学位論文

Mechanisms for embryonic gene activation in the 1-cell mouse embryos

（マウス 1 細胞期胚における胚性遺伝子の発現制御機構）

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Abbreviations

3-dA: 3'-deoxyadenosine

3-dG: 3'-deoxyguanosine

BL1: Butyrolactone I

BrdU: Bromodeoxyuridine

BrUTP: Bromouridine triphosphate

cdk: cyclin dependent kinase

CHX: cycloheximide

DMSO: Dimethylsulfoxide

EGA: embryonic genome activation

GV: Germinal vesicle

H3K9: Histone H3 lysine 9

HAT: Histone acetyl transferase

HDAC: Histone deacetylase

IVF: *in vitro* fertilization

MII: metaphase II

PB: physiological buffer

PBS: Phosphate buffer saline

PI: propidium iodide

pRb: Retinoblastoma protein

Ros: Roscovitine

TBP: TATA box binding protein

TSA: Trichostatin A

Abstract

The gene expression pattern in the differentiated oocytes is reprogrammed during meiosis and fertilization. This reprogramming requires the deletion of the gene expression program in the maternal genome of the oocytes and the initiation of a new program in the embryonic genome of the totipotent embryos after fertilization. In mice, it is known that embryonic gene activation (EGA) occurs in the 1-cell stage. In this study, I investigated the mechanism regulating EGA in the mouse 1-cell embryos.

In the chapter 1, I investigated the involvement of cyclyn A2/cdk2 in the regulation of EGA. Cyclin A2 protein was accumulated in the nucleus between 6 and 12 h after fertilization during which transcription is initiated in the 1-cell embryos. The accumulation was inhibited by 3'-deoxyadenosine, suggesting that poly(A) tail elongation triggers the initiation of translation from maternally accumulated cyclin A2 mRNA after fertilization. When cdk2 activity and the nuclear accumulation of cyclin A2 were inhibited by the treatment with cdk2 inhibitors and the microinjection of diced small interference RNA (d-siRNA), respectively, the increase of transcriptional activity was prevented during 1-cell stage. These treatments did not prevent the DNA replication. These results suggested that cyclin A2/cdk2 regulates EGA independently of cell cycle event in the 1-cell embryos.

In the chapter 2, to investigate the mechanism by which cyclin A2/cdk2 regulates EGA, I examined the phosphorylation of retinoblastoma protein (pRb), because pRb is one of the substrate for cyclin A2/cdk2. Immunocytochamistry with the antibodies recognizing the site-specific phosphorylation of pRb showed that the phosphorylation levels of cdk2 target sites in pRb increased during 1-cell stage. Microinjection with cyclin A2 d-siRNA and recombinant cyclin A2/cdk2 protein demonstrated that the phosphorylation state of Thr356, one of the cdk2 target site in pRb, was regulated by cyclin A2/cdk2. These results suggested that cyclin A2/cdk2

catalizes pRb during 1-cell stage. It is known that pRb functions by assembling multiprotein complexes as a negative regulator of gene expression and that this function is deregulated by its phosphorylation. Therefore, pRb phosphorylation by cyclin A2/cdk2 would lead to the deregulation of the repressive state of gene expression in the 1-cell embryos. This mechanism may be involved in the initiation of EGA.

In the chapter 3, I examined the changes in acetylation levels of core histone H3 and H4 during 1-cell stage and the involvement of cyclin A2/cdk2 in these changes. Several lines of evidence suggested that these histones are hyperacetylated at the promoter region of actively transcribed genes but are hypoacetylated at silent ones. Thus, histone acetylations have been shown to play important roles in the regulation of gene expression. Immunocytochemistry with anti-acetylated histones revealed that the acetylation levels of lysine 9 on histone H3 (H3K9), lysine 14 on histone H3 and lysine 5 on histone H4 significantly increased between 6 and 12 h after insemination during which EGA is initiated. Inhibition of cdk2 activity by the treatment with cdk2 inhibitors prevented the increase in acetylation of H3K9, suggesting that acetylation of H3K9 was induced by the cdk2. However, in the embryos in which cyclin A2 synthesis was inhibited by the microinjection of d-siRNA, acetylation level of H3K9 was not changed, suggesting that cyclin E/cdk2 but not cyclin A2/cdk2 may be responsible for the acetylation of H3K9.

Taken together with these results, I propose the following mechanism for EGA in the 1-cell mouse embryos. After fertilization, newly synthesized cyclin A2 forms an active complex with cdk2 which phosphorylates pRb. This phosphorylation of pRb deregulates transcriptionally repressive state which has been maintained by pRb. Transcriptionally repressive state is thus deregulated by the dissociation of transcriptional repressor and/or acetylation of core histones, which leads to the initiation of EGA.

General introduction

The fully grown oocytes are finally differentiated germ cells. Following fertilization, the embryo is a totipotent cell that can differentiate to any kind of cells. This dramatic change entails reprogramming of gene expression. Reprogramming requires the deletion of the gene expression program in the maternal genome before or just after fertilization, and then newly program is initiated in the embryonic genome. During oocyte growth, mRNA are transcribed actively from maternal genome. However, the transcriptional activity decreases before oocytes are fully grown to the germinal vesicle (GV) stage at which transcription is almost ceased. This transcriptionally silent state continues until the completion of meiosis after fertilization. The embryonic gene activation (EGA) occurs at species-specific timing (Braude et al., 1988; Camous et al., 1986; Crosby et al., 1988; Hoffert et al., 1997; Kelk et al., 1994; Manes, 1973). In the mouse embryos, EGA is initiated in the 1-cell stage, several hours after pronuclear formation, as demonstrated by BrUTP incorporation (Aoki et al., 1997; Bounial et al., 1995) and the expression of reporter gene (Ram and Schultz, 1993) and transgene (Matsumoto et al., 1994).

The mechanisms for the initiation of EGA seems to be quite unique. Firstly, the initiation of EGA occurs from the embryonic genomes which have been transcriptionally silent. There is no other case that transcription is completely ceased at the interphase. In the somatic cells, mRNA are transcribed even at the G0 phase, although the level of transcription is decreased. Therefore, some specific mechanism would regulate the initiation of gene expression from the completely silenced genomes in the 1-cell embryos. Secondly, EGA is associated with reprogramming of gene expression program. During meiotic maturation, and /or fertilization, gene expression program in the oocytes is reconstituted to generate the new program for totipotent 1-cell embryos. Therefore, the gene expression pattern in the embryos should greatly

differ from that in the oocytes. Finally, the stimulation for the signal to the activation of gene expression is different between 1-cell embryos and somatic cells. In the somatic cells, the stimulation from various cytokines are transduced to the nucleus *via* their receptors to modulate the gene expression pattern. However, no cytokine stimulation seems to be involved in the initiation of EGA. Only the stimulation with fertilization seems to be required for it. Furthermore, parthenogenetically activated embryos also can initiate EGA. The initiation of EGA thus seems to be regulated by the mechanisms specific for 1-cell embryos.

Little is known about the mechanisms for initiation of EGA, although it has been reported that 1-cell mouse embryos have some transcription factors and RNA polymerase II (RNAP II) activity. For example, nuclear concentrations of transcription factor, Sp1 and TATA-box binding protein, TBP increase during 1-cell stage (Worrad et al., 1994). In the 1-cell embryos, the RNAP II dependent transcriptional activity was decreased by the drug treatment that inhibits the phosphorylation of RNAP II subunits (Miyara et al., 2003). Although these reports suggest that transcriptionally permissive state is established during 1-cell stage, all of the factors reported here are generally required for the gene expression in most types of cells. Thus, there has been no report describing the specific mechanisms for the EGA in the 1-cell mouse embryos.

Recently, it has been reported that poly(A) tail elongation of maternal mRNA are essential for the embryonic gene activation in the 1-cell mouse embryos. Treatment with 3'-deoxyadenosine to inhibit polyadenylation of maternal mRNA decreases transcriptional activity in the 1-cell embryos (Aoki et al., 2003). This result led to the hypothesis that some maternal mRNAs are polyadenylated following fertilization, and that elongation of poly(A) tail trigger its translation. These newly synthesized proteins may induce initiation of EGA. This hypothesis is consistent with the experimental results that transcriptional activity and gene expression level were markably decreased by the inhibition of protein synthesis (Aoki et al 2003; Wang and

Latham, 1997).

It has been reported that the synthesis of some proteins from maternal mRNA are triggered by poly(A) tail elongation (Oh et al., 2000). Cyclin A2 is one of this kind of protein. The amount of cyclin A2 protein increased from 6 h to 12 h post insemination and translation of cyclin A2 was inhibited by 3'-deoxyadenosine which functions as a chain terminator of poly(A) tail elongation (Fuchimoto et al., 2001). According to these findings, cyclin A2 is a candidate for the regulator of EGA. During G1/S transition in the somatic cells, retinoblastoma protein (pRb) are phosphorylated by cyclin/cdk family including cyclin A2/cdk2. Phospho-pRb plays a role of transcriptional regulator *via* releasing transcription factor E2F-1 and histone deacetylase complex. Thus, the cyclin/cdk family play a role in regulating transcription indirectly during G1/S phase (Dyson, 1998; Sherr, 1996).

In this thesis, to understand the mechanisms for the initiation of EGA in the 1-cell mouse embryos, I investigated the involvement of cyclin A2/cdk2 in the initiation of EGA. The mechanisms by which cyclin A2/cdk2 activates the embryonal genome, *i.e.* phosphorylation of pRb and acetylation of histones, were also investigated.

Materials and Methods

In vitro fertilization and culture of embryos

Female ddY mice, 21-23 days of age and mature ICR male mice were purchased from SLC Japan Inc. (Shizuoka, Japan). To obtain metaphase II arrested oocytes (MII oocytes), female mice were superovulated by injection of 5 IU of pregnant mare's serum gonadotrophin (Teikoku zouki, Tokyo, Japan) followed 48 h later with 5 IU of human chorionic gonadotrophin (hCG) (Teikoku zouki). MII oocytes were isolated from the ampullae of oviducts 15.5 h post hCG injection and stored in Whitten's medium (Whitten, 1971). Sperm were obtained from cauda epididymis of ICR male mice and incubated with Whitten's medium into CO_2 incubator occupied with humidified atmosphere of 5 % CO_2 / 95 % air at 37 ℃, for 2 h. The MII oocytes were inseminated with the capacitated sperm which had been incubated for 2-4 h. Six hours after insemination, successfully fertilized oocytes, those containing male and female pronucleus were washed three times with glucose-free CZB medium (Chatot, 1989). These embryos were cultured with glucose-free CZB medium and used the experiments described below.

Insemination of *zona pellucida*-free oocytes

To remove the *zona pellucida*, oocytes were transferred to acidic mimimum essential medium compatible buffer (Acidic-MEMCO) (Evans et al., 1995) for just a few seconds. *Zona*-free oocytes were quickly washed three times with Whitten's medium containing 15 mg/ml of BSA and incubated for 1 h at 37 ℃ in the CO_2 incubator. The sperm stock that was obtained from cauda epididymis was diluted to a concentration of 15,000 sperm/ml with Whitten's medium containing 15 mg/ml of BSA. The diluted sperm solution was dropped to 10 μl and covered with mineral oil. *Zona*-free oocytes were transferred to sperm drops. Two oocytes were transferred to one sperm drop.

Treatment with cdk2 inhibitors and other chemicals

Four hours after insemination, the embryos were transferred to Whitten's medium containing 250 μg/ml of hyaluronidase (Sigma-Aldrich Co., St. Louis, MO, USA) and incubated for 5 min at 37 °C to remove cumulus cells. After washing three times with glucose-free CZB medium, these embryos were transferred to glucose-free CZB medium containing 100 μM of butyrolactone I (Wako Pure Chemical Industries, Ltd., Osaka, Japan) or 100 μM of roscovitine (Sigma-Aldrich Co.). The embryos were cultured until 12 h post insemination and they were subjected to *in vitro* transcriptional activity assay or immunocytochemistory. When the embryos were treated with aphidicolin (at a concentration of 3 μg/ml, Sigma-Aldrich Co.), cycloheximide (10 μg/ml, Sigma-Aldrich Co.), 3'-deoxyadenosine (2 mM, Sigma Chemical Co., St. Louis, MO, USA) and 3'-deoxuguanosine (2 mM, Sigma Chemical Co.), they were collected 1 h after insemination and transferred to glucose-free CZB medium containing these chemicals.

In vitro transcriptional activity assay

In vitro transcriptional activity assay was conducted as described by Aoki et al (Aoki et al, 1997). The 1-cell mouse embryos were collected from culture medium and washed three times quickly with physiological buffer (PB) containing 1 mM of $MgCl_2$, 100 mM of CH_3COOK, 30 mM of KCl, 1 mM of DTT, 0.2 mM of PMSF, 1 mM of Na_2ATP, 10 mM of Na_2HPO_4 and 50 units/ml of RNasin (Promega Corporation, Madison, WI, USA) (Jackson et al., 1993; Ferreria and Carmo-Fonseca, 1995). Then the plasma membrane was permeabilized with 0.05 % Triton X-100 in PB for only a few seconds until the shape of embryos began to be flat. Permeabilized embryos were washed three times with PB and incubated with transcription mixture containing 2 mM of ATP, 0.4 mM each of GTP, CTP, BrUTP, 1 mM of $MnCl_2$ and 50 mM of $(NH_4)_2SO_4$ in PB for 15

min at 33 °C. The embryos were washed three times with PB. To stop the reaction, embryos were transferred to 0.2% Triton X-100 in PB and incubated for three minutes at room temperature. After washing three times with PB, embryos were fixed into PBS containing 3.7 % of paraformaldehyde (PFA. Wako Pure Chemical Industries, Ltd.) (PBS/PFA) for 1 h at room temperature. The fixed embryos were washed three times with PBS containing 3 mg/ml BSA. The incorporated BrUTP was detected by immunostaining. The embryos were incubated with 2 μg/ml of anti-bromodeoxyuridine monoclonal antibodies (Roche Diagnostics Corporation, Indianapolis, IN, USA) for 45 min at room temperature. Then, these embryos were washed three times with PBS containing 3 mg/ml BSA and incubated with 30 μg/ml of fluorestain isothiocyanate (FITC)-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 45 min at room temperature. The embryos were washed three times with PBS containing 1 mg/ml BSA (PBS/BSA) and mounted with VectaShield (Vector Laboratories, Inc., Burlingame, CA, USA). Fluorescence was detected using a Carl Zeiss 510 laser-scanning confocal microscope (Carl Zeiss Co., Ltd., Oberkochen, Germany). The intensity of fluorescence was quantified using NIH-Image software (National Institute of Health, Bethesda, MD, USA). On the image file, a circle was handwritten along the outline of nucleus and the mean value of the intensity of fluorescence in the circle was measured. Then, this the circle was moved into cytoplasmic area and the mean value of fluorescence intensity was measured as a back ground. The net signal value was calculated by subtracting back ground signal value from the nucleoplasmic signal value. Then, the net signal value was multiplied by the nucleoplasmic area. Since 1-cell embryos formed both male and female pronucleus, the relative intensity of fluorescence in these pronuclei were combined into the fluorescence intensity for 1-cell embryos in each embryo.

Immunoblotting

MII stage oocytes were isolated from the ampullae of oviducts. One-cell embryos were collected at 12 h after insemination. Collected oocytes and embryos were transferred to PBS containing 250 μg/ml of hyaluronidase (Sigma-Aldrich Co.) and incubated for 5 min at 37 °C to remove cumulus cells. These oocytes and embryos were washed three times quickly with PBS containing 3 mg/ml polyvinylpyrrolidone (PBS/PVP) (Nacalai Tesque. INC, Kyoto, Japan). Two hundreds oocytes and embryos were collected in 10 μl of RIPA buffer consisting of 150 mM NaCl, 1 % NP-40, 0.5 mM EDTA, 50 mM Tris, 100 μM sodium orthovanadate, 0.1 % sodium dodecylsulfate (SDS), 1 mM dithiothreitol, 1 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin A and 100 μg/ml phenylmethylsulfonyl fluoride, (pH 8.0) and stored at -80 °C. These samples were subjected to SDS-PAGE with a 10 % of polyacrylamide gel and then transferred to polyvinylidene difluoride (PVDF) membrane. Following transfer, PVDF membrane was blocked with 4 % of Block Ace™ Powder (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) in TTBS consisting of tris-bufferd saline (TBS) including 0.1 % of Tween 20® (ICN Biomedicals Inc., Ohio, USA) for 1 h at room temperature. PVDF membrane was washed three times with TTBS for 10 min, and incubated with 1:400 dilution of anti-cdk2 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA., USA; catalog number sc-163) for 15 h at 4 °C. Then, membrane was washed three times with TTBS for 10 min, and incubated with 1:1000 dilution of anti-rabbit Ig, horseradish peroxidase linked whole antibody (Amersham Biosciences, Buckinghamshire, UK; catalog number NA934V) for 1 h at room temperature. Immunodetection was performed with an enhanced chemiluminescence system (Amersham Biosciences; catalog number RPN2135).

Immunostaining

The collected oocytes and embryos were washed three times with PBS/BSA and fixed in PBS/PFA for 1 h at room temperature followed by removal of the *zona*

pellucida using Acidic-MEMCO. The fixed oocytes and embryos were permeabilized with 0.5 % Triton X-100 for 15 min at room temperature and then washed three times with PBS containing 3 mg/ml BSA. The oocytes and embryos were incubated with first antibody for 1 h at room temperature or over night at 4 °C. Following this reaction, oocytes and embryos were washed three times with PBS/BSA and incubated with 30 μg/ml of fluorescein isothiocyanate (FITC)-conjugated anti-rabbit or anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) for 1 h at room temperature. After washing three times with PBS/BSA, oocytes and embryos were incubated in PBS/BSA containing 50 μg/ml RNase, DNase-free (Roche Diagnostics Corporation) for 30 min at 37 °C. The embryos were washed three times with PBS/BSA and mounted with VectaShield containing 0.5 mg/ml of propidium iodide (Sigma-Aldrich Co.). Fluorescence was detected using a Carl Zeiss 510 laser-scanning confocal microscope. The intensity of fluorescence was quantified using NIH-Image as described in the previous section.

