage (chapter 3), it was suggested that PrlR-Jak2-Stat5 signaling pathway was involved in the regulation of the preimplantation development *via* the expression of their downstream genes before the 4-cell stage. PrlR knockout mice exhibited reduced rates of fertilization and pre-implantation development, but did not show any disorder of male reproductive function (Ormandy et al., 1997; Binart et al., 2003).

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 (Pedersen et al., 1986; Robson et al., 2001), these cytokine receptors appear to regulate cellular differentiation and proliferation at these developmental stages by activation of the Jak2-Stat5 signaling pathway. In somatic cells, IL-5R mediates the differentiation of mature B-1

and B-2 cells from splenic B cells (Takatsu, 1998). In TNFR-null mice, early hematopoietic progenitor cells fail to differentiate into dendritic cells (Zhang et al., 1997).

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 (Robertson et al., 2000). They consist of a cytokine-specific α–chain, which binds the ligand with low affinity, and a common beta-chain (Geijsen et al., 2001). In ß–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 (Robb et al., 1995). GM-CSF knockout mice showed reduced rates of pre-implantation development (Robertson et al., 2001). These facts suggest that GM-CSFR is functional in morula stage embryos. However, IL-5 knockout mice did not show any defect in reproduction (Robertson et al., 2000). 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 results of the treatment with GM-CSF protein certified that GM-CSFR was functional in the preimplantation embryos. GM-CSF increased the nuclear localization of Stat5A, but not Stat5B in the morula stage (Figure 4-2). These results strongly suggest that GM-CSF/GM-CSFR signaling pathway mediated the Stat5A activation, but not Stat5B in the morula stage, which is consistent with the report that GM-CSFR preferentially activates Stat5A, but not Stat5B in human blood monocytes (Rosen et al., 1996).

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 (Pantaleon et al., 1997), 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 (Markham and Kaye, 2003), GHR may function by mediating the proliferation of cells in the 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 (Hara and Miyajima, 1996). 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 regulates selectively the proliferation of the cells of the inner cell mass at the blastocyst stage (Pampfer et al., 1994). These results suggest that TNFR is involved in cell differentiation after the morula stage.

The phosphorylation on the serine-residues of Stat5 was detected in the preimplantation embryos (Figure 4-3). In somatic cells, it was shown that the serine-phosphorylation of Stat5s affects the binding activity to the promoter (Beuvink et al., 2000). In COS-1 and COS-7 cells, prolactin and growth hormone regulate the serine-phosphorylation of Stat5s (Yamashita et al., 1998; Beuvink et al., 2000; Park et al., 2001; Yamashita et al., 2001). Therefore, their receptors should be expressed to regulate the serine-phosphorylation. In the results of RT-PCR, the expression level of PrlR was high before the 4-cell stage, while that of GHR increased after the 2-cell stage, and then decreased in the blastocyst stage (Figure 4-1). These results suggest that PrlR regulate the serine-phosphorylation of Stat5 before the 4-cell stage, while GHR regulate it between the 4-cell and morula stages. The mechanisms regulating the phosphorylation of serine residue seems to thus alter during preimplantation development.

In the results of immunocytochemistry, the uneven localization of serine-phosphorylated Stat5 in the edge of the nucleolus was observed from the late 1 cell to 4-cell stage (Figure4-3). This uneven localization was not observed in the immmunocytochemistry for Stat5A or Stat5B which was examined by using the antibodies recognizing them independently of their phosphorylation states (Figure 1-1 and 1-2 in chapter 1). It suggests that two forms of phosphorylated Stat5s, tyrosine-phosphorylated Stat5s and dual phosphorylated Stat5s on serine and tyrosine residues are present in the nucleus from the late 1-cell to 4-cell stage. These two forms of phosphorylated Stat5s have different specificities for their target gene promoters (Beuvink et al., 2000). Between the late

1-cell and morula stages, serine-phosphorylated (dual phosphorylated) Stat5s seems to regulate the expressions of the genes those are different from those regulated by tyrosine-phosphorylated Stat5s.

In conclusion, these results suggest that various cytokine receptors are involved in the regulation of development at various stages during the preimplantation development *via* the gene expression regulated by Jak2-Stat5 signaling pathway and PS-Stat5.

