Preimplantation-embryo-specific cell cycle regulation is attributed to the low expression level of retinoblastoma protein

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Abstract **It is known that a characteristic of the mammalian preimplantation-embryo-specific cell cycle is the substantially shortened G1-phase, although the regulation mechanisms of the unique cell cycle remain unclear. In the present study, we first examined the presence of retinoblastoma (RB) tumor suppressor gene product throughout mouse preimplantation embryo development and found that the RB expression was down-regulated between the four-cell and morula stages. Furthermore, the overexpression of RB protein in the mouse embryos during this phase inhibited their development significantly. These results suggest that the absence of RB protein contributes to the preimplantation-embryo-specific cell cycle.**

Key words: RB; Preimplantation embryo; Cell cycle; Microinjection

1. Introduction

Cell cycle progression in preimplantation embryo development is independent of exogenous mitogens; i.e. embryos can undergo cleavage in vitro without the addition of any growth factors or serum [1]. The period of one cycle is shorter in embryos than in somatic cells, and the cleaved blastomeres can enter the next M-phase without any growth in volume [2,3]. These differences between the embryonic cell cycle and the somatic cell cycle would be related to the shortened G1-phase in preimplantation embryo development. At present, however, the molecular mechanisms of the shortened G1-phase in preimplantation embryos have not yet been clarified.

In somatic cells, the cell cycle progression is triggered by extracellular signals [4,5]. When cells are stimulated by a mitogen, signal transduction pathways such as the mitogen activated protein kinase cascade activate and induce the expression of various genes, including D-type cyclins [6,7]. The cyclin D synthesis and assembly with their catalytic partners, CDK4 and CDK6, depend on mitogenic stimulations [8–11], and cyclin D-dependent kinases phosphorylate some substrates whose modification is required for the G1/S transition. Retinoblastoma (RB) protein is one of the major targets phosphorylated by cyclin D/cdk 4,6 [9–14].

The RB gene product (pRB) works as a negative growth regulator that constrains the progression of the cell cycle past the G1/S transition, and the successful passage from the G1- to the S-phase requires the inactivation of the pRB function [4,15–17]. In G1-phase, pRB binds to and represses the E2Fs, a family of transcriptional regulators, which can transactivate genes that are important for S-phase entry [18,19]. When pRB is inactivated by phosphorylation by cyclin D-dependent kinases, the genes required for the G1/S transition are highly transcribed, and the cells enter S-phase [19,20]. It is postulated, therefore, that pRB is absent or inactivated continuously in preimplantation embryos so that they may enter the S-phase immediately after the end of the previous M-phase.

In mouse preimplantation embryos, it was reported that a high RB mRNA level in the one-cell stage decreased during the first and second mitotic cell cycles, and almost no RB mRNA remained in the late two-cell stage [21]. Although this report is compatible with the postulate described above, it represents the only research on RB in preimplantation embryos, and at present no information is available regarding the period after the four-cell stage in the RB mRNA level or the period throughout preimplantation embryo development in the RB protein level. In the present study, we examined the presence of RB mRNA and RB protein during the mouse preimplantation embryo development by RT-PCR and immunological techniques, respectively, and showed that RB was down-regulated in mouse preimplantation embryos. Furthermore, we tried to express pRB in mouse embryos in order to examine whether pRB repressed the mouse preimplantation embryo development.

2. Materials and methods

2.1. In vitro fertilization and embryo culture

Embryos were prepared as described previously [22]. Briefly, female BDF1 mice (SLC Japan Inc., Tokyo, Japan) were superovulated by intraperitoneal injection of 7.5 IU pregnant mare serum gonadtropin, followed 48 h later by 7.5 IU human chorionic gonadtropin (hCG). Ovulated oocytes were collected 15–16 h after hCG injection from the ampullae of the oviducts into a modified Krebs–Ringer bicarbonate solution (TYH medium) [23]. Spermatozoa were obtained from the cauda epididymis of male BDF1 mice (SLC Japan Inc.), preincubated for 1–2 h at 37°C in TYH medium, and added to the medium containing the oocytes at a concentration of 150 sperm/μl. After 2 h, the eggs were treated with 0.05% hyaluronidase (Sigma, St. Louis, MO, USA) for a few minutes to remove cumulus cells. The eggs were cultured for up to 120 h after insemination in 5% CO_2 in air at 37°C in Whitten's medium (WM) [1]. The formation of pronuclei was examined at 6 h after insemination, and only embryos having two pronuclei were cultured further. The experiments were conducted in accordance with the guide for the care and use of laboratory animals of the College of Agriculture, University of Tokyo.