The first antibodies used for immunocytochemistry were as followings: the antibodies against pRb (Santa Cruz Biotechnology, Inc., Santa Cruz, CA., USA; catalog number sc-50), phosphorylated Ser780 of pRb (Cell Signaling Technology Inc., Beverly, MA., USA; catalog number 9307.), phosphorylated Ser249/Thr252, Thr356, Ser807/811, Thr821/826 of pRb (Santa Cruz Biotechnology, Inc., catalog number sc-16671, sc-16837, sc-16670 and sc-16669, respectively.), acetylated lysine 5, 12 of histone H4, acetylated lysine 14 of histone H3 (Upstate Biotechnology, Inc., Lake Placid, NY., USA; catalog number 06-759, 06-761 and 06-911, respectively.), acetylated lysine 9 of histone H3 (Cell Signaling Technology, Inc., catalog number 9671.), TFIID (TBP), cyclin A2 (Santa Cruz Biotechnology, Inc., catalog number sc-204, sc-751, respectively.). To prepare the working solutions of first antibodies, every antibody was diluted at a 1:100 with PBS/BSA. The embryos were incubated with each antibody solution except anti-cyclin A2 antibody for 1 h at a room temperature. For anti-cyclin A2 antibody, incubation

was performed over night at 4 °C.

Detection of DNA synthesis

DNA synthesis was detected by BrdU incorporation. The embryos were collected 9 h after insemination and transferred to glucose-free CZB medium containing 10 μM of 5-Bromo-2'-deoxyuridine (BrdU, Roche Diagnostics Corporation) and incubated for 1 h at 37 °C in CO_2 incubator. Following BrdU incorporation, embryos were washed three times with PBS/BSA and fixed in PBS/PFA for 1 h at room temperature. Then, these embryos were incubated with 0.5 % Triton-X 100 in PBS/BSA for 15 min at room temperature. After washing three times with PBS/BSA, embryos were transferred to 2N-HCl containing 3 mg/ml of polyvinylpyrrolidone (PVP, Sigma-Aldrich Co.) and incubated for 1 h at 37 °C for denaturing of DNA. To neutralization, HCl-treated embryos were transferred to borate buffer (pH 8.5) containing 3.82 mg/ml of $Na_2B_4O_7$-$10H_2O$, 9.92 mg/ml of H_3BO_3, 2.34 mg/ml of NaCl and incubated 1 h at room temperature. Neutralized embryos were washed three times with PBS/BSA and subjected to immunostaining with 2 μg/ml of anti-bromodeoxyuridine monoclonal antibodies (Roche Diagnostics Corporation) for 1 h at room temperature. Following procedures for immunostain are conducted as describe in the previous section.

Microinjection of cyclin A2/cdk2 protein into 1-cell embryos

The oocytes were collected 1 h after insemination. Collected embryos were transferred to Whitten's medium containing 250 μg/ml of hyaluronidase (Sigma-Aldrich Co.) and incubated for 5 min at 37 °C to remove cumulus cells. Microinjection was performed on an inverted microscope (ECLIPSE TE300, Nicon Corporation, Tokyo, Japan) using a micromanipulator (Narishige Co., Ltd., Tokyo, Japan) and microinjector (IM300, Narishige Co., Ltd.). After washing three times with Whitten's medium, 10-20

pl of cyclin A2/cdk2 active protein (0.2 μg/μl) (Upstate Biotechnology, Lake Placid, NY, USA. catalog number 14-448) were injected into cytoplasm of 1-cell embryos using borosilicate glass capillaries (GC100 TF-10, Harvard Apparatus Ltd., Kent, UK). For the experimental control, the cyclin A2/cdk2 protein which had been inactivated by boiling for 10 min were injected. Protein injected embryos were cultured with glucose-free CZB medium, collected 6 h after insemination and subjected to immunostaining. The embryos microinjected with proteins were collected 10 h after insemination and subjected to *in vitro* transcriptional activity assay.

Cloning of mouse cyclin A2

Total mRNA were isolated from two hundreds of MII oocytes by using ISOGEN (Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. Isolated mRNA were reverse-transcribed in a 21 μl of reaction mixture containing 1 μl of oligo (dT) primer (500 mg/ml, GIBCO BRL, Bostone, MA, USA), 4 μl of x5-reaction buffer, 2 μl of DTT (0.1 M), 1 μl of dNTP mixture (four nucleotides, each at a concentration of 10 mM, GIBCO BRL), 1 μl of RNasin (40 units/ml, Promega Corporation) and 10 units of Super Script II RNase H- Reverse Transcriptase (GIBCO BRL) at 42 °C for 50 min.

Amplification of cyclin A2 cDNA by PCR was carried out in a 25 μl of reaction mixture containing 2.5 μl of x10 PCR reaction buffer, 1.5 μl of 25 mM-$MgCl_2$, 2.5 μl of 2.5 mM-dNTP mixture, 50 pmol of sense and antisense-primers, 1.25 units of Ampli Taq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA) and 2 μl of cDNA solution. The following primers were used: mouse cyclin A2 sense primer, 5'-CCG CGA TGC CGG GCA CCT-3', mouse cyclin A2 antisense primer, 5'-TCA CAC ACT TAG TGT CTC TG-3'. Followed by heating at 95 °C for 10 min, PCR was performed through 40 cycles consisting of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 60 s, and a final extention at 72 °C for 10 min. The amplified products were separated on 2 % agarose gel electrophoresis and visualized by staining with

ethidium bromide. The anticipated size (1237 bp) of band was cut out, and the cDNA fragments were eluted from the gel. The eluted cDNA were subcloned into pCR II vector (Invitrogen Corporation, Carlsbad, CA, USA). DNA sequences were determined by the dideoxy method using the DNA sequencing system of Applied Biosystems Japan Ltd., Tokyo.

Preparation of diced siRNA (d-siRNA)

Small interference RNA (siRNA) were prepared by dicing the double strand RNA (dsRNA) for whole cording region of mouse cyclin A2. The dsRNA was prepared as following, the cDNA of cyclin A2 coding region as a template for dsRNA were obtained by PCR. PCR was performed with pCR II plasmid including whole cording region of cyclin A2 as a template using *Pfu* DNA polymerase (Promega Corporation) and cyclin A2 sense / antisense primers in the following condition. Following the initial heat treatment at 95 °C for 1 min, PCR was performed through 15 cycles consisting of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 90 s, and a final extention at 72 °C for 10 min. The amplified cDNA product was separated on 2 % agarose gel electrophoresis and visualized. The anticipated size (1237 bp) of band was cut out, and the cDNA fragments were eluted from the gel. This first PCR product was amplified by the second PCR using *Pfu* DNA polymerase and following primers: cyclin A2 T7-sense primer, 5'-TAA TAC GAC TCA CTA TAG GTA CTC CGC GAT GCC GGG CAC CT-3', cyclin A2 T7-antisense primer, 5'-TAA TAC GAC TCA CTA TAG GTA CTT CAC ACA CTT AGT GTC TCT G-3'.

The second PCR was performed through 25 cycles in the same conditions of first PCR reaction.

Both of the cyclin A2 sense RNA and antisense RNA were transcribed from purified second PCR product. *In vitro* transcription was performed in a 20 μl of reaction mixture containing 2 μl of x10 reaction buffer, 2 μl of 10 mM-NTP mixture, 40

units of RNasin (Promega Corporation), 1 μg of second PCR products and 20 units of T7 RNA polymerase (Roche Diagnostics GmbH, Mnnheim, Germany) for 1 h at 37 °C. Followed by transcriptional reaction, 20 units of DNase I (RNase-free, Roche Diagnostics GmbH) were added to the reaction mixture and incubated 30 min at 37 °C. To stop the enzymatic reaction, 2 μl of 0.2 M-EDTA were added. After purification of single strand RNAs (ssRNAs) by ethanol precipitation, 60 μg of ssRNAs were collected in RNase-free microcentrifuge tube. This tube was transferred to 250 ml of boiled water and left for 1-1.5 h to allow the water to cool down to the room temperature. The dsRNA thus obtained were diced by using BLOCK-iT™ Dicer RNAi Kits (Invitrogen Corporation). The 60 μg of dsRNA were diced to siRNA in a 300 μl of the reaction mixture containing reaction buffer and 60 units of dicer enzyme for 16 h at 37 °C. To verify the integrity of diced reaction, diced siRNA (d-siRNA) were separated on 4 % agarose gel electrophoresis with 10 bp DNA step ladder maker (Promega Corporation) and visualized by staining with ethidium bromide. Then, the d-siRNA were purified using the BLOCK-iT™ RNAi purification reagents and spin cartridges supplied in BLOCK-iT™ kit (Invitrogen Corporation). The purified d-siRNA were injected to MII oocytes. The procedure for microinjection was described following section.

For the experimental control, d-siRNA against rabbit alpha globin was prepared. The cDNA of rabbit alpha globin were obtained by reverse transcription of the mRNA (Sigma Cenical Co.) and amplified with *Pfu* DNA polymerase (Promega Corporation) using following primers: globin sense primer, 5'-CCC GCT GAC AAG ACC AAC AT-3' and globin antisense primer, 5'-CGA TAT TTG GAG GTC AGC AC-3'. PCR was performed through 15 cycles consisting of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 45 s, and a final extention at 72 °C for 5 min. The anticipated size (413 bp) of band was cut out, and these cDNA fragments were subjected to PCR again through 25 cycles using *Pfu* DNA polymerase and following primers: Globin T7-sense primer, 5'-TAA TAC GAC TCA CTA TAG GTA CTC CCG CTG

ACA AGA CCA ACA T-3', Globin T7-antisense primer, 5'-TAA TAC GAC TCA CTA TAG GTA CTC GAT ATT TGG AGG TCA GCA C-3'. In vitro transcription and dicing reaction were performed as the same procedures for cyclin A2.

Microinjection of d-siRNA into MII stage oocytes

MII oocytes were isolated from the ampullae of oviducts 15.5 h post hCG injection and stored in human tubal fluid (HTF) medium containing 5.93 mg/ml of NaCl, 0.35 mg/ml of KCl, 0.049 mg/ml of $MgSO_4$-$7H_2O$, 0.054 mg/ml of KH_2PO_4, 0.76 mg/ml of $CaCl_2$-$2H_2O$, 2.1 mg/ml of $NaHCO_3$, 0.5 mg/ml of Glucose, 3.4 μl of DL-Lactic acid (sodium salt, Sigma Chemical Co.), 0.037 mg/ml of pyruvic acid (sodium salt, Sigma Chemical Co.), 0.075 mg/ml of penicillin G (potassium salt, Sigma-Aldrich Co.) and 0.05 mg/ml of streptomycin sulfate (Sigma-Aldrich Co.). Then, MII oocytes were transferred to HTF medium containing with 250 μg/ml of hyaluronidase (Sigma-Aldrich Co.) and incubated for 5 min at 37 °C to remove cumulus cells. After washing three times with hyaluronidase-free HTF medium, 10-20 pl of d-siRNA (180 μg/ml) including 150 mM KCl were microinjected to cytoplasm using borosilicate glass capillaries (GC100 TF-10, Harvard Apparatus Ltd., Kent, UK). Microinjection was performed on an inverted microscope (ECLIPSE TE300, Nicon Corporation) using micromanipulator (Narishige Co., Ltd.) and the microinjector (IM300, Narishige Co., Ltd.). The capillary for microinjection was hold by microelectrode holder (World Precision Instruments, Inc., Sarasota, FL., USA., catalog number MEH7W10). This microelectrode holder is designed to provide an electrical coupling between d-siRNA solution-filled glass pipettes and high input impedance microelectrode amplifiers (Nihon Kohden Corporation, Tokyo, Japan, catalog number MEZ-8301). High input impedance induced the penetration of leading edge of capillary into *zona pellucida* and cell membrane. This procedure yielded a high survival rate of microinjected embryos (511/787; 64.9 %).

Statistical analysis

Statistical analysis was performed by student's t-test. All statements of significance were based on a probability level of < 0.01.

Chapter 1

Cyclin A2 / cdk2 involves in the regulation of embryonic gene activation in the mouse 1-cell embryos.

Abstract

In mice, embryonic gene activation (EGA) occurs during the 1-cell stage. In this chapter, I examined the involvement of cyclin A2/cdk2 in the regulation of EGA. Immunocytochemistry with anti-cyclin A2 antibody revealed that newly synthesized cyclin A2 was accumulated in the nucleus between 6 and 12 h after insemination during which transcription is initiated. Inhibition of poly(A) tail elongation by 3'-deoxyadenosine prevented this accumulation, suggesting that poly(A) tail elongation triggers the initiation of translation from maternally accumulated cyclin A2 mRNA after fertilization. Since cyclin A2 regulates cdk2 activity, I examined the involvement of cyclin A2/cdk2 in the regulation of EGA. The treatment with cdk2 inhibitors reduced the transcriptional activity in the 1-cell embryos. To clarify the involvement of cyclin A2 in the EGA, cyclin A2 synthesis was inhibited by the microinjection with cyclin A2 diced small interference RNA (d-siRNA). The microinjection of d-siRNA inhibited the nuclear accumulation of cyclin A2 and reduced the transcriptional activity, although DNA synthesis was not inhibited. In addition, microinjection of recombinant cyclin A2/cdk2 protein increased the transcriptional activity. These results suggested that cyclin A2/cdk2 regulates EGA independently of cell cycle event in the 1-cell embryos.

Introduction

Embryonic gene activation (EGA) is critical event for the preimplantation development in mammals. In the mouse, EGA occurs during the 1-cell stage (Aoki et al., 1997; Bounial et al., 1995). Although it has not been well elucidated about the molecular basis for the mechanism by which EGA occurs in the 1-cell stage, it was reported that poly(A) tail elongation of maternally inherited mRNA were essential for the initiation of EGA (Aoki et al., 2003). It is well known that poly(A) tail elongation of mRNA is tightly coupled with the activation of translation. During the oocyte maturation in *Xenopus*, cytoplasmic polyadenylation of maternal mRNA is the key mechanism for the control of its translation (Boaz et al., 1996; Hake and Richer 1994; Mendez et al., 2000). In the mouse oocytes, microinjected mRNA was actively translated when it was polyadenylated (Vassalli et al., 1989). After fertilization, the initiation of translation of Spin and cyclin A2 is associated with poly(A) tail elongation (Fuchimoto et al., 2001; Oh et al., 2000). Thus, polyadenylation of maternal mRNA stimulates translation. These findings suggest that some maternal mRNA are polyadenylated following fertilization, resulting in the initiation of their translation. These newly synthesized proteins may control the initiation of EGA in the 1-cell embryos.

Cyclin A2 is a good candidate for such a protein involved in the regulation of EGA. The maternal cyclin A2 mRNA is polyadenylated following fertilization, and the amount of cyclin A2 protein increases during 1-cell stage (Fuchimoto et al., 2001). Inhibition of poly(A) tail elongation decreased the accumulation of cyclin A2 protein. In the somatic cells, cyclin A2 is essential for the cell cycle regulation. During G1/S stage, cyclin A2, as well as cyclin D and E, binds to cyclin dependent kinase (cdk) and phosphorylates retinoblastoma protein (pRb). This phosphorylation by these cyclin/cdk complexes induces conformational changes of pRb and releases the

transcription factor, E2Fs from pRb complex. Free E2Fs direct the gene expression involved in S-phase entry and S-phase progression. Thus, the cyclin/cdk families regulate gene expression for the cell cycle progression.

The purpose of this chapter is to clarify the role of cyclin A2/cdk2 in the initiation of EGA in the mouse 1-cell embryos. For this purpose, I investigated the effect of cdk2 inhibitors and the inhibition of cyclin A2 synthesis on the transcriptional activity of the 1-cell embryos. I also examined the effect of microinjection of active cyclin A2/cdk2 protein on the transcription.

Results

Effects of 3'-deoxyadenosine and cycloheximide on the accumulation of cyclin A2 in the nucleus of mouse 1-cell embryos

A previous report showed by immunoblotting that cyclin A2 protein was not detected in the oocytes and that it appeared following fertilization (Fuchimoto et al., 2001). Its amount increased during 1-cell stage and this increase was prevented by 3'-deoxyadenosine (3-dA), an inhibitor of polyadenylation of mRNA (Fuchimoto et al., 2001). However, immunoblotting approach did not provide the information of the nuclear localization. This information is important, since cyclin A2 functions in the nucleus (Jackman et al., 2002; Pines and Hunter, 1991). Therefore, I immunostained 1-cell embryos with anti-cyclin A2 antibody to examine the pronuclear localization of cyclin A2. Since a previous study showed that embryonic gene expression is initiated at mid-S phase (Aoki et al., 1997), I collected the embryos 6 and 12 h after insemination: in the culture system in my laboratory, the embryos are in G1 and S/G2 phases at 6 and 12 h after insemination, respectively (Figure 1-1; Aoki et al., 1992; Aoki et al., 1999). I also confirmed that BrUTP incorporation was detected in none of the embryos at 6 h, in a very small part of embryos at 8 h and in all embryos at 12 h (Figure 1-2). The results of immunostain showed that, the amount of cyclin A2 in the pronuclei prominently increased between 6 and 12 h (Figure 1-4).

To investigate whether the nuclear accumulation of cyclin A2 occurs after its *de novo* synthesis induced by polyadenylation of maternal mRNA, 1-cell embryos were cultured in the presence of 3-dA or cycloheximide (CHX). The results showed that the accumulation of cyclin A2 was inhibited by the treatment with 3-dA (Figure 1-4). The amount of cyclin A2 in the nucleus was significantly smaller in the embryos treated with 3-dA than 3-dG and no treatment ($P < 0.01$). The treatment with CHX also significantly decreased the amount of accumulated cyclin A2 when compared to the

treatment with DMSO and no treatment ($P < 0.01$). These results are consistent with the previous report in which the amount of cyclin A2 was examined by immunoblotting (Fuchimoto et al., 2001), supporting the hypothesis that maternal cyclin A2 mRNA is polyadenylated after fertilization and this polyadenylation stimulates the translation of cyclin A2. Once cyclin A2 was translated, the protein seemed to be accumulated rapidly in the pronucleus. It is known in somatic cells that the newly synthesized cyclin A2 binds to pre-existing cdk2, which allow them to get into nucleus. Therefore, I examined by immunoblotting that cdk2 is present before cyclin A2 synthesis in early 1-cell stage. The results showed that cdk2 protein is already expressed before fertilization, and that its amount was not changed after fertilization (Figure 1-3).

Effects of cdk2 inhibitors and the microinjection of cyclin A2 diced siRNA on EGA in the mouse 1-cell embryos

To examine whether the cyclin A2/cdk2 is involved in the regulation of EGA, 1-cell embryos were cultured with the roscovitine (Ros) or butyrolactone I (BL1), potent inhibitors of cdks. The effects of these inhibitors on transcriptional activity were investigated 12 h after insemination by *in vitro* transcriptional activity assay. The results showed that the transcriptional activity was reduced by the treatment with the inhibitors (Figure 1-5). The amount of incorporated BrUTP was significantly smaller in the embryos treated with Ros than 0.16 % DMSO ($P < 0.01$). The treatment with BL1 also significantly decreased the incorporation of BrUTP when compared to the treatment with 1.00 % DMSO ($P < 0.01$). These results suggested that cyclin A2/cdk2 regulates the initiation of EGA.