Table 4-1. PCR primers

Target gene		Sequences	Product size
Prolactin receptor (long form)	Sense	5' - CATCACAGTAAATGCCACGAACGAA -3'	586bp
	Antisense	5' - GGCACTCAGCAGTTCTTCAGACTTG -3'	
Growth hormone receptor	Sense	5' - ACCCCAGGATCTATTCAGCT -3'	205bp
	Antisense	5' - ATGTCTCCACGAATCCCGGT -3'	
Tumor necrosis factor receptor	Sense	5' - GTACTGCGCCTTGAAAACCC -3'	348bp
	Antisense	5' - GATGCTTGGAGTTTGGCTGG -3'	
IL-3 receptor (alpha chain)	Sense	5' - AGGAAGGGCAGGGACATCTT -3'	245bp
	Antisense	5' - TCACGCCAGAACATCCGGTA -3'	
IL-5 receptor (alpha chain)	Sense	5' - CTAGCGTGAGGACCATTCTG -3'	210bp
	Antisense	5' - TCCTTCCCAACAAGCCAGGT -3'	
GM-CSF receptor (alpha chain)	Sense	5' - CTGCTCTTCTCCACGCTACT -3'	230bp
	Antisense	5' - TCCTGAACCAGCAGCGGCAA -3'	
rabbit alpha globin	Sense	5' - GCAGCCACGGTGGCGAGTAT -3'	257bp
	Antisense	5' - GTGGGACAGGAGCTTGAAAT -3'	