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2.2. RT-PCR

Total RNA was isolated from 50 eggs or embryos using TRIzoL reagent (Gibco BRL) according to the manufacturer's instructions except for the addition of 20 μg of glycogen and 20 pg of enhanced green fluorescent protein (EGFP) mRNA, as carrier and internal control, respectively. The EGFP mRNA was transcribed in vitro using the T7 Cap-Scribe kit (Roche). The RNA samples were reverse-transcribed into cDNA using SuperScript II (Gibco BRL) and random hexamers (Takara) in a final volume of 20 μl reaction mixture. The primers used for the amplification of the mouse RB sequences were as follows: 5′-CAACCCCCCCAAACCACTGA-3′ (sense) and 5′-CCAGATGTAGGGGGTCAGGA-3′ (antisense). The primers for EGFP sequences were described previously [24]. The reaction was performed with either 33 cycles for RB or 24 cycles for EGFP. Each cycle consisted of 30 s of denaturation at 94°C, 30 s of annealing at either 58°C for RB or 60°C for EGFP, and 60 s of extension at 72°C, followed by a final extension cycle at 72°C. The PCR products were separated by electrophoresis in 2.0% agarose, stained with ethidium bromide, and photographed under ultraviolet light. For semiquantitative analysis, EGFP mRNA was added before the isolation of total RNA. This served as an internal control to evaluate the efficiency of RNA extraction and RT. The appropriate number of cycles of PCR amplification was determined from the exponential range of amplification for each set of primers.

2.3. Immunoblotting

One hundred embryos in 1.6 μl of saline supplemented with 0.1% polyvinylpyrrolidone were treated with 0.4 μl of 5× Laemmli's Buffer, denatured immediately by boiling for 5 min, and then stored at −80°C until use. Samples were subjected to modified SDS–PAGE [22] on a 7.5% polyacrylamide gel and transferred to Immobilon P (Millipore, Bedford, MA, USA; pore size 0.2 μm). Following transfer and blocking for 1 h in 3% skimmed milk in phosphate-buffered saline (PBS), the membrane was incubated overnight at 4°C with the anti-RB antibodies (14001A; Pharmingen) diluted 1:300 in blocking solution. Bound antibodies were visualized by using horseradish peroxidase-conjugated anti-mouse IgG+IgM (Jackson Immunoresearch Laboratories) diluted 1:2000 in PBS, followed by enhanced chemiluminescent detection (ECL kit; Amersham).

2.4. Immunofluorescence

Embryos were fixed in 4% para-formaldehyde for 30 min and then permeabilized with 0.5% Triton X-100 in PBS for 10 min. Blocking was performed with 5% bovine serum albumin (BSA) in PBS for 1 h at room temperature. The embryos were incubated with anti-RB antibodies (14001A; Pharmingen) diluted 1:100 in blocking solution at 4°C overnight, and then incubated with secondary rhodamine-conjugated goat anti-mouse IgG (Sigma) diluted 1:100 in 5% BSA–PBS for 2 h at room temperature. Samples were finally mounted and observed under Zeiss confocal laser microscope for the detection of RB.

2.5. Plasmid construction

The RB expression vector pBARB, which contains β-actin promoter and RB cDNA, was kindly provided by Dr. Takahashi (Kyoto University). To obtain more effective expression plasmid, the open reading frame of RB was subcloned into the *Bgl*II site of pCXN (kindly provided by Dr. Miyazaki, Osaka University) which contains CMV-IE enhancer, β-actin promoter and α-globin 3′ flanking sequence. The pCX-EGFP (a gift from Dr. Okabe, Osaka University) was used as an expression control.