To ensure these results, I microinjected diced small interference RNA (d-siRNA), which had been prepared by dicing the full length of double strand cyclin A2 mRNA, into the MII stage oocytes. Twelve hours after insemination, the pronuclear accumulation of cyclin A2 and transcriptional activity was examined.

Immunostaining with anti-cyclin A2 antibody showed that microinjection of cyclin A2 d-siRNA significantly decreased the accumulation of cyclin A2 in the pronuclei ($P < 0.01$) (Figure 1-6A, B). This effect was specific, since no effect was observed in the accumulation of TATA binding protein (TBP) whose amount in the pronucleus had been reported to increase after fertilization (Worrad et al., 1994). Furthermore, the microinjection of rabbit globin d-siRNA did not affect the nuclear accumulation of cyclin A2.

In the embryos microinjected with cyclin A2 d-siRNA, transcription was prominently reduced. The transcriptional activity was significantly lower ($P < 0.01$) in the embryos microinjected with cyclin A2 d-siRNA than the control embryos withiout microinjection and those with rabbit globin d-siRNA (Figure 1-6C), which strongly suggests that cyclin A2 regulates EGA in the 1-cell embryos.

Since cyclin A2 is essential for the progression of S phase in the somatic cells, it is possible that the reduction of cyclin A2/cdk2 activity by the cdk inhibitors or cyclin A2 –d-siRNA induced the cessation or retardation of S phase progression, which led to the reduction of transcription in the 1-cell embryos. It has been reported that when DNA synthesis was completely blocked by the treatment with aphidicolin, EGA occurred but the transcriptional activity decreased by 35% (Aoki et al., 1997). To address this possibility, I analyzed the effects of cdk inhibitors and cyclin A2 d-siRNA on the DNA synthesis in the 1-cell embryos. As shown in the Table 1-1, DNA synthesis was not inhibited by the treatment with cdk inhibitors or microinjection with cyclin A2 d-siRNA, as the incorporation of BrdU was detected in all embryos subjected to those treatments, although no incorporation was observed in the embryos treated with aphidicolin. These results demonstrated that cyclin A2/cdk2 is involved in the regulation of EGA by the mechanism independent of cell cycle events.

Effect of the microinjection of cyclin A2/cdk2 protein on EGA in the mouse 1-cell

embryos

To further confirm the involvement of cyclin A2/cdk2 in the regulation of EGA, I microinjected recombinant cyclin A2/cdk2 protein into the cytoplasm of 1-cell embryos 1 h after imsemination. Following culture, the embryos were collected 10 h after insemination for *in vitro* transcriptional activity assay. Figure1-7 shows that injection of active cyclin A2/cdk2 protein increased transcriptional activity. The embryos microinjected with active protein showed significantly higher activity than those with heat-inactivated protein (P <0.01).

Discussion

After fertilization, embryonic genome is activated in two phases (Schultz, 1993). In the first phase, initiation of EGA occurs in the male and female pronuclei during the 1-cell stage (Aoki et al., 1997; Bounial et al., 1995). Following condensation of male and female chromosomes at the first mitosis, zygotic nucleus is formed and the second wave of EGA occurs during the 2-cell stage (Schultz, 1993). Although a number of reports have demonstrated that embryos become competent to transcribe their genes during the 1-cell stage (Majumder et al., 1993 ; Ram and Schultz, 1993; Rastelli et al., 2001; Wang et al., 2001), the mechanism by which the embryonic genes are activated remains to be elucidated.

In this chapter, I examined the involvement of cyclin A2/cdk2 in EGA, as a candidate for the factor regulating the initiation of EGA in the 1-cell embryos. The results showed that the transcriptional activity was decreased in the embryos in which the activity and nuclear accumulation of cyclin A2/cdk2 was inhibited by the treatment with the cdk inhibitors and the microinjection of cyclin A2 d-siRNA, respectively (Figure 1-5, 1-6C). Furthermore, the injection of recombinant active cyclin A2/cdk2 increased transcriptional activity (Figure 1-7). These results suggest that cyclin A2/cdk2 is involved in the initiation of EGA in the 1-cell embryos.

Nuclear accumulation of cyclin A2

In a previous report, an immunoblotting analysis showed that cyclin A2 protein was not detected in the unfertilized oocytes, and that it abruptly appeared before the onset of DNA replication after fertilization (Fuchimoto et al., 2001). This appearance was prevented by inhibiting protein synthesis or polyadenylation by cycloheximide or 3'-deoxyadenosine. In this chapter, I have demonstrated that the nuclear accumulation of cyclin A2 increases during G1 and S phase in the 1-cell

embryos, which was also prevented by cycloheximide or 3'-deoxyadenosine (Figure 1-4). Since the appearance and the nuclear accumulation of cyclin A2 protein was thus well correlated with each other, the protein would be transported into the nucleus soon after it was synthesized.

In the somatic cells, the nuclear accumulation of cyclin A2 during S phase has been well reported. In HeLa cells, only a small amount of cyclin A2 was detected in the nucleus at early S phase and then it increased during S and G2 phases (Pines and Hunter, 1991). The nuclear accumulated cyclin A2 seemed to form a complex with cdk2, since it was reported that the association with cdk2 is necessary for the transport of cyclin A2 to the nucleus (Jackman et al., 2002; Maridor et al., 1993). Therefore, in the 1-cell embryos, the nuclear accumulated cyclin A2 would be also associated with cdk2.

During G1 phase, it is known that cyclin E also binds to and activates cdk2 in the somatic cells (Sherr, 1996). However, cyclin E was not detected in the mouse 1-cell embryos by immunoblotting analysis (Iwamori, 2004). Therefore, cdk2 activity seems to be regulated by binding with cyclin A2 but not cyclin E during 1-cell stage.

Cdk2 inhibitors prevented EGA

To examine the involvement of cyclin A2/cdk2 in the regulation of EGA in the 1-cell embryos, I used cell division cycle 2 (cdc2)/cdk2 selective inhibitors, roscovitine (Ros) and butyrolactone I (BL1). These inhibitors prominently decreased the transcriptional activity in the 1-cell embryos (Figure 1-5), suggesting the involvement of cdk2 in the initiation of EGA. Although these inhibitors also affect some other enzymes, they could be used as cdk2-specific inhibitors in this study by following reasons. Firstly, comparison of 50% inhibitory concentration (IC_{50}) in *in vitro* kinase assay revealed that Ros is highly specific for cdc2, cdk5 and cdk2 but not other G1 cyclin dependent kinases, including cdk4 and cdk6, or other many protein kinases, *e.g.*

protein kinase A and C, myosin light-chain kinase, casein kinase 2, insulin receptor tyrosine kinase, *et cetera* (Kitagawa et al., 1994; Meijer et al., 1997): there are more than 100-fold differences in IC_{50} values. BL1 also showed profound inhibitory effects on cdc2 and cdk2 but little effects on the other kinases, *e.g.* mitogen-activated protein kinase, protein kinase C, cyclic-AMP dependent kinase, casein kinase II, *et cetera* (Kitagawa et al., 1993): the IC_{50} values for cdc2 and cdk2 were 40 times higher than other kinases (Kitagawa et al., 1993; Someya et al., 1994). Secondly, although Ros and BL1 efficiently inactivate cdc2 kinase as well as cdk2, as described above (Ktagawa et al., 1994; Meijer et al., 1997), cdc2 kinase is activated only at M phase but not interphase at which EGA was analyzed in this study (Choi et al., 1991). Therefore, their effects on cdc2 kinase could be neglected in this study. Finally, cdk5, which was isolated as cdc2-related kinase, was preferentially expressed in non-proliferating organs such as brain and lung (Tsai et al., 1993). Its expression has not been reported in the preimplantation embryos. The cdk5 null mutant embryos are alive until around the time of parturition. They exhibited neuronal defects, suggesting that cdk5 plays a role in neurogenesis (Ohshima et al., 1996). It is thus unlikely that cdk5 functions in the early preimplantation embryos. Taken together, Ros and BL1 would specifically inhibit cdk2 in the 1-cell embryos and a marked decrease of transcription in the embryos treated with these inhibitors suggests that cdk2 activity is essential for the initiation of EGA.

Inhibition of cyclin A2 synthesis by d-siRNA

To inhibit cyclin A2 synthesis following fertilization, I employed RNAi approach in which d-siRNA was microinjected into the embryos. The results showed that the microinjection of d-siRNA significantly reduced the nuclear accumulation of cyclin A2 in the 1-cell embryos (Figure 1-6A, B).

Several reports had demonstrated that RNA interference (RNAi) was operative

in the mouse oocytes and preimplantation embryos (Svoboda et al., 2001, 2004; Wianny and Zernicka-Goetz, 1999). Although in these reports, selective mRNA degradation was achieved successfully by double strand mRNA (dsRNA) and dsRNA expression vector, it took for at least 20 h after their microinjection. However, in my experiment, much earlier effect of RNAi is required because the *de novo* synthesis of cyclin A2 protein starts as early as 6 h after insemination (Figure 1-4; Fuchimoto et al., 2001). d-siRNA would exert its effect in a shorter time than dsRNA, since it takes some extra time for dsRNA to be diced into d-siRNA in the embryos after microinjected.

Generally, one of the best ways to know the function of a gene of interest is to produce the null mutant mice by gene targeting. It was previously reported that the homozygous cyclin A2 null mutant developed normally until postimplantation, around day 5.5 p.c. (Murphy et al., 1997). However, gene targeting approach does not provide the information about the necessity of the targeted gene in the early preimplantation development, since considerable amounts of maternal transcripts and proteins which were synthesized from heterogenic mother genes still remain in the embryos after fertilization (Schultz, 1993). Especially, in the 1-cell mouse embryos, most part of transcript and protein are derived from maternal gene, since the level of transcription from embryonic gene is still low at this stage. Furthermore, even in the blastocyst stage, cyclin A2 protein was detected in its null mutant embryos (Murphy et al., 1997). Thus, microinjection of d-siRNA, which was employed in this study, is the best way to investigate the function of cyclin A2 in the 1-cell embryos.

Cyclin A2 functions as a transcriptional regulator independently of cell cycle

Cyclin families are named “cyclin” because their expressions depend on cell cycle progression (Girard et al., 1991; Pagano et al., 1992; Resnitzky et al., 1995; Zindy et al., 1992). Therefore, the investigations for the functions of cyclin families have been centered on their cell cycle regulation. However, it has not been fully understood

how cell cycle progression is regulated by cyclin families. Recently, retinoblastoma protein (pRb) was identified as a substrate for cyclin/cdk families (Sherr, 1996). Since it had been known that phosphorylation of pRb was involved in the transcriptional regulation (Harbour and Dean, 2000a, 2000b; Nevins et al., 1997; Swanton, 2004; Vandel et al., 2001), a number of studies have investigated the function of cyclin/cdk families in the cell cycle regulation *via* transcriptional control. These studies have demonstrated that the phosphorylation of pRb by cyclin A2/cdk2 induced the dissociation of a complex of pRB and the proteins containing LXCXE motif which is the one of pRb binding motif (Zarkowska and Mittnacht, 1997). Release of these LXCXE motif containing proteins from pRb induced deregulation of suppressive state for gene expression, since most of pRb binding proteins function as repressors of gene expression (Frolov and Dyson, 2004; Harbour and Dean, 2000a). Thus, cyclin A2/cdk2 regulates transcription *via* phosphorylation of pRb. Furthermore, it was reported that cyclin A2/cdk2 directly activates a transcription factor. Several reports demonstrated that general transcription factor Sp1 (named by the purification scheme that included Sephacryl and Phosphocellulose columns; Kadonaga et al., 1987) was associated with cyclin A2/cdk2 (Fojas de Borja et al., 2001; Haidweger et al., 2001) and phosphorylated by them (Fojas de Borja et al., 2001; Haidweger et al., 2001). Phosphorylation of Sp1 increases its DNA binding affinity (Fojas de Borja et al., 2001; Haidweger et al., 2001). Sp1 binding sites, GC box and the related GT/CACC box are present in many promoters and other regulatory sequences of tissue-specific and ubiquitous genes (Philipsen and Suske, 1999), suggesting that cyclin A2/cdk2 is implicated with the expression of numerous genes *via* regulating Sp1 activity.

Global transcriptional activity is coupled with the first round of DNA replication. Its inhibition did not prevent the initiation of EGA but inhibited global transcriptional activity slightly (Aoki et al., 1997). Therefore, it is possible that the inhibition of cyclin A2/cdk2 function by cdk2 inhibitors and d-siRNA prevented DNA

replication, which led to the decrease in the transcriptional activity. However, this scenario can be excluded, since DNA replication normally occurred in the embryos treated with cdk inhibitors and microinjected with d-siRNA (Table 1-1). These results suggest that cyclin A2/cdk2 regulate the initiation of EGA independently of DNA replication in the 1-cell embryos.

Table 1-1. **Effects of cdk2 inhibitors and the microinjection of diced siRNA on DNA synthesis in the mouse 1-cell embryos**

Treatment	No. of experiment	No. of embryos		
		Total analyzed	BrdU signal	
			+	-
No treatment	3	125	125	0
DMSO [a]	3	85	85	0
Roscovitine [b]	3	65	65	0
Butyrolactone I [b]	3	69	69	0
Globin d-siRNA	2	21	21	0
Cyclin A2 d-siRNA	2	15	15	0
Aphidicolin [c]	2	12	0	12

[a] DMSO was added to culture medium at a concentration of 0.16 or 1.00 %, since it was used as the solvent for roscovitine (Ros) and butyrolactone I (BL1) and the final concentrations of DMSO were 0.16 % and 1.00 % for Ros and BL1.

[b] The concentration of Ros and BL1 was 100 μM.

[c] The concentration of Aphidicolin was 3 μg/ml.

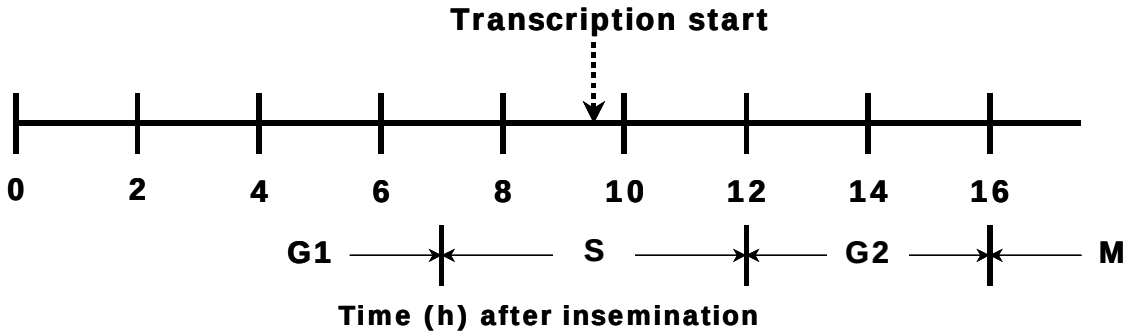


Figure 1-1. Time schedule for the events of cell cycle during mouse 1-cell stage.

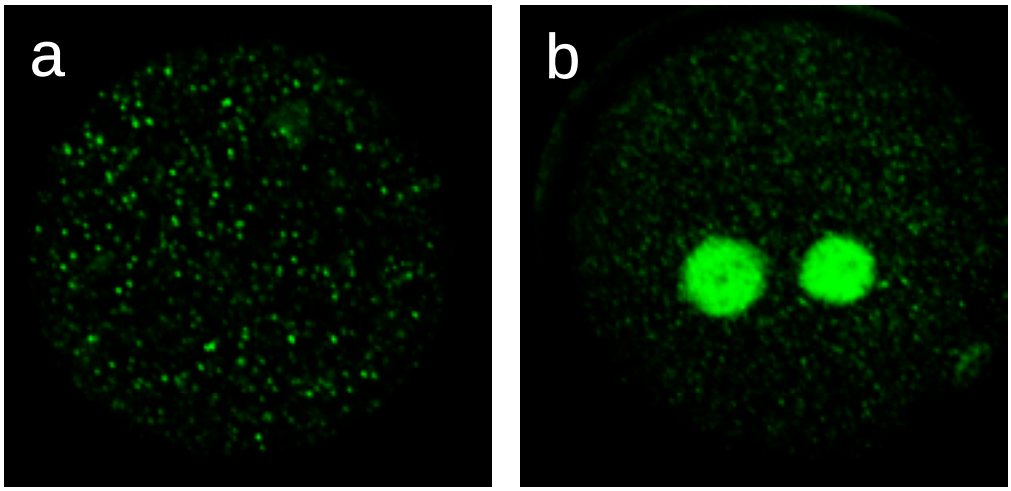


Figure 1-2. **Incorpotated BrUTP by mouse 1-cell embryos.** Confocal microscopic images of the mouse 1-cell embryos immunostained with anti-BrdU antibody. One-cell embryos were collected 6 (a) and 12 (b) h after insemination and permeabilized. Permeabilized embryos were incubated with transcription mixture containing BrUTP. Incorporated BrUTP by 1-cell embryos were detected by immunostain with anti-BrdU antibody.

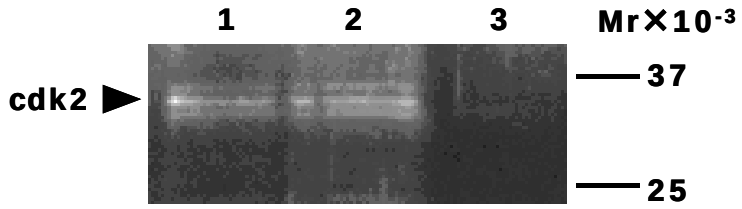


Figure 1-3. **Immunoblotting analysis for cdk2 in the unfertilized oocytes and 1-cell embryos.** Unfertilized oocytes were isolated from the ampullae of oviducts. One cell embryos were collected 12 h post insemination. Two hundreds of unfertilized oocytes (lane 1) and 1-cell embryos (lane 2) were used. Ten μl of CZB medium in which the embryos were cultured was used (lane 3). These samples were subjected to SDS-PAGE and probed with anti-cdk2 antibody. Arrow head indicates cdk2. Molecular mass standards are indicated to the right of the lanes.