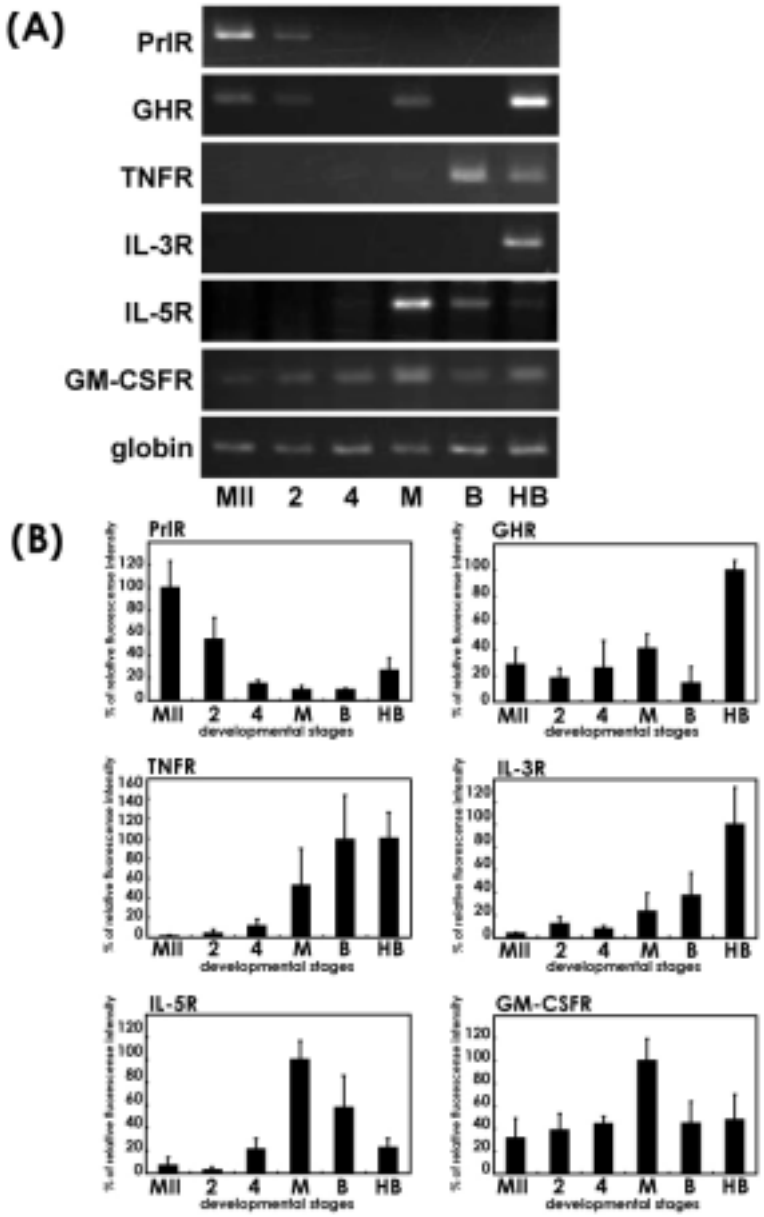

Figure 4-1. Expression patterns of cytokine receptors during pre-implantation development in the mouse. Total RNA samples were isolated from oocytes at the MII stage (MII) and from embryos at the 2-cell (2), 4-cell (4), morula (M), blastocyst (B), and hatched blastocyst (HB) stages, which corresponds 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 with SEM.