2.6. Microinjection

The purified plasmid DNAs were microinjected into the pronuclei of embryos at 6–9 h after insemination using an Eppendorf pressure microinjecter and sterile pipets. About 10 pl of the DNA solution containing around 5–10 pg/μl was injected per zygote. Microinjection was performed using a WR-50 microinjection apparatus (Narishige). The samples were dissolved in injection buffer (20 mM HEPES–KOH, pH 7.4, 120 mM KCl). The embryos were kept in M2 medium (M7167; Sigma) during the injection periods. After injection, the embryos were washed in WM three times and then cultured in WM at 37°C, in an atmosphere of 5% CO_2 in air.

2.7. Observation of EGFP expression in the embryos

The expression of EGFP in the embryos was observed for fluorescence by a fluorescence stereomicroscope (MZ FL III, Leica) at 24, 48, 72, and 96 h after insemination. The embryos were examined their developmental stage and counted according to the distinguishing EGFP expression.

2.8. Statistical analyses

The data were analyzed by Student's *t*-test. Differences at $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. RB expression in mouse preimplantation embryos

First, we examined whether endogenous RB was expressed during mouse preimplantation embryo development. When RT-PCR was performed using a set of primers specific for the mouse RB sequence, RB transcripts were detected before two-cell embryos and decreased during the first mitotic cell cycle (Fig. 1A, lanes 1–3) as described previously [21]. Although RB mRNA was not detected between the four-cell embryo and early blastocyst embryo at 96 h of culture, RB was transcribed again in the late blastocyst stage embryo at 120 h (Fig. 1A, lanes 4–8). When the immunoblotting was performed using anti-RB monoclonal antibody, clear bands specific for pRB were detected before 24 h of culture at approximately 105 kDa (Fig. 1B, lanes 1–3). In contrast, no bands were detected from the late two-cell embryo at 36 h to the early blastocyst embryo at 108 h of culture (Fig. 1B, lanes 4–10). In the late blastocyst stage at 120 h, however, a clear band specific for pRB was detected (Fig. 1B, lane 11). The presence of pRB in the blastocyst but not in the earlier stage embryos was also shown by immunocytochemical analyses (Fig. 1C). These results suggest that there were no or few RB expressions in mouse embryos from the late two-cell to the early blastocyst stages.

3.2. Overexpression of pRb in mouse embryos

Next we tried to overexpress pRB in mouse embryos before the morula stage. As it has been reported that the transient protein expression rate of mouse early embryos injected with a vector-DNA into the pronucleus was less than 50% of that of the manipulated embryos [24,25], we attempted to distinguished the embryo expressing pRB from the non-expressing embryos. For this purpose, pCAG-EGFP and pCAG-RB were co-injected into pronuclei and it was determined whether EGFP could be used as the marker of pRB expression or not. The expression of EGFP was observed within 15–18 h after injection (24 h after insemination, Fig. 2A) and the expression rate was about 50% of that of the embryos injected with plasmids (Fig. 2B). The expression rate increased gradually and reached 70% at 63–66 h after injection (72 h after insemination). The high expression rate was maintained until 96 h after insemination (Fig. 2B). There were no differences in the EGFP expression rate of embryos between the only pCAG-EGFP injected group and the group co-injected with pCAG-EGFP and pCAG-RB (Fig. 2B). When EGFP-positive and -negative embryos in the co-injected groups were classified and subjected to immunoblotting against pRB, a clear pRB signal was detected only in the EGFP-positive embryos (Fig. 2C). Furthermore, when immunofluorescent staining against pRb was performed in the co-injected embryos at 48 h after insemination, the pRB signal was also obtained only in the EGFP-positive embryos (Fig. 2D). These results suggest that EGFP might be a powerful marker of protein expression.