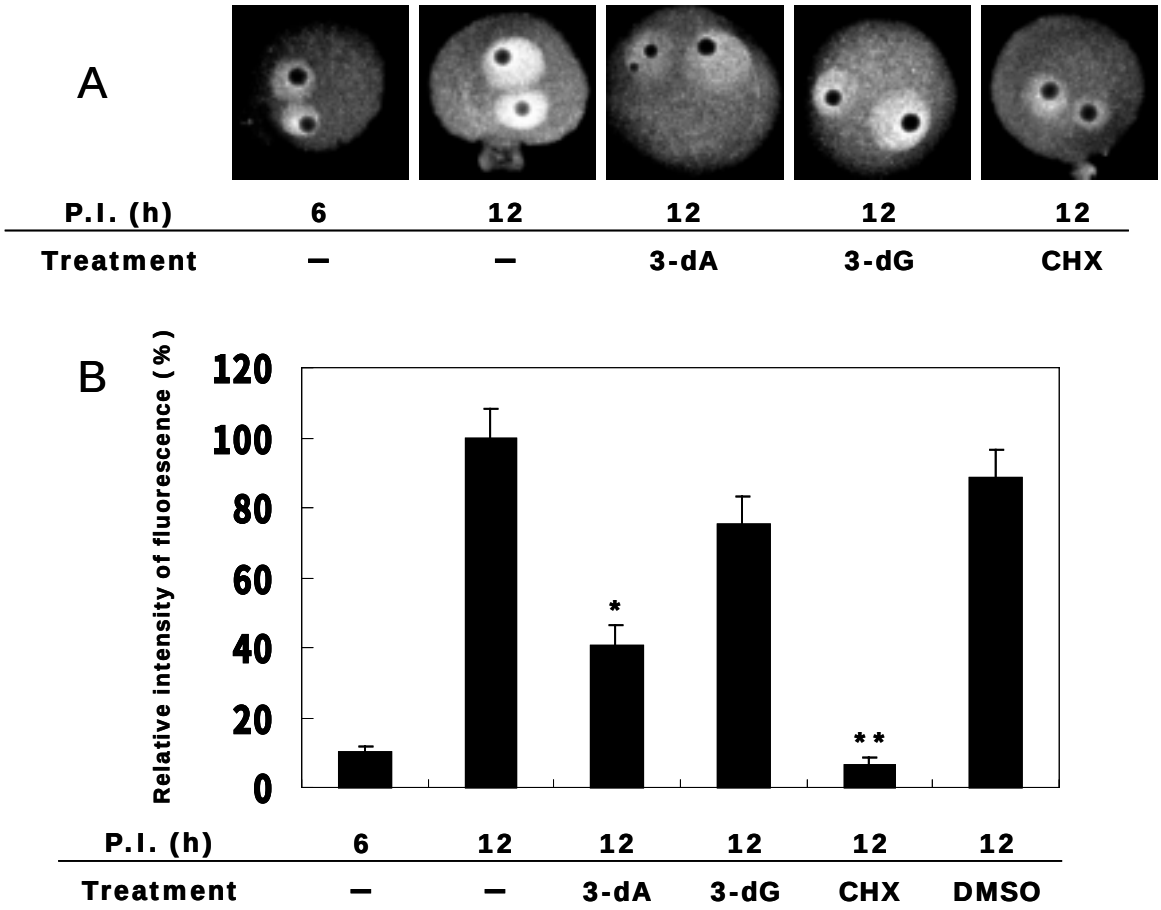


Figure 1-4. **Changes of pronuclear concentration of cyclin A2 during 1-cell stage and the effects of the treatment with 3'-deoxyadenosine (3-dA) and cycloheximide (CHX).** (A) Confocal microscopic images of the mouse 1-cell embryos immunostained with anti-cyclin A2 antibody. One-cell embryos treated with 3-dA, 3'-deoxyguanosine (3-dG) and CHX were collected 12 h post insemination (P.I.) and immunostained with anti-cyclin A2 antibody. The chemicals were dissolved with dimethylsulfoxide (DMSO). Control embryos without the treatment were collected 6 and 12 h P.I.. (B) The relative intensity of pronuclear fluorescence was analyzed as described in *Materials and Methods*. The relative fluorescence in the embryos treated with 3-dA and CHX. The intensity of fluorescence was measured in the embryos after immunostaine with anti-cyclin A2 antibody. The average value in the control embryos (12 h,

non-treatment) was set as 100%. The experiment was conducted twice, similar results were obtained in each case. The numbers of embryo examined were: 32, 29, 19, 27, 15 and 14 for the control embryos at 6 h, 12 h, and the embryos treated with 3-dA, 3-dG, DMSO, and CHX, respectively. The columns and bars represent means ± s.e.m. An asterisk (*) indicates significant dufference from the value at the 3-dG (Student's *t* test $P < 0.01$). Double asterisk (**) indicate significant dufference from the value at the DMSO (Student's *t* test $P < 0.01$).

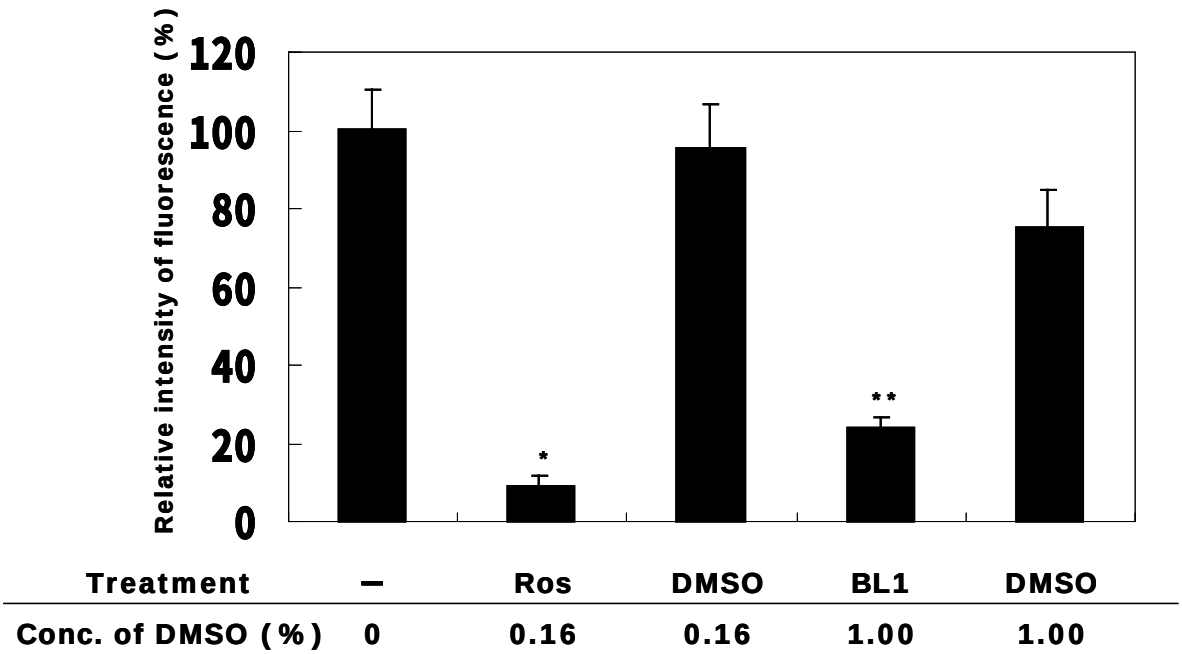
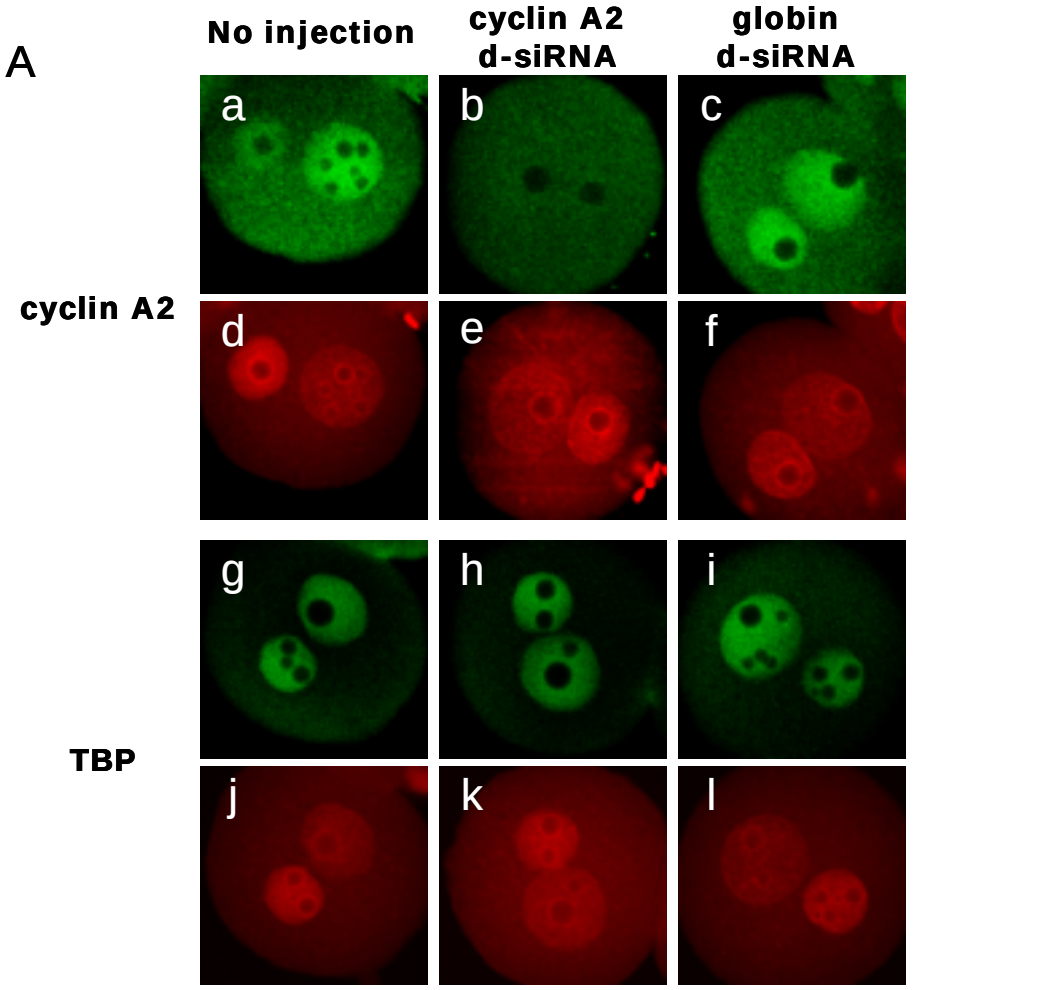


Figure 1-5. **Effects of roscovitine (Ros) and butyrolactone I (BL1) on BrUTP incorporation by the mouse 1-cell embryos.** The embryos were collected 4 h after insemination and transferred to the culture medium containing Ros or BL1. DMSO was used as the solvent for Ros and BL1 and the final concentrations of DMSO in the culture medium were 0.16 % and 1.00 % for Ros and BL1, respectively. DMSO and cdk2 inhibitors were not added in culture medium for the control embryos (treatment minus). The embryos were collected 12 h after insemination and subjected to *in vitro* transcriptional activity assay. The intensity of fluorescence was analyzed as described in *Materials and Methods*. The average value in the control embryos was set as 100%. The experiments for Ros and BL1 were conducted three times and twice, respectively. Similar results were obtained in each case. The total numbers of embryo examined were: 39, 33, 37, 21 and 26 for the control, Ros, 0.16 % DMSO, BL1, and 1 % DMSO, respectively. The columns and bars represent means ± s.e.m. An asterisk (*) indicates significant dufference from the value at the 0.16 % DMSO (Student's *t* test $P < 0.01$).

Double asterisk (**) indicate significant dufference from the value at the 1.00 % DMSO (Student's *t* test $P < 0.01$).


A
No injection
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globin d-siRNA
cyclin A2
TBP
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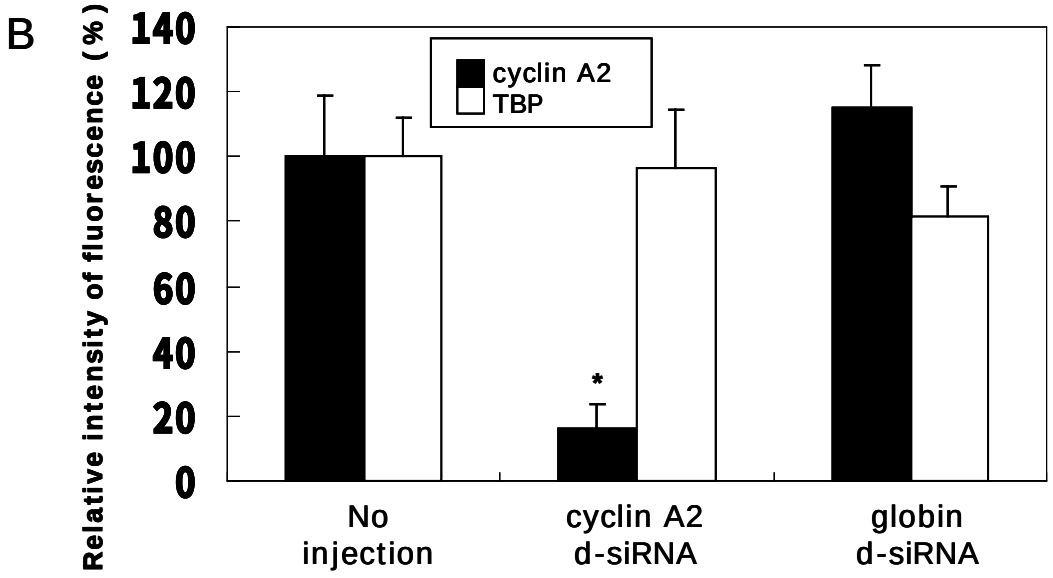
B
Relative intensity of fluorescence (%)
140
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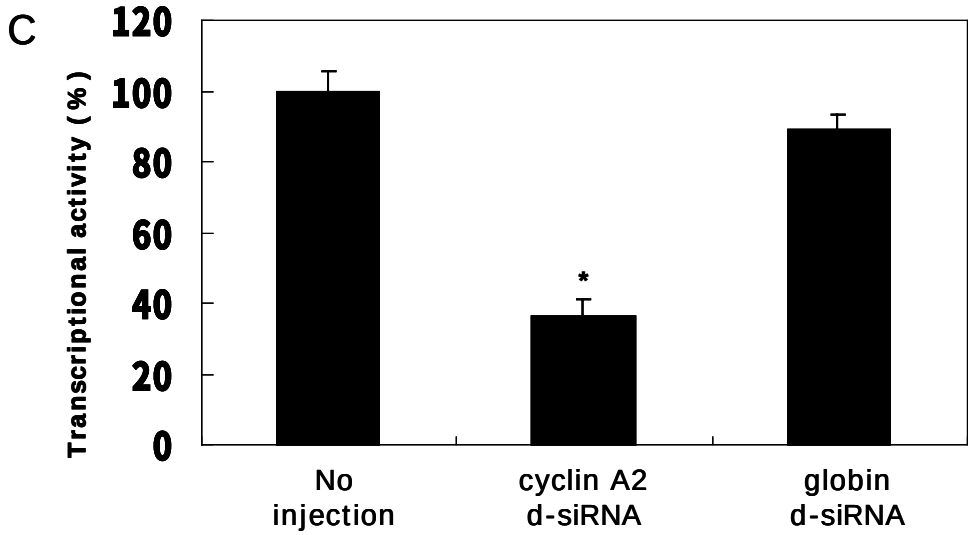


Figure 1-6. **Inhibition of cyclin A2 accumulation in the nucleus by cyclin A2 diced siRNA (d-siRNA).** (A) Confocal microscopic images of the mouse 1-cell embryos immunostained with anti-cyclin A2 antibody (a-c) and anti-TBP antibody (g-i). DNA was stained with propidium iodide (d-f, j-l). MII oocytes were microinjected with cyclin A2 d-siRNA (b, h) or globin d-siRNA (c, i) and collected 12 h after insemination and immunostained with the antibodies. (B) The relative intensity of pronuclear fluorescence in the embryos injected d-siRNA. The intensity of fluorescence was measured in the embryos after immunostaining with anti-cyclin A2 and anti-TBP antibodies. The average value in the control embryos with no injection was set as 100 %. The experiments of microinjection were perform three times in which the embryos were immunostained with ant-cyclin A2 antibody in every case and with ant-TBP antibody twice. Similar results were obtained in each case. The total numbers of embryo examined were: 11, 10, 17, 8, 14 and 11 for control/cyclin A2 immunostain, control/TBP immunostain, globin d-siRNA injection/cyclin A2 immunostain, globin d-siRNA injection/TBP immunostain, cyclin A2 d-siRNA injection/cyclin A2 immunostain and cyclin A2 d-siRNA injection/TBP immunostain, respectively. The

columns and bars represent means ± s.e.m. An asterisk (*) indicates significant dufference from the value at the no injection and globin d-siRNA injection (Student's *t* test $P < 0.01$). (C) Effects of the microinjection of cyclin A2 d-siRNA on BrUTP incorporation in the mouse 1-cell embryos. MII oocytes were microinjected with cyclin A2 d-siRNA or globin d-siRNA, collected 12 h after insemination and subjected to *in vitro* transcriptional activity assay. The intensity of fluorescence was analyzed as described in *Materials and Methods*. The average value in no injected embryos was set as 100 %. The experiments were conducted twice. Similar results were obtained in each case. The total numbers of embryos examined were: 10, 31 and 37, for no injection, cyclin A2 d-siRNA injection and globin d-siRNA injection, respectively. The columns and bars represent means ± s.e.m. An asterisk (*) indicates significant dufference from the value at the no injection and globin d-siRNA injection (Student's *t* test $P < 0.01$).

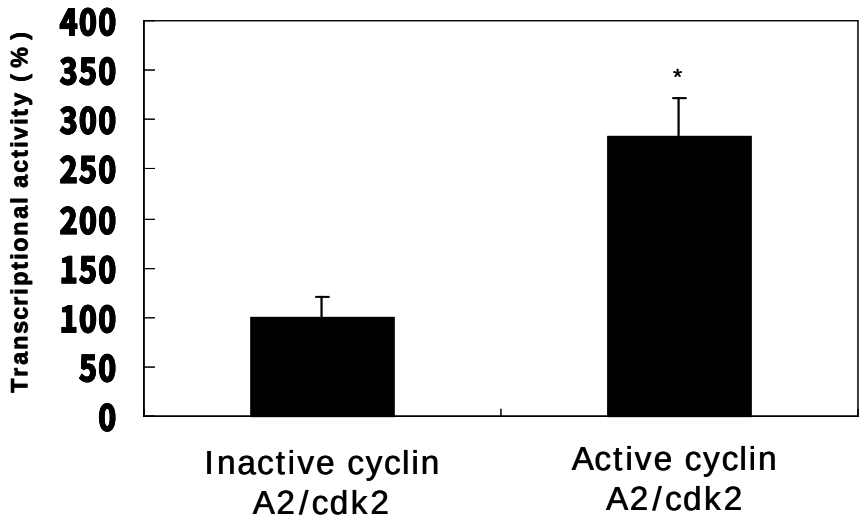


Figure 1-7. **Effects of cyclin A2/cdk2 protein injection on BrUTP incorporation in the mouse 1-cell embryos.** One-cell embryos were microinjected with active cyclin A2/cdk2 protein or inactive one, collected 10 h after insemination and subjected to *in vitro* transcriptional activity assay. The intensity of fluorescence was analyzed as described in *Materials and Methods*. The average value in the embryos microinjected with inactive cyclin A2/cdk2 protein was set as 100 %. The experiments were conducted three times. Similar results were obtained in each case. The total numbers of embryo examined were 34 and 29 for inactive protein injection and active protein injection, respectively. The columns and bars represent means ± s.e.m. An asterisk (*) indicates significant dufference from the value at the inactive cyclin A2/cdk2 injection (Student's *t* test $P < 0.01$).