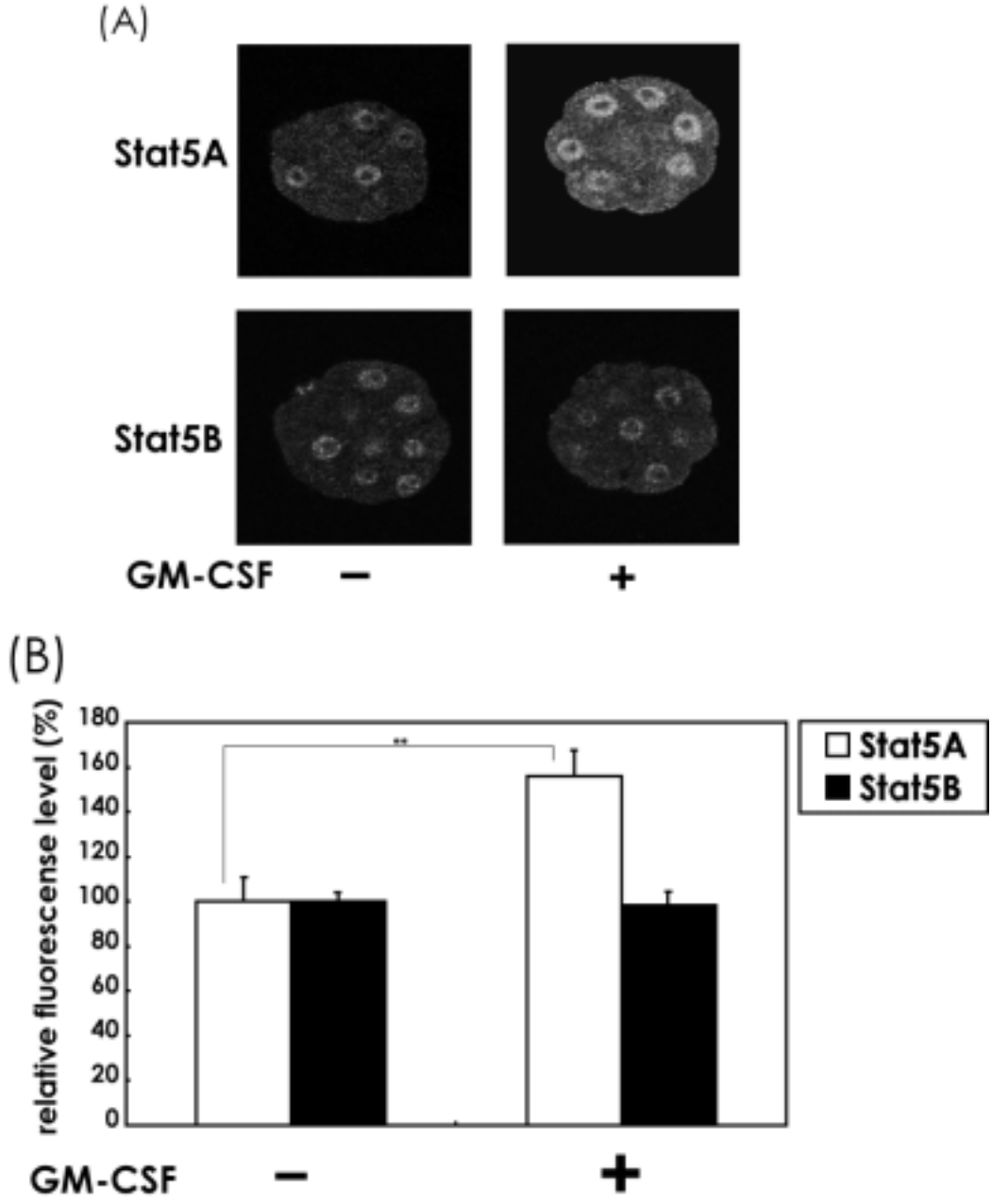

Figure 4-2. The 1 h incubation with GM-CSF led to the increase of the localization of Stat5A, but not Stat5B, in the nucleus of the mouse morula stage embryos.
Quantification of nuclear Stat5s in the 2-cell stage embryos treated with Ag490 or SU6656. (A) The embryos were cultured with GM-CSF ligand from 71 to 72 h after insemination, followed by immunocytochemistry with anti-Stat5A antibody or anti-Stat5B antibody. (B) Quantification of the relative fluorescence intensities of nucleus. The averaged values of controls were set as 100% and the values of others were calculated relative to this value. The results are presented as the mean with SEM. n=20. The open and closed columns represent the Stat5A and Stat5B. **:$P < 0.01$

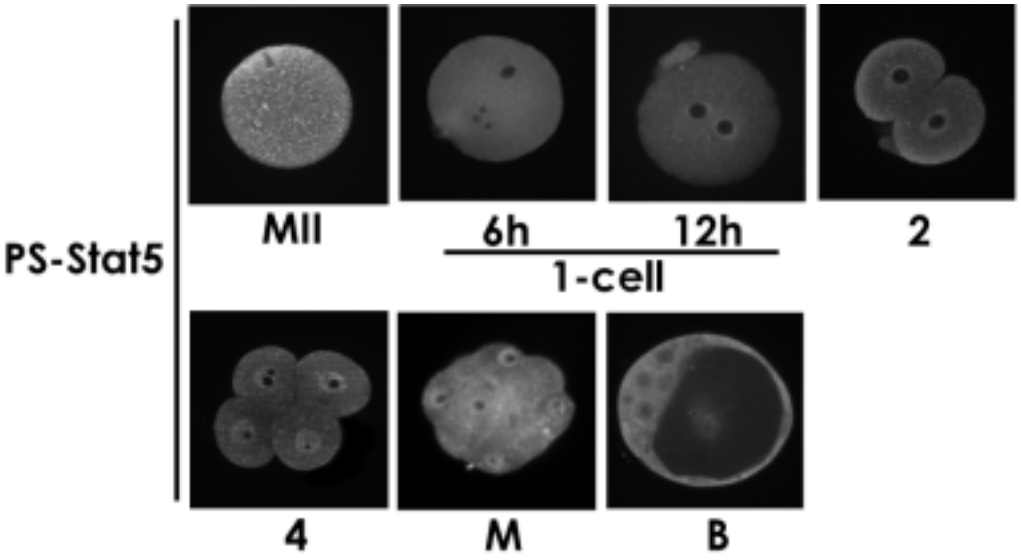

Figure 4-3. Localization of serine residue-phosphorylated Stat5 (PS-Stat5) proteins in the preimplantation embryos.
The immunofluorescent confocal microscopy of mice oocytes and preimplantation embryos stained with anti-PS-Stat5 antibody.
The oocytes at MII stage (MII) and the embryos at the stages of 1-cell (6 and 12 h), 2-cell (2), 4-cell (4), morula (M) and blastocyst (B) collected 6, 12, 31, 48, 72 and 96 h after insemination,respectively, were stained with specific antibody for phospho serine (726/730) residues of Stat5.

General Discussion

In this thesis, I showed that Stat5 signaling pathway was activated throughout the preimplantation development. A diagram of Stat5s activation mechanisms in the preimplantation embryos is drawn as Figure D-1. In this diagram, I characterized two types of signaling pathways activating Stat5s, Jak-Stat5 and SFKs-Stat5 signaling pathways, during the preimplantation development.