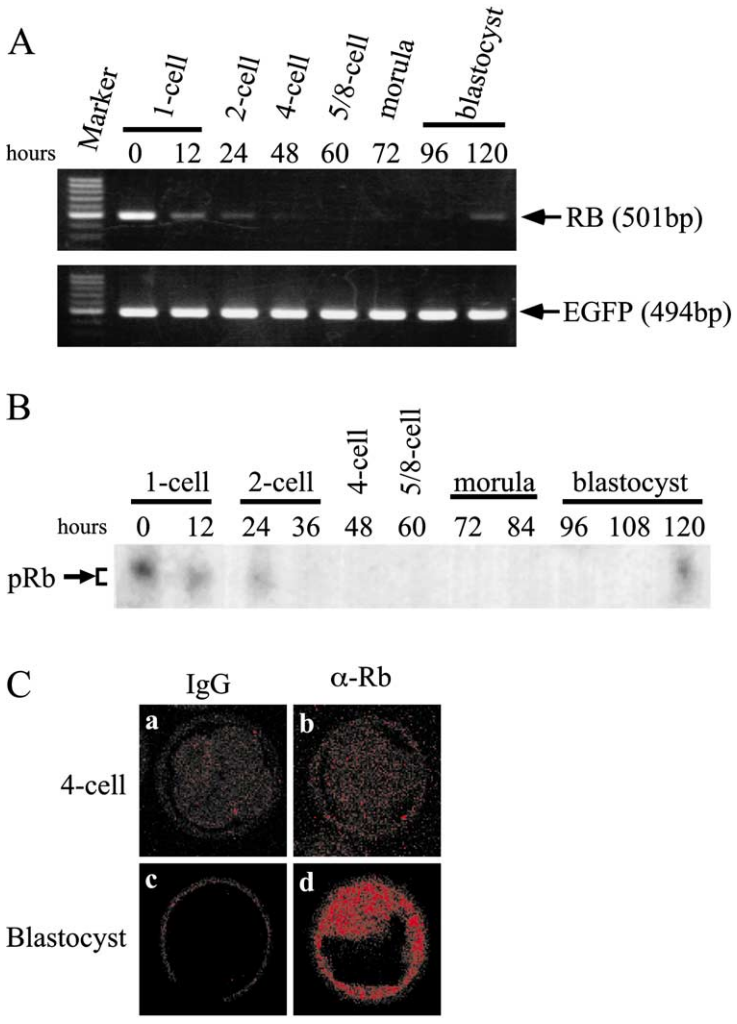

Fig. 1. RB expression during preimplantation development of the mouse embryo. A: PCR products amplified from cDNA prepared from the RNA from each 50 of oocytes or embryos at indicated stages. RB cDNA was amplified using specific primers. EGFP was the internal control for semiquantitative analysis. B: Each 100 unfertilized oocytes or embryos at the indicated stages were collected and subjected to immunoblotting with an anti-RB antibody. Arrow indicates RB-specific band. C: Embryos cultured for 48 h (a,b) and 108 h (c,d) were collected and stained with a control mouse IgG (a,c) and an anti-RB antibody (b,d). The antibodies were visualized with an anti mouse IgG antibody conjugated with rhodamine.

3.3. *Effect of pRb expression on mouse preimplantation embryo development*

As there were no differences in the rate of EGFP-negative embryos between the group injected with pCAG-EGFP alone and the group co-injected with pCAG-EGFP and pCAG-RB, their rates are combined in Fig. 3 as EGFP-negative. Most of the EGFP-negative embryos developed into the two-cell, four-cell, morula and blastocyst stages at 24, 48, 72 and 96 h of culture, respectively. The frequencies of these embryos are shown in Fig. 3. The EGFP-positive embryos injected with pCAG-EGFP alone and the EGFP-negative embryos were equally developed. However, the development of the EGFP-positive embryos co-injected with pCAG-RB, which might overexpress pRB, was deteriorated in comparison with the EGFP-negative embryos and EGFP-positive embryos injected with pCAG-EGFP alone, and most of embryos expressing pRB arrested before the morula stage (Fig. 3). These results suggest that pRB repressed the cell cycle progression during preimplantation embryo development.