Chapter 2

Phosphorylation of retinoblastoma protein (pRb) by cyclin A2/cdk2

Abstract

In chapter 1, I showed that cyclin A2/cdk2 was involved in the regulation of EGA. In this chapter, to clarify the mechanisms by which cyclin A2/cdk2 regulates EGA, I investigated the changes of retinoblastoma protein (pRb) phosphorylation state during 1-cell stage. pRb is one of the substrate for cyclin/cdk families, possessing 16 potential phosphorylation sites for cyclin/cdk complexes. Immunostaining with antibodies recognizing the site-specific phosphorylation of pRb revealed that phosphorylation levels of cdk2 target sites, Ser249/Thr252, Thr356, Ser807/811 and Thr821/826 increased during 1-cell stage, although Ser780, a cdk4 target site, was not phosphorylated. These results suggested that cdk2 was activated during 1-cell stage and catalyzed pRb. To examine whether the phosphorylation of pRb depends on cyclin A2 synthesis during 1-cell stage, cyclin A2 d-siRNA was microinjected into the embryos. The results showed that inhibition of cyclin A2 synthesis decreased phosphorylation levels of Thr356 during 1-cell stage, suggesting that *de novo* synthesis of cyclin A2 is necessary for the phosphorylation of pRb during 1-cell stage. Supporting this hypothesis was that microinjection of recombinant cyclin A2/cdk2 increased the phosphorylation level of Thr356 in pRb. Since it is known that pRb function as a suppressor of gene expression, its phosphorylation leads to deregulation of the suppression of gene expression. Therefore, newly synthesized cyclin A2 would activate cdk2 to phosphorylate pRb, which results in the deregulation of the suppression of genes by pRb. This may be the mechanism for the initiation of EGA.

Introduction

It has been known that the initiation of embryonic gene activation (EGA) is regulated by the newly synthesized proteins following fertilization (Aoki et al, 2003; Wang and Latham, 1997). In chapter 1, I showed that cyclin A2, which is newly synthesized after fertilization, was involved in the regulation of EGA. In this chapter, the mechanisms by which cyclin A2/cdk2 regulates EGA are investigated. To this end, the substrate for cyclin A2/cdk2, which would be a regulator of gene expression, should be identified.

Retinoblastoma protein (pRb) is a good candidate for it. pRb is one of the substrate for cyclin/cdk families, possessing 16 potential phosphorylation sites for cyclin/cdk complexes (Barrientes et al, 2000; Driscoll et al, 1999). It is expressed ubiquitously (Buchkovich et al., 1989) and was also detected in the mouse 1-cell embryos (Iwamori et al, 2002). In the mammalian somatic cells, cyclin D/cdk4, cyclin D/cdk6, cyclin E/cdk2 and cyclin A2/cdk2 phosphorylate pRb during G1 and S phase (Akiyama et al., 1992; Harlow, 1994; Lin et al., 1991; Meyerson and Matsushime et al., 1994; Sherr, 1996; Zarkowska and Mittnacht, 1997). In the cell cycle, pRb functions by assembling multiprotein complexes on the promoters of target genes as a negative regulator of cell proliferation. A large number of proteins including transcription factors and the components of chromatine-associated complexes have been identified as pRb binding proteins (Frolov and Dyson, 2004; Harbour and Dean, 2000a). Most of these proteins function as repressors of gene expression. It is known that cyclin/cdk complexes phosphorylate pRb to inactivate it, which results in the activation of genes that have been suppressed by pRb. Therefore, it is possible that pRb suppresses the gene expression in the early 1-cell stage. Upon the accumulation of newly translated cyclin A2 in the late 1-cell stage, cdk2 would be activated and phosphorylate pRb, which results in the deregulation of the suppression of genes by pRb. This may be the

mechanism for the initiation of EGA.

There are many proteins which are associated with pRb. Throughout cell cycle, pRb regulates transcription by recasting its binding partners (DeGregori, 2004). In addition, its partners seem to be different in each tissue to regulate the tissue specific gene expression pattern. For instance, E1A-like inhibitor of differentiation 1 (EID-1) which was identified as pRb binding protein is preferentially expressed in adult heart and skeletal muscle (MacLellan et al, 2000; Miyake et al., 2000). Overexpression of EID-1 in skeletal muscle cells resulted in muscle-specific gene repression, suggesting that EID-1 function as transcriptional regulator for muscle-specific genes (MacLellan et al, 2000). Since there are a large number of pRb binding proteins, the mechanisms by which pRb regulates transcription are not fully understood.

The phosphorylation of pRb induces its conformational changes which affect the binding affinity with pRb partners (Driscoll et al, 1999; Zarkowska T and Mittnacht S, 1997). In the somatic cells, phosphorylation level of pRb is changed in a cell cycle-dependent manner. At the G0 and G1 phases of cell cycle, unphosphorylated form is detected, whereas at the S and G2/M phases, multiple phosphorylated forms are detected (Buchkovich et al. 1989; DeCaprio et al, 1992). Cyclin A2/cdk2 efficiently phosphorylate Thr5, Ser249/Thr252, Thr356, Ser807/811 and Thr821/826 on pRb as examined by *in vitro* kinase assay, whereas Ser780 was mainly phosphorylated by cyclin D/cdk4 (Kitagawa et al., 1996). In particular, the phosphorylation of Thr356, Ser807, Ser811 or Thr821 induce a drastic change in the conformation of pRb (Driscoll et al., 1999). Thus, phosphorylation by cdk2 is involved in the conformational changes of pRb, suggesting that cdk2 activity affect the recasting of pRb binding partners. However, it is not fully understood which sites are preferentially phosphorylated by cyclin A2/cdk2 *in vivo*.

There is no literature which investigated the phosphorylation state of pRb or

the association of pRb with EGA in the mouse preimplantation embryos. In this chapter, to clarify the involvement of pRb phosphorylation in the regulation of EGA, I investigate the changes in the phosphorylation state of pRb during 1-cell stage using the antibodies that recognize site specific phosohorylated pRb.

Results

To examine whether retinoblastoma protein (pRb) is the substrate of cyclin A2/cdk2 in the mouse 1-cell embryos, I investigated the phosphorylation state of pRb by immunostaining the embryos with the antibodies against site-specific phosphorylated forms of pRb. It has been known that there are many phosphorylated sites in pRb and that Ser249/Thr252, Thr356, Ser807/811 and Thr821/826 are preferentially phosphorylated by cyclin A2/cdk2 (Kitagawa et al., 1996). As shown in figure 2-1, phosphorylation levels of these sites significantly increased between 6 and 12 h ($P < 0.01$) during which the increase in the nuclear localization of cyclin A2 was observed (Figure 1-4), although total amount of pRb, which was examined by using the antibody recognizing both phosphorylated and unphosphorylated pRb, was not changed. On the other hand, the phosphorylation of Ser780 on pRb, which has been known as the site that is phosphorylated by cyclin D/cdk4 but not cyclin A2/cdk2 (Kitagawa et al., 1996), was not detected in the 1-cell embryos (Figure 2-1C). After cleavage, a faint signal of phosphorylation was detected in the nucleus at the early 2-cell stage and then the signal increased at the late 2-cell stage.

To examine whether the increase of phosphorylations during 1-cell stage were dependent on cdk2 activity, 1-cell embryos were cultured in the presence of roscovitine (Ros). The results showed that the phosphorylation levels of Ser249/Thr252, Thr356, Ser807/811 and Thr821/826 were significantly decreased by the treatment with Ros ($P < 0.01$), but that total amount of pRb was not changed (Figure 2-1, A, B). These results suggested that cyclin A2/cdk2 catalyzed pRb as a substrate around the time of EGA in the 1-cell embryos.

The results described above and in chapter 1 suggest that cyclin A2 is synthesized during G1/S phase (6-12 h after insemination), which leads to the activation of cdk2 by forming their complex and that the activated cyclin A2/cdk2

increases the phosphorylation levels of pRb. To address this scenario, I firstly inhibited the synthesis of cyclin A2 by treating the embryos with cycloheximide (CHX), a protein synthesis inhibitor, and examined for the phosphorylations of pRb. Figure 2-2 shows that the treatment with CHX decreased the phosphorylation of pRb. The phosphorylation levels of Ser249/Thr252, Thr356 and Ser807/811 were significantly lower in the embryos treated with CHX when compared to the ones treated with DMSO ($P < 0.01$). Secondly, to specifically inhibit the synthesis of cyclin A2, the embryos were microinjected with cyclin A2 diced small interference RNA (d-siRNA). In the microinjected embryos, phosphorylation level of Thr356 was prominently reduced. Its phosphorylation level was significantly lower than the control embryos without microinjection and those microinjected with rabbit globin d-siRNA ($P < 0.01$) (Figure 2-3), which strongly suggests that cyclin A2/cdk2 regulates phosphorylation of Thr356 in the 1-cell embryos. To further confirm the involvement of cyclin A2/cdk2 in the phosphorylation of Thr356, I microinjected recombinant cyclin A2/cdk2 protein into the cytoplasm of 1-cell embryos 1 h after insemination. Following culture, the embryos were collected 6 h after insemination for immunocytochemistry. Figure 2-4 shows that the microinjection of active cyclin A2/cdk2 protein increased phosphorylation level of Thr356. The embryos microinjected with active protein showed significantly higher phosphorylation level than those microinjected with heat-inactivated protein ($P <0.01$).

Discussion

In this chapter, I showed that the phosphorylation levels of retinoblastoma protein (pRb) on the cyclin A2/cdk2 target sites increased between 6 and 12 h during which embryonic gene expression was initiated. These phosphorylations would be regulated by cyclin A2/cdk2, since phosphorylation levels of pRb were decreased by the treatment with Ros treatment. Furthermore, the phosphorylation level of Thr356 was decreased in the embryos microinjected with cyclin A2 d-siRNA, suggesting that newly synthesis of cyclin A2 was involved in the regulation of Thr356 phosphorylation. These results suggested that pRb was phosphorylated by cyclin A2/cdk2 during mouse 1-cell stage.

Regulation of pRb phosphorylation in the 1-cell embryos

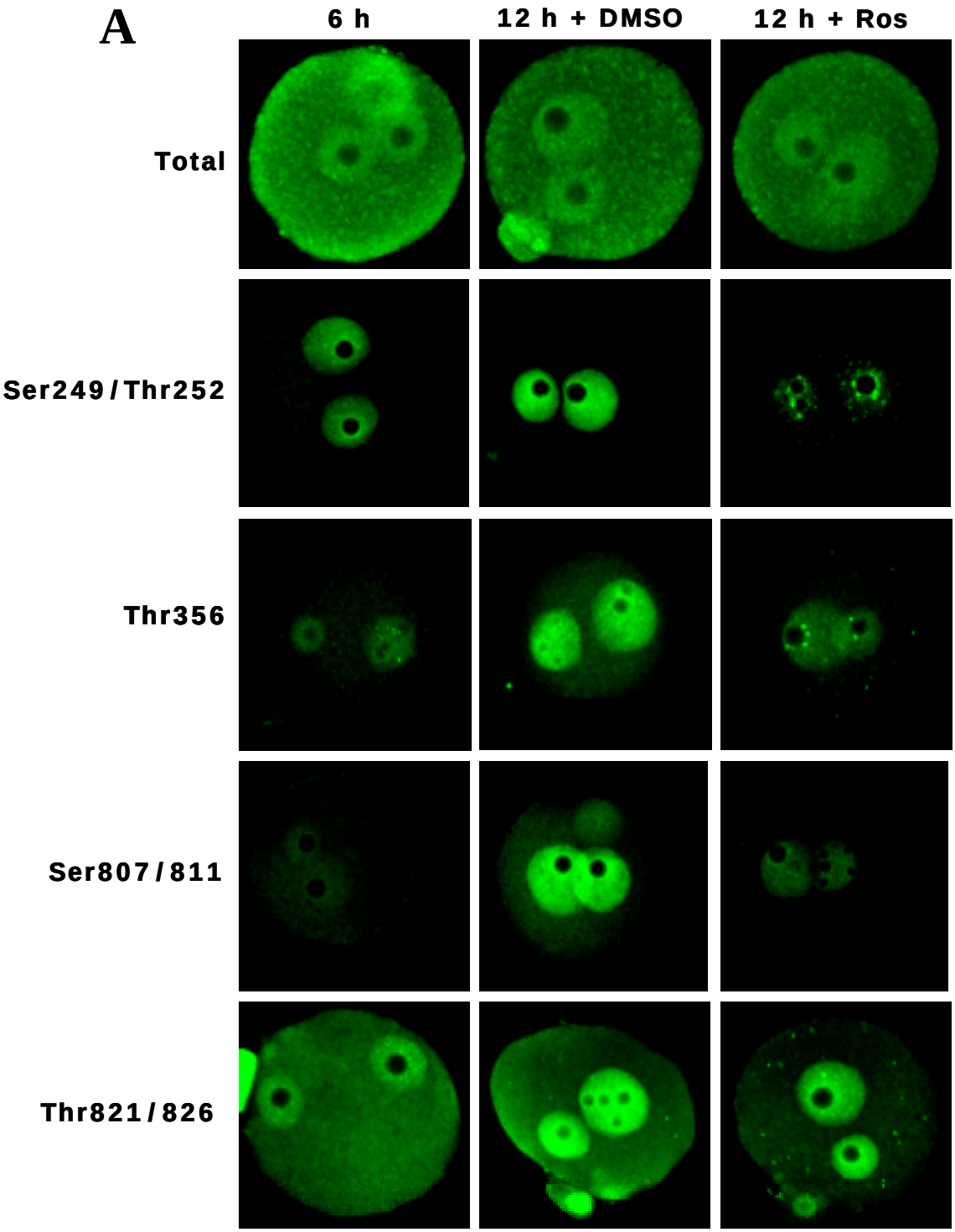
It is well known that cyclin/cdk families phosphorylate pRb in a cell cycle dependent manner (Buchkovich et al. 1989; DeCaprio et al, 1992). In the somatic cells, pRb is phosphorylated during G1/S phase by three types of cyclin/cdk families, cyclin D/cdk4, cyclin E/cdk2 and cyclin A2/cdk2 (Akiyama et al., 1992; Harbour et al., 1999; Lin et al., 1991; Zarkowska and Mittnacht, 1997). In the 1-cell embryos, however, cyclin D/cdk4 would not be activated, since Ser780, a cdk4 target site, was not phosphorylated. Since it was reported that both cyclin D and cdk4 were accumulated in the pronucleus of the 1-cell embryos (Kohoutek and Hample, 2004), the activity of cdk4 would be inhibited by cdk inhibitors during 1-cell stage. In the somatic cells, the kinase activity of cdk4 is regulated by the cdk activators, cyclin D and $p34^{SEI-1}$, and the cdk inhibitors (CKIs), INK4 family members (Sherr, 2001; Sugimoto et al., 1999, 2002). Cdk4 is activated by forming a complex with cyclin D and $p34^{SEI-1}$ (Sugimoto et al., 1999, 2002), whereas it is inhibited by the association with INK4 family including $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$ (Chan et al., 1995; Guan et al., 1994; Hannon and Beach,

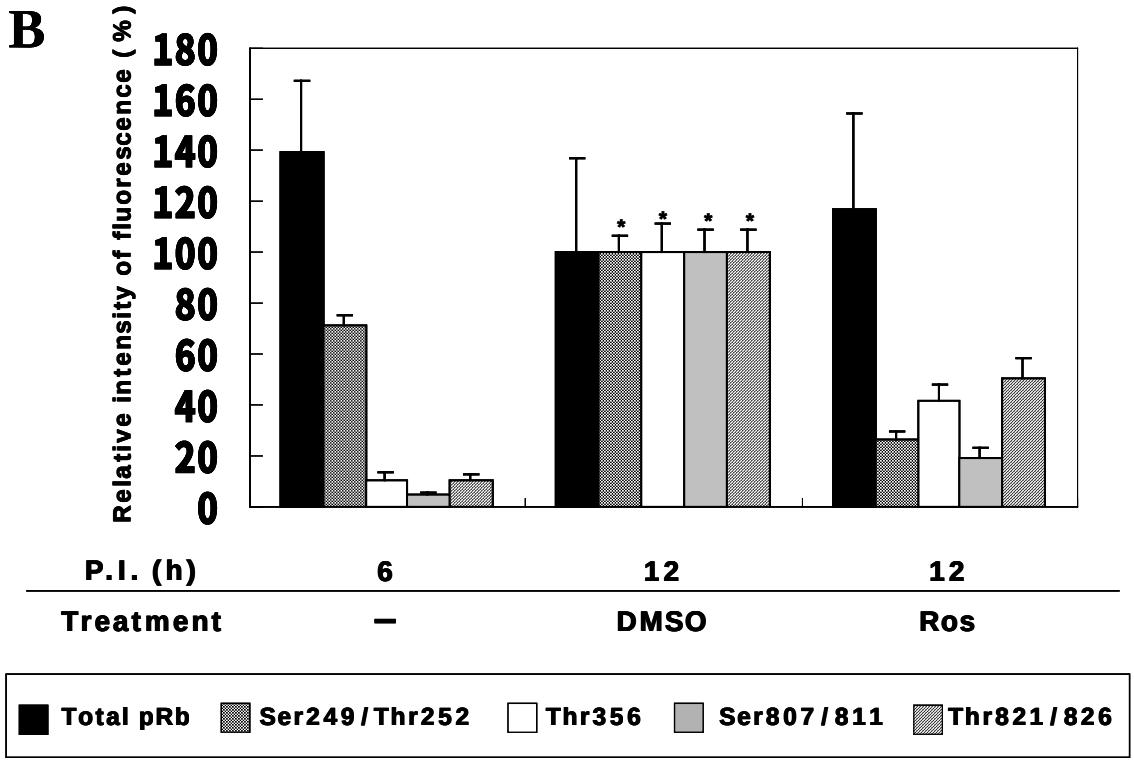
1994; Hirai et al., 1995; Serrano et al., 1993; Sherr, 2001). The activity of cdk4 would be inhibited by INK4 families in the 1-cell embryos, resulting in the unphosphorylated state of Ser780 (Figure 2-1C). On the other hand, it is known that cdk2 activity is inhibited by the association with Cip/Kip family including $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ (Lee et al., 1995; Matsuoka et al., 1995; Polyak et al., 1994; Toyoshima and Hunter, 1994). Although it has been reported that $p27^{Kip1}$ was expressed in the mouse oocytes, it was rapidly degraded following fertilization and disappeared until 6 h post insemination (Kohoutek and Hample, 2004). Taken together with my finding that cyclin A2 was newly synthesized and accumulated in the pronucleus between 6 and 12 h post insemination (Figure 1-4), cdk2 seems to be activated by the degradation of its inhibitor followed by the synthesis of its activator. Thus, cdk2 activity would be increased dependently on the newly synthesis of cyclin A2, which leads to the increase in the phosphorylation level of pRB during 1-cell stage.