Jak-Stat5 signaling pathway is essential for the activation of Stat5A in the 2-cell, morula and the TE/ICM of blastocyst stage, and of Stat5B in the 1-cell, 2-cell, and ICM of blastocyst stage (Figure 2-1 and 2-2 in chapter 2). This pathway is likely to regulate the development between the 2- and 4-cell stages, and the morula and blastcysts stages (Figure 2-8 in chapter 2). Various cytokine receptors those activate Jak-Stat5 signaling pathway, *i.e.* PrlR, IL-5R, GM-CSFR, IL-3R, TNFR and GHR, are expressed during the preimplantation development (Figure 4-1 in chapter 2), and some of these receptors are involved in the proliferation and/or differentiation in the preimplantation embryos (Pampfer et al., 1994; Robertson et al., 2001; Binart et al., 2003; Markham and Kaye, 2003).

Stat5s are also activated *via* SFKs-Stat5 signaling pathway. Since SFKs-Stat5 signaling pathway was required for the activation of Stat5A in the TE and ICM of blastocysts and Stat5B in the ICM of blastocysts (Figure 2-1 and 2-2 in chapter 2), it is expected that the cytokine receptor which activates SFKs-Stat5 signaling pathway is expressed in the blastocyst stage. Indeed, it was reported that the expression of EGFR, a cytokine receptor that activate SFKs-Stat5 signaling pathway, is expressed in the both of the TE and ICM of blastocysts (Dardik et al., 1992). Because EGF, a ligand protein of EGFR, increases the rate of preimplantation development in mouse (Paria and Dey, 1990; Goldman et al., 1993; De La Fuente et al., 1999), it is suggested that SFKs-Stat5 signaling pathway is involved in the preimplantation development *via* EGFR.

In the 4-cell stage, neither Ag490 nor SU6656 affected the translocation of Stat5s into the

nucleus or the preimplantation development (Figure 2-1, 2-2 and 2-8 in chapter 2), which suggests neither Jaks nor Src family kinases activates Stat5s in this stage. Other upstream kinases that are not inhibited by Ag490 and SU6656, *e.g.* Jak1 and Tyk2, may activate Stat5s. However, it is also possible that Stat5s is redundantly activated by both of Jak and Src family kinases. The results in chapter 4 showed that both of cytokine receptors for Jak-Stat5 and SFKs-Stat5 signaling pathways are expressed in the 4-cell stage. It was also reported that the expression of EGFR, one of SFKs-Stat5 stimulating cytokine receptor, increases after the 4-cell stage (Wiley et al., 1992; Olayioye et al., 1999). These results suggest that both of Jak-Stat5 and SFKs-Stat5 signaling pathways activated by these receptors redundantly activate Stat5s in the 4-cell stage.

Various cytokine receptors are expressed during the preimplantation development. Although all of them activate Stat5, the difference in the upstream cytokine receptors is likely to regulate the gene expression in different manners by changing the DNA binding preference of Stat5s. It was reported that the change in the DNA binding preference of Stat5s affected the expression of target genes (Yu et al., 1999; Friedrichsen et al., 2003). As a candidate for the mechanism changing the DNA binding preference, I showed the phosphorylation on the serine-residues of Stat5s which was changed during the preimplantation development (Figure 4-3 in chapter 4). In addition to the modification on the serine-residues of Stat5s, other mechanism is likely to affect the DNA binding preference of Stat5s: that is the chaperone for Stat5. It was reported that ERBB4, an upstream cytokine receptor of Stat5 signaling pathway, is involved in the gene expression as a chaperone for Stat5A in the nucleus (Williams et al., 2004). This report shows that when the intracellular domain of ERBB4 is digested, it moves into the nucleus to associate with Stat5A, which leads to the change in the DNA binding preference of Stat5. This report suggests that cytokine receptors that move into the nucleus could affect the gene expression by working as a nuclear chaperone of Stat5s. EGFR, a member of ERBB family and a Stat5 activating cytokine receptor, is also localized in the nucleus and affect the gene expression (Olayioye et al.,

1999; Carpenter, 2003). It was also shown that the nuclear EGFR binds to the cyclin D1 promoter that is a binding target of Stat5s (Lin et al., 2001), suggesting that EGFR is also functional as a nuclear chaperone of Stat5s. The nuclear localization of PrlR and GHR was also observed (Perrot-Applanat et al., 1997; Pantaleon et al., 1997). In the nucleus, PrlR and GHR may also work as chaperones of Stat5s. Therefore, the change in the expression of the cytokine receptors is likely to have profound effects on the gene expression, *via* the function of several cytokine receptors as Stat5s chaperone, during the preimplantation development.