4. Discussion

In this report, we showed for the first time the temporal pattern of the expression of RB at the mRNA and protein levels throughout mouse preimplantation embryo development. The expression of RB has been reported in the mouse from immature oocytes to the two-cell stage embryos at the mRNA level only by the RT-PCR method [21]. That earlier report showed that the RB mRNA remained at a high level until 12 h after fertilization and decreased thereafter to a basal level at the two-cell stage 32 h after fertilization. Our present findings agreed well with this previous result. Furthermore, we showed that RB mRNA expression did not begin again until the blastocyst stage, when the first differentiation appeared. In addition, we examined RB expression at the protein level and obtained the same results, which showed the absence of pRB from the late two-cell to the mid-blastocyst stages. These results suggest the relationship of the absence of pRB to the preimplantation-embryo-specific cell cycle progression. In mammalian preimplantation embryos, the G1-phase is sub-

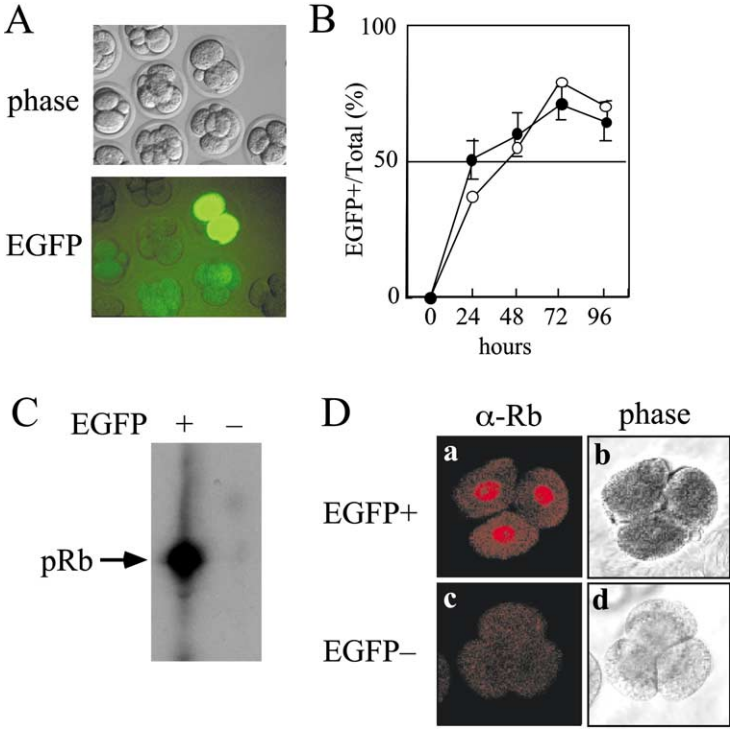

Fig. 2. Expression of exogenous RB protein in embryos injected with the vector DNAs into the pronucleus. A: A typical example of the co-injected embryos cultured for 56 h. The same field was examined under normal light (upper panel) and EGFP excitation light (lower panel). B: The rate of EGFP-positive embryos microinjected with pCAG-EGFP alone (open circle) and co-injected with pCAG-EGFP and pCAG-RB (closed circle). Embryos were cultured in WM until 96 h after DNA microinjection and examined under the EGFP excitation light (488 nm) at the indicated culture periods. C: Detection of exogenous RB protein in embryos injected with the mixture of pCAG-EGFP and pCAG-RB. Embryos were cultured in WM for 72 h (63–66 h after microinjection), then EGFP-positive (left) and -negative (right) embryos were subjected to immunoblotting with an anti-RB antibody. Arrow indicates the RB-specific band. D: Typical examples of the EGFP-positive (a,b) and -negative (c,d) embryos collected at 48 h after insemination (39–42 h after microinjection) and stained with an anti-RB antibody (a,c). The embryos were examined by phase contrast microscopy (b,d) or immunofluorescent microscopy for detecting the anti-RB antibody with an anti mouse IgG antibody conjugated with rhodamine.

stantially shortened, and DNA synthesis is started soon after mitosis [21,26], which indicate the presence of all of the molecules required for the G1/S transition during the preceding M-phase or throughout the cell cycle. In somatic cells, the expression of the molecules required for the G1/S transition is regulated by the transcription factor, E2F, which is inhibited by pRB binding [18,19]. The absence of pRB from the four-cell stage to the morula stage in mouse embryos, therefore, might induce the constitutive activation of E2F and the accumulation of these molecules in order to start DNA synthesis soon after the decondensation of chromosomes.