The mechanism by which pRb phosphorylation regulated EGA

Hypophosphorylated pRb functions as a transcriptional negative regulator for the cell cycle progression, and its functions are lost when pRb is phosphorylated, (Sherr, 1996). Thus, the function of pRb as a transcriptional repressor is coupled with its phosphorylation state (Harbour et al., 1999). The phsphorylation of pRb changes the binding affinity for its binding partners. The hyper-phosphorylation of pRb in G1 phase induces the release of the LXCXE motif proteins that are themselves, or are associated with, various repressors for gene expression (Dunaief et al., 1994; Magnaghi-Jaulin et al., 1998; Zarkowska and Mittnacht, 1997). For instance, in the early G1 phase, unphosphorylated pRb recruits histone deacetylase 1 (HDAC1) associating with LXCXE motif protein to repress transcription (Brehm et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998). During G1/S phase, pRb is phosphorylated by cyclin A2/cdk2, which leads to the release of HDAC1 from pRb, accompanying with

the dissociation of LXCXE motif protein from pRb (Zarkowska and Mittnacht, 1997). Dissociation of HDAC1 from pRb induces the acetylation of core histones around the promoter region, which induces the conformational changes of chromatin structure to trigger the transcription (Ferreira et al., 2001). Thus, cyclin A2/cdk2 inactivates pRb that functions as a transcriptional repressor. In the 1-cell embryos, cdk2 target sites in pRb were phosphorylated between 6 and 12 h post insemination during which EGA was initiated (Figure 2-1). These results suggested that pRb was inactivated by cyclin A2/cdk2 followed by the dissociation of various repressors including HDAC1. This mechanism would induce the expression of the genes that have been repressed by pRb.


A
6 h
12 h + DMSO
12 h + Ros
Total
Ser249/Thr252
Thr356
Ser807/811
Thr821/826


B
Relative intensity of fluorescence (%)
180
160
140
120
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40
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0
P.I. (h)
6
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Thr821/826

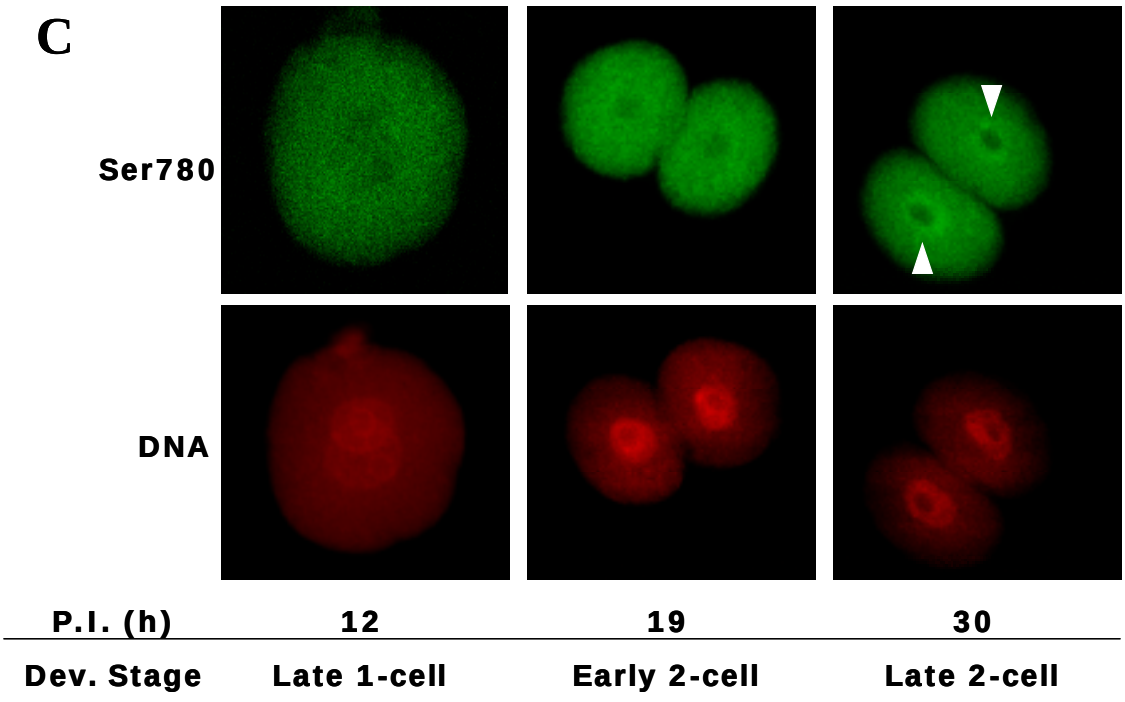
C
Ser780
DNA
P.I. (h)
12
19
30
Dev. Stage
Late 1-cell
Early 2-cell
Late 2-cell

Figure 2-1. **Phosphorylation of pRb by cdk2 during 1-cell stage.** The embryos were transferred to the CZB medium containing roscovitine (Ros) or DMSO. DMSO was used as the solvent for Ros and the final concentrations of DMSO in the culture medium were 0.15 %. The embryos were collected 6 and 12 h post insemination (P.I.) and immunostained with the antibodies against pRb (Total), phospho Ser249/Thr252-pRb (Ser249/Thr252), phospho Thr356-pRb (Thr356), phospho Ser807/811-pRb (Ser807/811) and phospho Thr821/826-pRb (Thr821/826). (A) Confocal microscopic images of the mouse 1-cell embryos immunostained with anti-pRb antibody or site-specific anti-phospho pRb antibodies. (B) The relative intensities of pronuclear fluorescence in the embryos immunostained with anti-pRb antibody and site-specific anti-phospho pRb antibodies. The intensity of fluorescence was analyzed as described under *Materials and Methods*. The average value in the embryos treated with DMSO was set as 100%. The experiments for phospho Ser249/Thr252, Thr356 and Ser807/811 pRb were conducted three times, and those for phospho Thr821/826 and total pRb were twice. Similar results were obtained in each case. Eight or more embryos were analyzed in any experimental groups. The columns and bars represent means ± s.e.m. Asterisks (*) indicate significant differences from the value at the 6 h P.I. and Ros (Student's *t* test $P < 0.01$). (C) Confocal microscopic images of the mouse embryos immunostained with anti-Ser780 pRb antibody (Ser780). DNA was stained with propidium iodide. One-cell embryos, early 2-cell and late 2-cell embryos were collected 12, 19 and 30 h P.I. and immunostained with anti-Ser780-pRb antibody. The experiment was conducted three times. Similar results were obtained in each case.

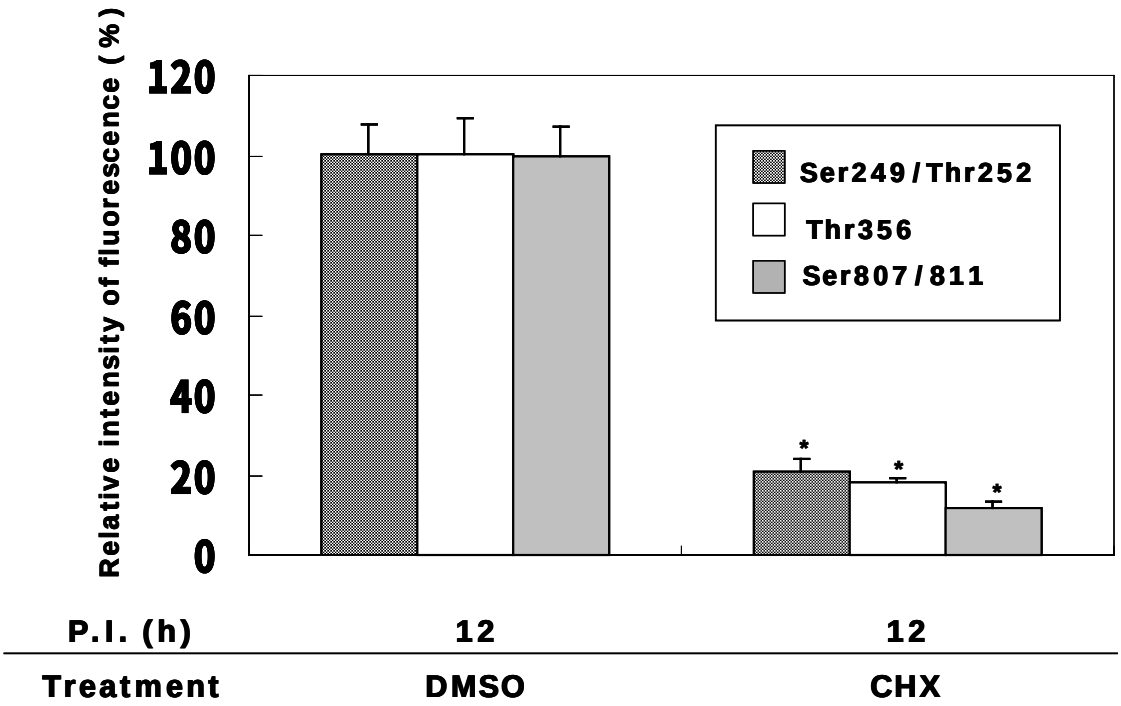

Figure 2-2. **Effect of cycloheimide (CHX) on the site-specific phosphorylation of pRb in the mouse 1-cell embryos.** The embryos were collected 1 h after insemination and transferred to the culture medium containing 10 μg/ml CHX. DMSO was used as the solvent for CHX and the final concentrations of DMSO in the culture medium were 0.16 %. The embryos were collected 12 h post insemination (P.I.) and subjected to immunostaining with the site-specific anti-phospho pRb antibodies (Ser249/Thr252, Thr356 and Ser807/811). The intensity of fluorescence in the pronucleus was analyzed as described under *Materials and Methods*. The average value in the embryos treated with DMSO was set as 100%. The experiments were conducted twice and similar results were obtained in each case. The total numbers of embryo examined for each phosphorylation site were 35 or more. The columns and bars represent means ± s.e.m. Asterisks (*) indicate significant differences from the value at DMSO (Student's *t* test $P < 0.01$).

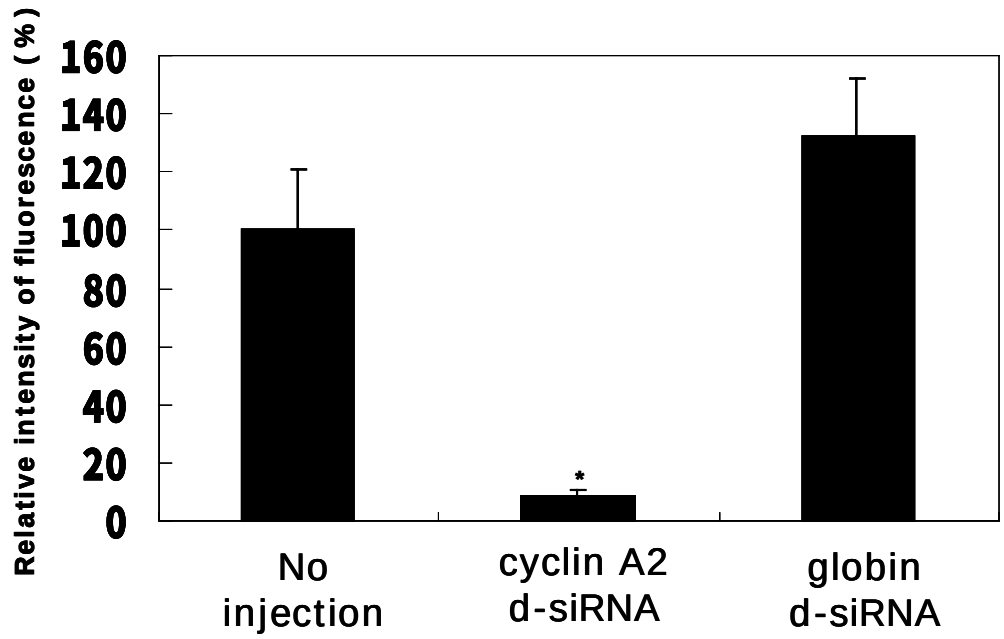

Fig. 2-3. **Effects of cyclin A2 d-siRNA microinjection on the phosphorylation of Thr356 on pRb in the mouse 1-cell embryos.** MII stage oocytes were microinjected with mouse cyclin A2 d-siRNA or rabbit alpha-globin d-siRNA. Twelve hours after insemination, they were immunostained with anti-Thr356 pRb antibody. The intensity of fluorescence in the pronucleus was analyzed as described under *Materials and Methods*. The average value in the control embryos with no injection was set as 100 %. The experiments were conducted twice and similar results were obtained in each case. The total numbers of embryos examined were 11, 9 and 10 for no injection, cyclin A2 d-siRNA injection and globin d-siRNA injection, respectively. The columns and bars represent means ± s.e.m. An asterisk (*) indicates significant dufference from the value at the no injection and globin d-siRNA injection (Student's *t* test $P < 0.01$).

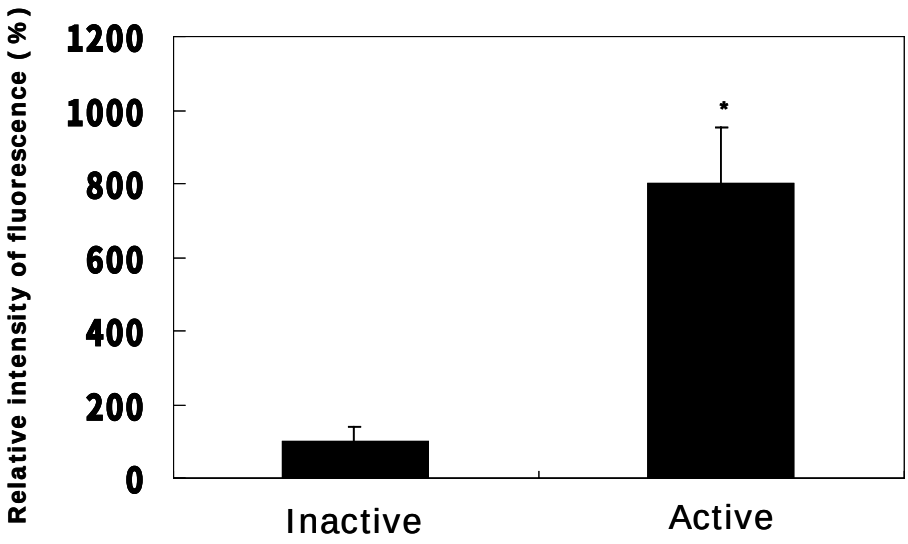

Figure 2-4. **Effects of cyclin A2/cdk2 protein microinjection on phosphorylation of Thr356 on pRb in the mouse 1-cell embryos.** One-cell embryos were microinjected with active cyclin A2/cdk2 protein (active) or inactive one (inactive) at 1 h after insemination. They were collected 6 h and subjected to immunostaining with anti-Thr356 pRb antibody. The intensity of fluorescence was analyzed as described under *Materials and Methods*. The average value in the embryos microinjected with inactive cyclin A2/cdk2 protein was set as 100 %. The experiments were conducted three times and similar results were obtained in each case. The total numbers of embryos examined were 18 and 24 for inactive protein injection and active protein injection, respectively. The columns and bars represent means ± s.e.m. An asterisk (*) indicates significant dufference from the value at the inactive cyclin A2/cdk2 injection (Student's *t* test $P < 0.01$).

Chapter 3

Acetylation levels of core histones increase during 1-cell stage.

Abstract

In the chapter 1 and 2, I suggested that pRb phosphorylation by cyclin A2/cdk2 is involved in the regulation of EGA. It has been known that pRb phosphorylation is involved in the regulation of histone acetylation and that histone acetylation play important roles in the regulation of gene expression. In this chapter, I examined the changes in histone acetylation levels during 1-cell stage and the involvement of cyclin A2/cdk2 in these changes. Immunocytochemistry with anti-acetylated histones revealed that the acetylation levels of lysine 9 on histone H3 (H3K9), lysine 14 on histone H3 and lysine 5 on histone H4 significantly increased between 6 and 12 h after insemination during which EGA is initiated. Inhibition of cdk2 activity by the treatment with cdk2 inhibitors prevented the increase in acetylation of H3K9, suggesting that acetylation of H3K9 was induced by the cdk2. However, in the embryos in which cyclin A2 synthesis was inhibited by the microinjection of d-siRNA, acetylation level of H3K9 was not changed, suggesting that cyclin E/cdk2 but not cyclin A2/cdk2 may be responsible for the acetylation of H3K9.

Introduction

Histone acetylation has been shown to play important roles in the regulation of gene expression (Grunstein, 1997; Jenuwein and Allis, 2001; Strahl and Allis, 2000; Turner, 1998). In general, histones are hyperacetylated at the promoters of active transcribed genes but are hypoacetylated at silent ones. The level of acetylation is regulated in the balance between histone acetyltransferases (HATs) and deacetylases (HDACs) activities, and various transcription factors recruit them to regulate transcription *via* modulating histone acetylation level. Although it was reported that histone H3 and H4 were acetylated and the localization of acetylated histones were changed in the nucleus during 1- and 2-cell stages in mice (Adenot et al., 1997; Kim et al., 2003; Sarmento et al., 2004; Stein et al., 1997), the changes in acetylation levels between the early and late 1-cell stage have not been investigated.

In the chapter 1 and 2, I showed that embryonic gene activation (EGA) in the mouse 1-cell embryos was regulated by cyclin A2/cdk2 *via* site-specific phosphorylation of retinoblastoma protein (pRb). It was reported that pRb recruits and forms a complex with HDACs to repress transcription in the somatic cells (Brehm et al., 1998; Magnaghi-Jaulin et al., 1998). This complex is dissociated by the phosphorylation of pRb which is catalyzed by cdk2 (Takaki et al., 2004; Zarkowska and Mittnacht, 1997). These reports suggested that pRb phosphorylation by cdk2 released HDACs from promoter region, which results in the transcriptional activation. Therefore, it is possible that the phosphorylation of pRb, which is catalyzed by cyclin A2/cdk2, leads to the increase in acetylation levels of histones and that the increased acetylation levels induce EGA in the 1-cell embryos. However, there is no report which investigated the association of pRb with acetylation of histones in the mouse preimplantation embryos.