Taken together with the results in this thesis, it is suggested that complicated signaling pathways activating Stat5s are functional. Stat5s seems to be involved in the preimplantation development *via* regulating gene expression.

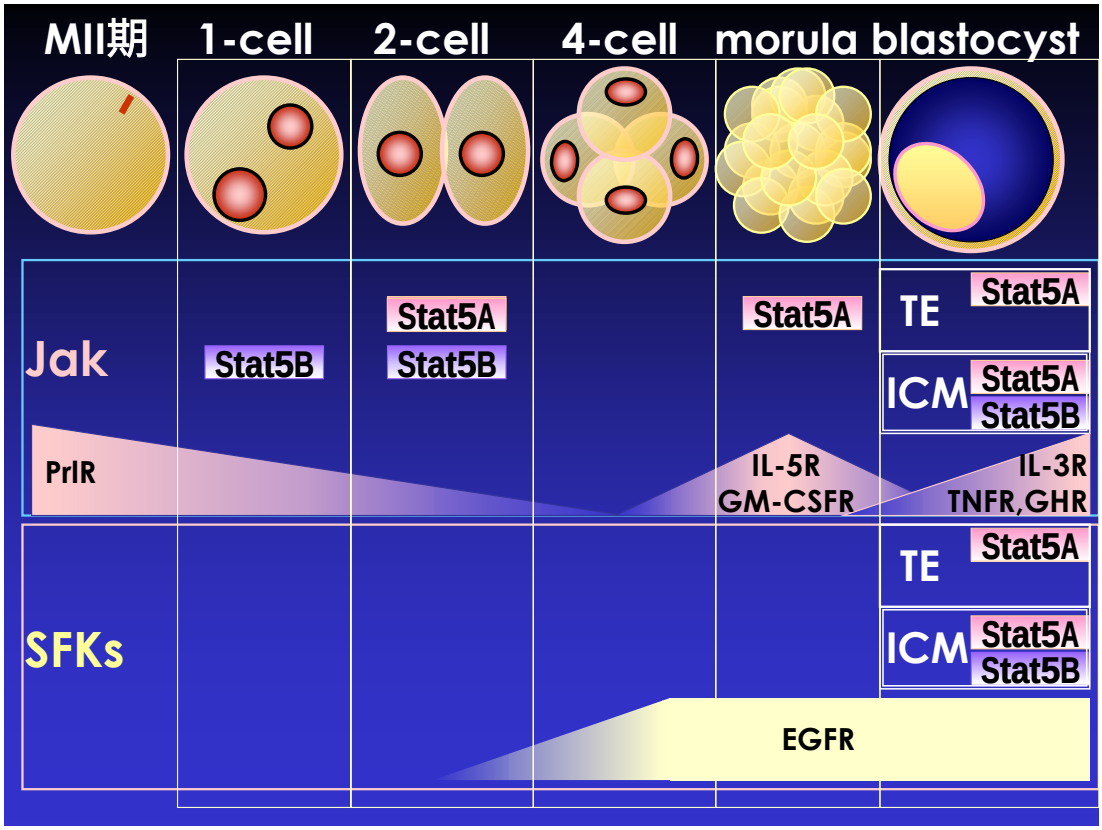

Figure D-1. Diagram of Stat5s activation mechanisms in the preimplantation embryos.

During the preimplantation development, Jak-Stat5 signaling pathway is essential for the activation of Stat5A in the 2-cell, morula and the TE/ICM of blastocyst stage, and of Stat5B in the 1-cell, 2-cell, and ICM of blastocyst stage. SFKs-Stat5 signaling pathway was required for the activation of Stat5A in the TE and ICM of blastocysts and Stat5B in the ICM of blastocysts. Various cytokine receptors for Jak-Stat5 and SFKs-Stat5 signaling pathways are expressed during preimplantation development.

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