In this chapter, to address this hypothesis, I examined the changes in acetylation levels of histone H3 and H4 during 1-cell stage and the effect of cdk2

inhibitor on them. Since, cdk2 activity is regulated by cyclin A2 synthesis during 1-cell stage, I also microinjected cyclin A2 d-siRNA into the embryos and examined its effect on histone acetylation to clarify the involvement of cyclin A2/cdk2 in the regulation of histone acetylations.

Results

I examined the changes in acetylation levels of histone H3 and H4 during 1-cell stage by immunocytochemistry with anti-acetylated histone antibodies. As shown in figure 3-1, acetylation levels of lysine 9 on histone H3 (H3K9), lysine 14 on histone H3 (H3K14), and lysine 5 on histone H4 (H4K5) significantly increased between 6 and 12 h after insemination ($P < 0.01$), although acetylation level of lysine 12 on histone H4 (H4K12) did not change.

Since H3K9, H3K14 and H4K5 were thus acetylated between 6 and 12 h during which the nuclear localization of cyclin A2 protein increased (Figure 1-1 and 1-4), the increase in histone acetylations during 1-cell stage is possible to depend on cyclin A2/cdk2 activity. To address this possibility, 1-cell embryos were cultured in the presence of roscovitine (Ros), cdk2 inhibitor, and examined for histone acetylations. The results showed that the acetylation of H3K9 was significantly decreased by the treatment with Ros ($P < 0.01$), but other lysine residues were not (Figure 3-1). These results suggested that cyclin A2/cdk2 increases the acetylation level of histone H3K9 around the time of EGA in the 1-cell embryos.

This increase in the acetylation level may be merely due to the increase in the amounts of histones constituting the chromosomes, but not due to the increase in the activity for histone acetylation, since DNA is replicated and histones constituting the chromosomes increase twice their amounts between 6 and 12 h (Figure 1-1). However, this possibility could be excluded by the experiment in which DNA replication was inhibited by aphidicolin. In the embryos treated with aphidicolin, the acetylation level of histone H3K9 still increased between 6 and 12 h to the level comparable with that in the control embryos treated with DMSO as a solvent for aphidicolin (Figure 3-2). This result suggests that the increase in acetylation level of H3K9 was independent of DNA replication, but that it was caused by the increase in the activity for histone acetylation.

During 1-cell stage, the activity for histone acetylation but not the amounts of histones seems to be rate-limiting in the acetylation of histones, since inhibiting the duplication of histones constituting chromosomes did not show significant effect on the acetylation level.

The results described above and in chapter 1 suggest that cyclin A2 is newly synthesized during G1/S phase (6-12 h after insemination), which leads to the activation of cdk2 by forming their complex and that the activated cyclin A2/cdk2 increases the acetylation level of H3K9. To address this scenario, the synthesis of cyclin A2 was inhibited by microinjecting cyclin A2 diced small interference RNA (d-siRNA) into the embryos. However, the inhibition of cyclin A2 synthesis did not show any effect on the acetylation level of H3K9. There was no significant difference between the embryos microinjected with cyclin A2 d-siRNA and globin d-siRNA (Figure 3-3), suggesting that cyclin A2 is not involved in the mechanism regulating H3K9 acetylation in the 1-cell embryos. On the contrary, microinjection of active cyclin A2/cdk2 protein increased the acetylation level of H3K9. The embryos microinjected with active protein showed significantly higher acetylation level than those microinjected with heat-inactivated protein (P <0.01) (Figure 3-4). Excess activity of cdk2 may phosphorylate some proteins which are not phosphorylated by cyclin A2/cdk2 in the physiological condition and activate them to induce H3K9 acetylation.

Discussion

Post translational modifications of core histone N-termini are involved in various cellular functions including gene expression, DNA replication, DNA repair, imprinting and cell cycle (Peterson and Laniel, 2004). Several lines of evidence suggested that histone acetylation has been shown to play important roles in the regulation of gene expression (Grunstein, 1997; Jenuwein and Allis, 2001; Strahl and Allis, 2000; Turner, 1998). It was reported that acetylation of histone H4 is involved in the recruitment of chromatin remodeling complex SWI/SNF on the promoter region, and acetylation of histone H3 recruits transcription factor TFIID (Agalioti et al., 2002). Thus, acetylation of histone H3 and H4 are involved in the recruitment of transcriptional regulating proteins, suggesting that these acetylations were tightly associated with the activation of gene expression. In this chapter, I showed that in the 1-cell stage of mouse embryos, the acetylation levels of H3K9, H3K14 and H4K5 increased between 6 and 12 h post insemination during which EGA is initiated (Figure 3-1). These acetylated histones would play roles in the recruitment of various transcription factors and their regulating factors to the promoter regions for activating gene expression.

On the promoter regions that are associated with E2F-Rb-HDAC1 complexes, cell cycle dependent dissociation of HDAC1 induces the acetylation of histone H3 and H4. During G0 to S phase transition, HDAC1 is dissociated from the promoter region with a progression of cell cycle, which leads to the increase in the acetylation levels of H3K9, H3K14, H4K5 and H4K12 (Ferreira et al., 2001; Nicolas et al., 2003). Thus, acetylation levels of histone H3 and H4 were regulated by the association of HDAC1 with promoter region. During G0 to S phase transition, phosphorylation levels of pRb is also dramatically changed. During G0 phase, pRb is hypophosphorylated, and then phosphorylated by cyclin/cdk families during G1/S phase. Thus, changes in the

acetylation levels of histones correlate with those in pRb phosphorylation states, suggesting that a common mechanism regulates these changes. It was reported that pRb phosphorylation by cdk2 induced the dissociation of pRb binding proteins including HDACs from pRb (Zarkowska and Mittnacht, 1997). These lines of evidence suggest that the phosphorylation of pRb by cdk2 is involved in the regulation of histone acetylation, although it remains unclear which site(s) should be phosphorylated to induce the dissociation of HDACs. In the 1-cell embryos, acetylation level of H3K9, H3K14 and H4K5 increased between 6 and 12 h post insemination during which the cell cycle is at G1/S phase (Figure 3-1). These changes in the acetylation levels were correlated with those in phosphorylation levels of pRb (Figure 2-1 and 3-1). Therefore, in the 1-cell embryos, acetylations of H3K9, H3K14 and H4K5 are also regulated by the dissociation of HDACs from promoter regions *via* pRb phosphorylation.

During oocyte growth, histone H3 and H4 are highly acetylated until the germinal vesicle (GV) stage. However, these histones are prominently deacetylated during meiosis (Kim et al., 2003). It was suggested that this deacetylation are regulated by HDAC1 (Kim et al., 2003). Therefore, during early 1-cell stage, HDAC1 would be still functional to suppress histone acetylations. To initiate acetylations during 1-cell stage, pRb would be phosphorylated by cyclin/cdk families in order to release HDAC1 from the promoter regions. The treatment of roscovitine (Ros), indeed, inhibited the acetylation of H3K9 (Figure 3-1A).

The cdk2 activity which is associated with the acetylation of H3K9 may not be regulated by cyclin A2 synthesis, since acetylation levels of H3K9 was not changed significantly when cyclin A2 synthesis was inhibited by the microinjection of cyclin A2 d-siRNA (Figure 3-3). However, in the embryos microinjected with recombinant cyclin A2/cdk2 protein, acetylation level of H3K9 was increased. This increase would be due to the microinjection of excess cyclin A2 protein, which resulted in the non-specific phosphorylation of some proteins or the sites of pRb which are not phosphorylated by

cyclin A2/cdk2 in the physiological condition. Since cdk2 is activated by the association with cyclin E as well as cyclin A2 (Sherr, 1996), cyclin E/cdk2 may regulate the acetylation of H3K9 during 1-cell stage. In terms of pRb phosphorylation, cyclin A2/cdk2 is also able to phosphorylate the sites which are preferentially phosphorylated by cyclin E/cdk2, although the phosphorylation sites by cyclin D/cdk4 and cyclin A2, E/cdk2 stringently demarcated each other (Kitagawa et al., 1996). Therefore, cyclin E/cdk2 but not cyclin A2/cdk2 may be responsible for the phosphorylation of the specific sites in pRb to induce the dissociation of HDACs. Alternately, in addition to cyclin E/cdk2, cyclin A2/cdk2 is also involved in the phosphorylation of these sites and these complexes function redundantly.

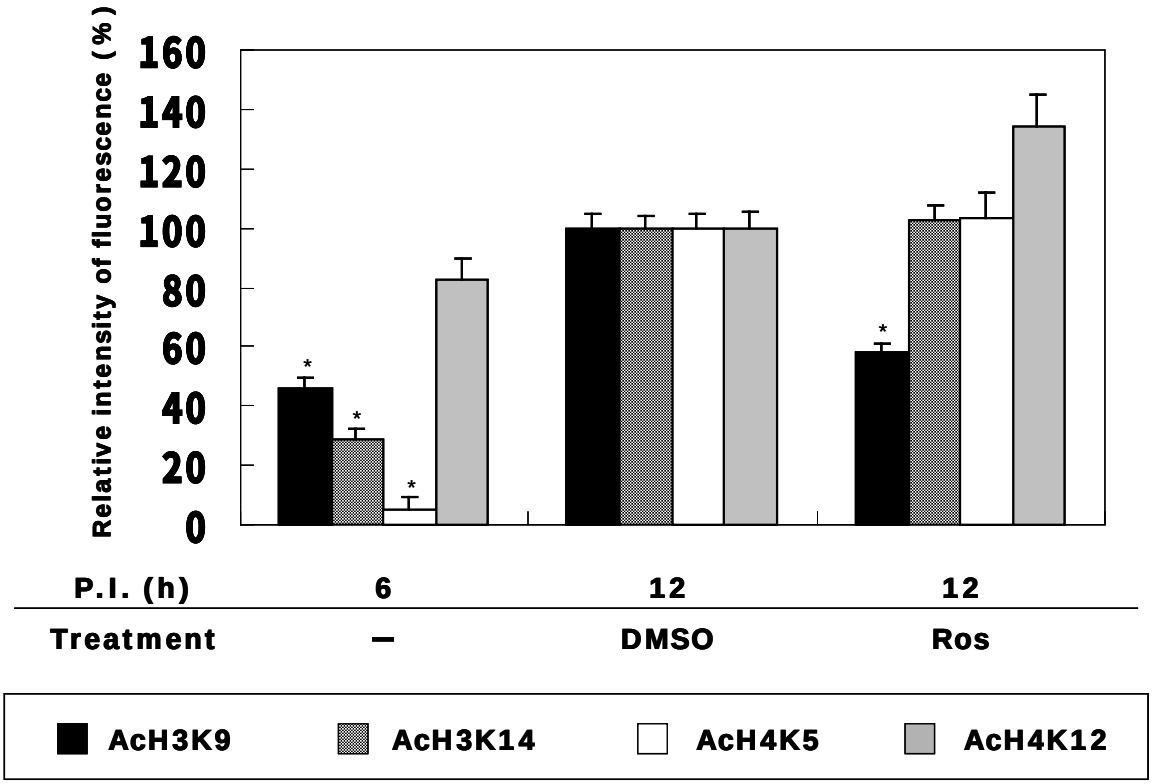

Figure 3-1. **Acetylation of histone H3 and H4 during 1-cell stage.** The embryos were transferred to the CZB medium containing roscovitine (Ros) or DMSO at 4 h post insemination (P.I.). DMSO was used as the solvent for Ros and the final concentrations of DMSO in the culture medium were 0.16 %. The embryos were collected 6 h and 12 h P.I. and immunostained with the antibodies against acetylated lysine 9 on histone H3 (H3K9), acetylated lysine 14 on histone H3 (H3K14), acetylated lysine 5 on histone H4 (H4K5) and acetylated lysine 12 on histone H4 (H4K12). The intensity of fluorescence was analyzed as described under *Materials and Methods*. The average value in the embryos treated with DMSO was set as 100%. The experiments for acetylated histone H3K9 and acetylated histone H4K12 were conducted three times, and those for acetylated histone H3K14 and acetylated histone H4K5 were twice. Similar results were obtained in each case. Ten or more embryos were analyzed in any experimental groups. The columns and bars represent means ± s.e.m. Asterisks (*) indicate

significant differences from the value at DMSO (Student's *t* test $P < 0.01$).

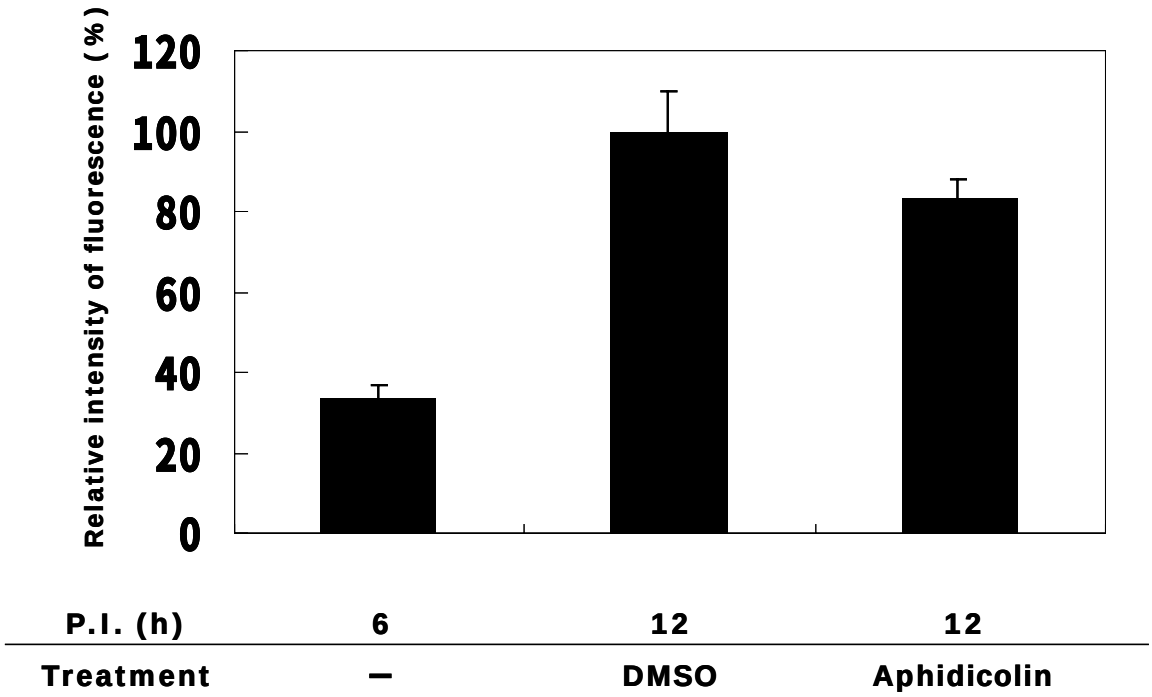

Figure 3-2. **Effect of aphidicolin on the acetylation of lysine 9 on histone H3 (H3K9) in the mouse 1-cell embryos.** The embryos were collected 1 h post insemination (P.I.) and transferred to the culture medium containing 3 μg/ml aphidicolin. DMSO was used as the solvent for aphidicolin and the final concentrations of DMSO in the culture medium were 0.15 %. The embryos were collected 6 h and 12 h P.I. and subjected to immunostaining with the anti-acetylated H3K9 antibody. The intensity of fluorescence in the pronucleus was analyzed as described under *Materials and Methods*. The average value in the embryos treated with DMSO was set as 100%. The experiments were conducted three times and similar results were obtained in each case. The total numbers of embryos examined were 30, 22 and 49 for the embryos collected 6 h P.I. and DMSO- and aphidicolin-treated embryos which were collected 12 h P.I., respectively. The columns and bars represent means ± s.e.m.

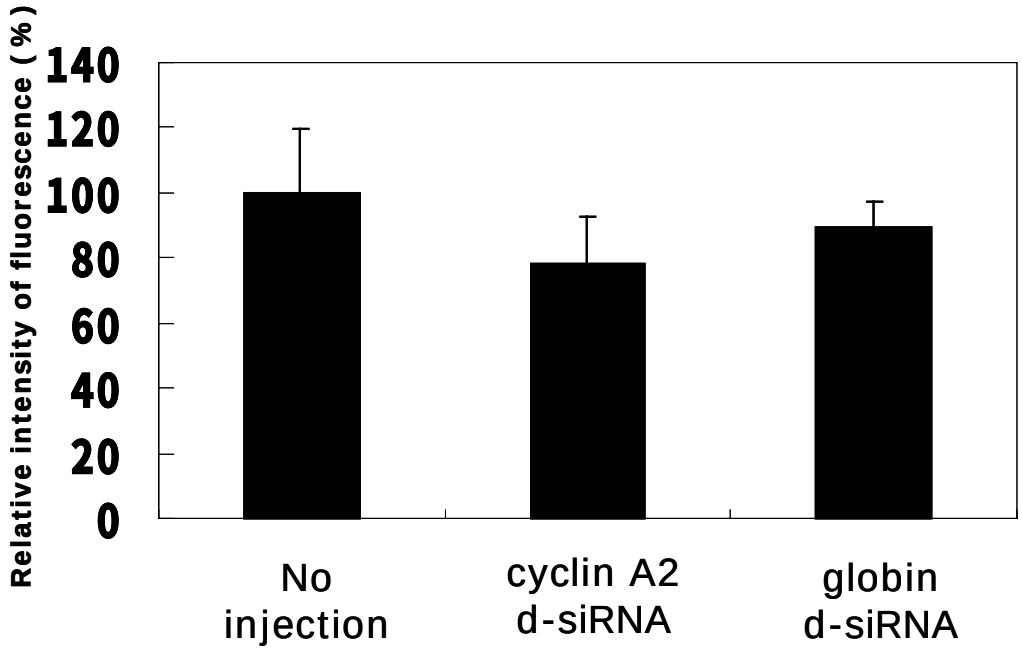

Fig. 3-3. **Effects of cyclin A2 d-siRNA microinjection on the acetylation of lysine 9 on histone H3 (H3K9) in the mouse 1-cell embryos.** MII stage oocytes were microinjected with mouse cyclin A2 d-siRNA or rabbit alpha-globin d-siRNA. Twelve hours post insemination, they were immunostained with anti-acetylated H3K9 antibody. The intensity of fluorescence in the pronucleus was analyzed as described under *Materials and Methods*. The average value in the control embryos with no injection was set as 100 %. The experiments were conducted twice and similar results were obtained in each case. The total numbers of embryos examined were 9, 10 and 10 for no injection, cyclin A2 d-siRNA injection and globin d-siRNA injection, respectively. The columns and bars represent means ± s.e.m.

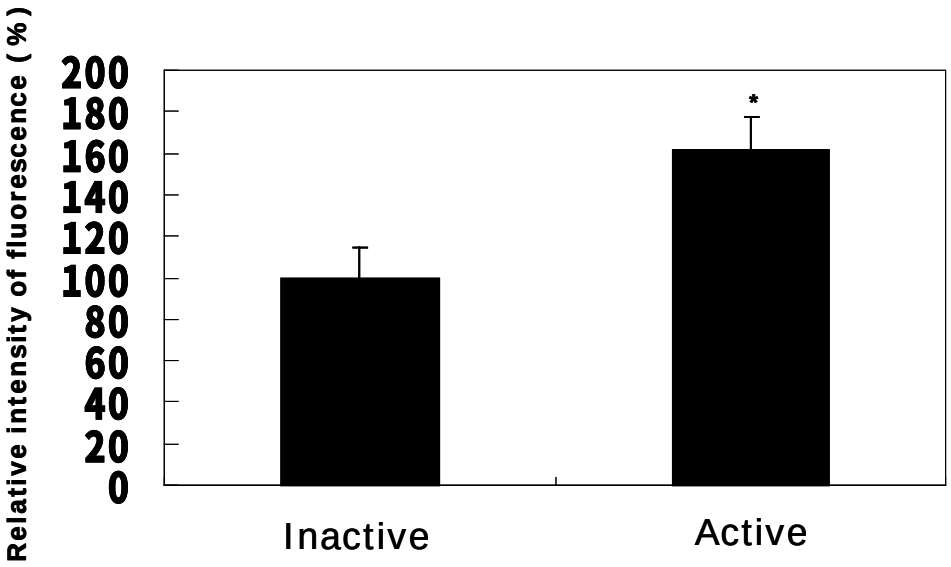

Figure 3-4. **Effects of cyclin A2/cdk2 protein microinjection on acetylation of lysine 9 on histone H3 (H3K9) in the mouse 1-cell embryos.** One-cell embryos were microinjected with active cyclin A2/cdk2 protein (active) or inactive one (inactive) at 1 h post insemination. They were collected 6 h and subjected to immunostaining with anti-acetylated H3K9 antibody. The intensity of fluorescence was analyzed as described under *Materials and Methods*. The average value in the embryos microinjected with inactive cyclin A2/cdk2 protein was set as 100 %. The experiments were conducted three times and similar results were obtained in each case. The total numbers of embryos examined were 30 and 37 for inactive protein injection and active protein injection, respectively. The columns and bars represent means ± s.e.m. An asterisk (*) indicates significant differences from the value at inactive cyclin A2/cdk2 injection (Student's *t* test $P < 0.01$).

General discussion

In this thesis, I demonstrated that cyclin A2/cdk2 regulates embryonic genome activation (EGA) in the mouse 1-cell embryos, independently of cell cycle progression. Cyclin families are named “cyclin” because their expressions depend on cell cycle progression (Girard et al., 1991; Pagano et al., 1992; Resnitzky et al., 1995; Zindy et al., 1992). Therefore, the investigations for the functions of cyclin families have been centered on their cell cycle regulation. Several lines of evidence indicated that cyclin A2/cdk2 is indispensable for the cell cycle progression. It is well known that cyclin A2/cdk2 associates with the mechanism monitoring the aberrant cell cycle progression. A large complex composed of minichromosome maintenance (MCM) protein, cdc6 and origin recognition complex (ORC) regulate the initiation of DNA replication (Fujita, 1999; Quintana and Dutta, 1999). These protein complexes are assembled on the chromatin before DNA replication. When this replication machinery is activated *via* those phosphorylations, DNA polymerase is recruited on the replication point. Following initiation of DNA replication, cdc6 is phosphorylated by cyclin A2/cdk2. This phosphorylation leads to the dissociation of cdc6 from chromosome, and then cdc6 is destroyed (Coverley et al., 2000; Petersen et al., 1999). Cyclin A2/cdk2 also phosphorylates MCM4 in the MCM4-MCM6-MCM7 DNA helicase complex, which results in the inactivation of its helicase activity (Ishimi et al., 2000). Cyclin A2/cdk2 thus inhibits the activity of MCM4 and induce degradation of cdc6 to prevent the aberrant re-replication of DNA.

Recently, in the somatic cells, it was also reported that cyclin A2/cdk2 regulates transcription. Cyclin A2/cdk2 associated with gene expression of house keeping genes *via* phosphorylation of transcription factor, Sp1 (Fojas de Borja et al., 2001; Haidweger et al., 2001). Progesterone receptor also interacts with cyclin A2 and recruits cyclin A2/cdk2 to progesterone-responsible promoters to stimulate

transcription (Narayanan et al., 2005). On this promoter, cyclin A2/cdk2 recruits histone acetyltransferase to regulate histone acetylation. This acetylation would play a role in preparing transcriptionally permissive state of chromatin structure. Thus, cyclin A2/cdk2 also functions as a transcriptional regulator as well as cell cycle regulator. Here, I have shown that cyclin A2/cdk2 regulates transcription in the 1-cell embryos. The treatment with cdk inhibitors and the microinjection of cyclin A2 d-siRNA to inhibit cyclin A2 synthesis reduced the transcriptional activity but not inhibited DNA replication. These results suggested that cyclin A2/cdk2 associates with EGA, independently of cell cycle progression.

Between the mouse 1-cell embryos and somatic cells, the mechanism regulating the translation of cyclin A2 is different. In the somatic cells, the amount of cyclin A2 protein is controlled at the transcription level of cyclin A2 mRNA during S phase (Yam et al., 2002). The mRNA encording cyclin A2 is transcribed in a cell cycle dependent manner (Girard et al., 1991; Henglein et al., 1994; Pagano et al., 1992; Pines and Hunter, 1989; Sherr, 1996; Zindy et al., 1992; Zwicker et al., 1995). The cyclin A2 mRNA level increased during S phase, which was followed by the increase in its protein level (Sherr, 1996; Yam et al., 2002). On the other hand, in the 1-cell embryos, cyclin A2 mRNA is stored as a maternal inherited mRNA which was accumulated before fertilization. Although the amount of cyclin A2 mRNA slightly decreases following fertilization, that of the protein increases during 1-cell stage (Fuchimoto et al., 2001). Thus, the amount of cyclin A2 protein is not controlled at the transcription level. A previous report (Fuchimoto et al., 2001) and my results showed that cyclin A2 mRNA is not translated before fertilization and begins to be translated by poly(A) tail elongation after fertilization. In the 1-cell embryos, the amount of cyclin A2 is thus controlled at the transcription level. This mechanism for the initiation of cyclin A2 synthesis after fertilization would be associated with the phenomenon specific for 1-cell embryos.

In this thesis, I suggested that cyclin A2/cdk2 regulates EGA *via* pRb

phosphorylation and histone acetylation, as a following scenario. Translated cyclin A2 rapidly binds to cdk2 and are transported into pronucleus during 1-cell stage. These cyclin A2/cdk2 phosphorylate pRb. pRb phosphorylation leads to the dissociation of transcriptional repressor including HDACs from promoter region. Thus, transcriptionally repressive state is deregulated by the dissociation of transcriptional repressor and/or acetylation of core histones, which leads to the initiation of EGA (Figure D-1). In addition to these mechanisms, however, there is a possibility that other networks of protein interaction would regulate EGA in the 1-cell embryos. Cyclin E/cdk2 but not cyclin A2/cdk2 may be responsible for the phosphorylation of the specific sites in pRb to induce the dissociation of HDACs, since cdk2 is activated by the association with cyclin E as well as cyclin A2 (Sherr, 1996). In my results, acetylation level of lysine 9 on histone H3 (H3K9) was decreased in the 1-cell embryos treated with cdk2 inhibitor but not in those microinjected with cyclin A2 d-siRNA. These results suggested that cdk2 activity was associated with acetylation of H3K9, although cyclin A2 was not. Since in addition to HDACs, many other chromatin modifiers and transcription factors have been shown to interact with pRb, some of these proteins may regulate EGA independently of histone acetylation. Recent reports demonstrated that transcription factor Sp1 is phosphorylated by cyclin A2/cdk2 and that the DNA binding affinity of Sp1 increases when it is phosphorylated (Fojas de Borja et al., 2001; Haidweger et al., 2001). Thus, cyclin A2/cdk2 may regulate EGA by phosphorylating multiple transcription regulating factors.

I have suggested that the newly synthesis of cyclin A2 is involved in the initiation of EGA and that the translation of cyclin A2 is regulated by poly(A) tail elongation of maternal mRNA after fertilization. This mechanism could give a good explanation for the changes in gene expression pattern from differentiated oocytes to totipotent embryos following fertilization. During oocyte growth, mRNA is actively transcribed from maternal genome including numerous oocyte specific genes, *e.g.* c-mos

and the *zona pellucida* genes (Liang et al., 1990; Propst et al., 1987; Ringuette et al., 1988). However, the transcription ceases when oocytes are fully grown to the germinal vesicle (GV) stage, and this transcriptionally silent state is maintained during meiotic maturation. After fertilization, the transcription is resumed, but the pattern of gene expression should be changed from that in the oocytes, since oocytes are differentiated cells expressing oocyte-specific genes but the 1-cell embryos are totipotent cells. To manage this change, some factors involved in the regulation of embryonic gene expression would appear after fertilization. This factor should not be present in the oocytes and newly synthesized in the 1-cell embryos after fertilization to make the difference in the gene expression pattern between the oocytes and 1-cell embryos. However, the early 1-cell stage embryos contain the same set of maternal inherited transcripts as that in the oocytes, since transcription is silenced before EGA. Although a recent report demonstrated that sperm bring a small amount of mRNAs (Ostermeier et al., 2004), these mRNAs are not required for the EGA, probed by parhenogenetic embryos in which active transcription occurs from zygotic genome (Aoki et al., 1997). Thus, when the differentiated oocytes and totipotent embryos use the same set of maternal mRNAs, what makes the difference in their properties? The mechanism which I showed in this thesis would be a good answer for this: maternal cyclin A2 mRNA is not translated before fertilization, but begins to be translated to synthesize the protein by poly(A) elongation after fertilization, which triggers the expression of genes from embryonic genome. Some of these genes may not have been expressed in the oocytes. The reprogramming of gene expression pattern between differentiated oocytes and totipotent 1-cell embryos could be thus accomplished. The proposed schematic view is summarized in Figure D-2.

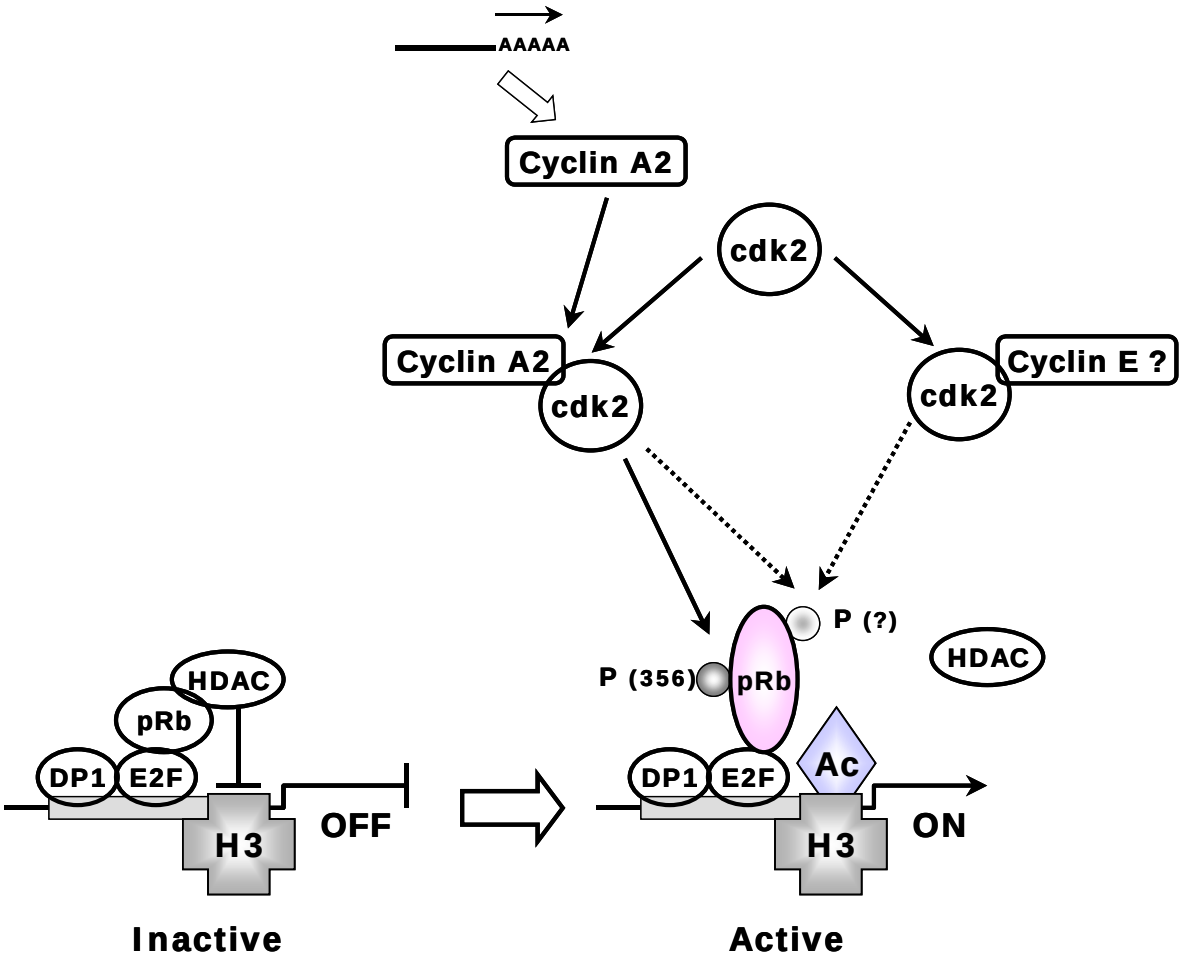

Figure D-1. A working hypothesis for the initiation of embryonic gene activation (EGA). Following fertilization, maternal mRNA encoding cyclin A2 is polyadenylated, which induces the initiation of translation. The newly synthesized cyclin A2 activates cdk2 to phosphorylate pRb. Once pRb is phosphorylated, its function as a repressor of gene expression is lost, which leads to the initiation of EGA. pRb phosphorylation may induce the dissociation of HDACs from the promoter regions. This phosphorylation of pRb may be regulated by cyclin E/cdk2, since cdk2 is activated by the association with cyclin E as well as cyclin A2 (Sherr, 1996). The dissociation of HDACs increases the acetylation level in core histones, which induces the change in chromatin structure from transcriptionally repressive state to permissive one.

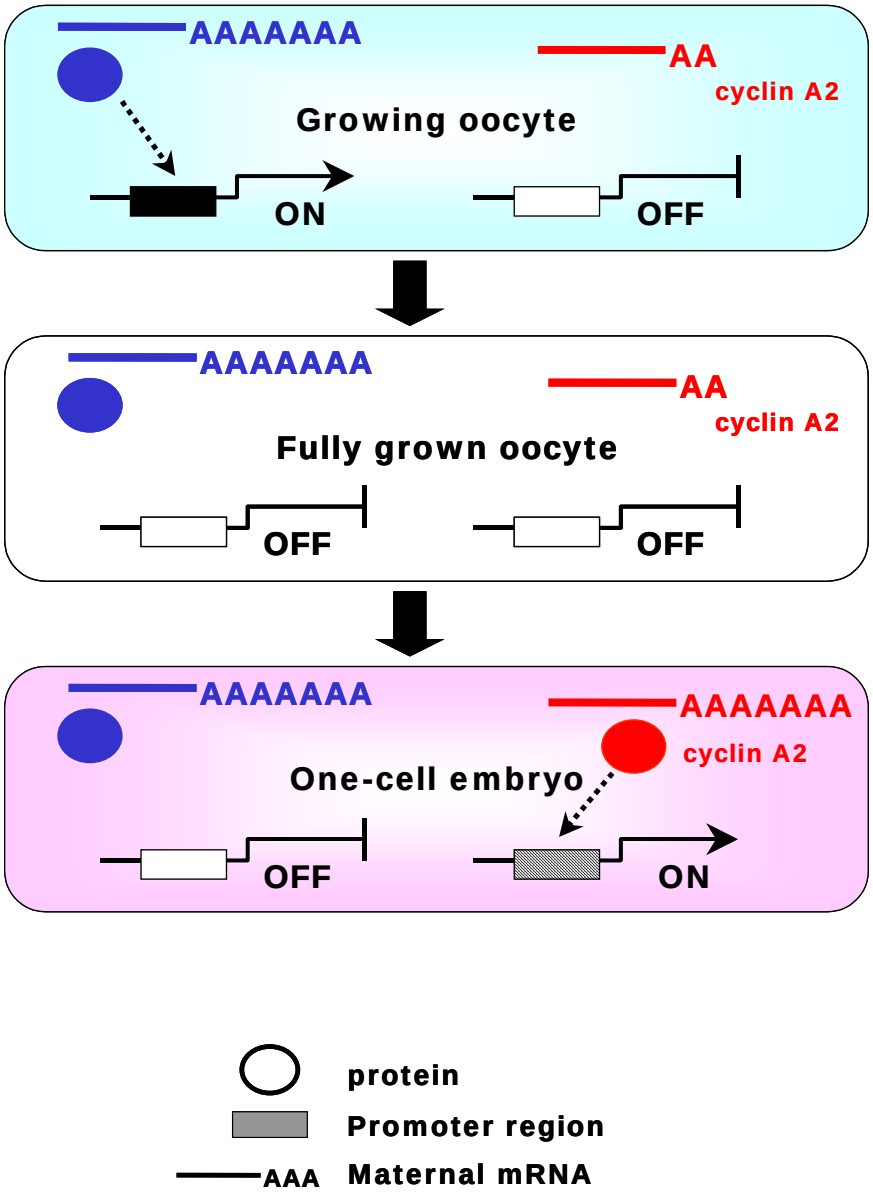

Figure D-2. A schematic view for the mechanism involved in the reprogramming of gene expression pattern. In the growing oocytes, mRNA is actively transcribed from maternal genome including numerous oocyte-specific genes. Cyclin A2 mRNA is also transcribed but not translated because of its short poly(A) tail. When oocytes are fully grown to the germinal vesicle stage, the transcription ceases by unknown mechanism. After fertilization, the poly(A) tail of cyclin A2 mRNA is elongated, which triggers the

translation of the mRNA. The newly synthesized cyclin A2 protein activates the genes which have not been transcribed in the oocytes.

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