There is a dramatic change in gene expression at the two-cell stage of mouse embryos, known as zygotic gene activation (ZGA), when the embryonic genome starts to be transcribed [27–29]. It has been well accepted that most proteins and RNAs present before ZGA are maternal factors and most of them are degraded at ZGA. The present study showed that RB was one of those maternal factors. Although RB was expressed during the two-cell stage, the G1-phase remains short in this embryonic cell cycle. This fact indicates that the inactivation of pRB function is not necessary for the start of the S-phase in the mouse embryo before ZGA, probably because all of the molecules required for the G1/S transition are also maternal factors and already present in the embryos as reported in Xenopus embryos [30].

Next, we tried to overexpress pRB during mouse preimplantation embryo development in order to clarify the relationship of absence of pRB to the preimplantation-embryo-specific cell cycle. It has been reported that the transient protein expression rate of mouse preimplantation embryos injected with a vector-DNA into the pronucleus was about 40% of the injected embryos [24,25]. To get more definitive results, therefore, we tried to select the embryos expressing pRB by co-injecting of the EGFP vector DNA with the RB vector DNA as an expression marker. In the present study, EGFP was expressed by 50% of embryos injected either with EGFP vector alone or an EGFP and RB vector mixture, and the expression rate was maintained of more than 50% thereafter. These results are quite compatible with the previous reports, thus showing the reliability of the present experiments. Furthermore, we found that only the EGFP-expressing embryos expressed pRB in the nucleus, which indicated the usefulness of the EGFP-vector co-injection as the marker of RB expression.

The preimplantation embryo development was heavily inhibited by the pRB expression, and the majority of the embryos expressing pRB could not develop over the four-cell stage. This inhibitory effect was specific for pRB because the development of embryos expressing only EGFP was compatible with that of embryos without protein expression. Most of the stopped embryos were morphologically not degenerated but were rather arrested at the two- to four-cell stages. This result is compatible with the hypothesis that maternal factors required for the G1/S transition are present in the one-cell

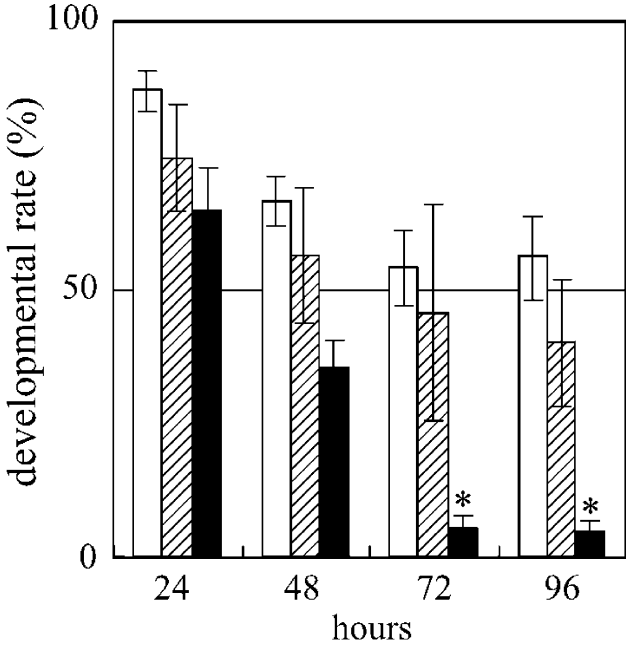

Fig. 3. Developmental rates of embryos expressed exogenous RB protein. Embryos were injected with pCAG-EGFP alone or the mixture of pCAG-EGFP and pCAG-RB, then cultured up to 96 h after the microinjection of DNAs. The rates of embryos developed to the two-cell, four-cell, morula, and blastocyst stages at 24, 48, 72, and 96 h, respectively, are shown. The white bar represents the rate of EGFP-negative embryos of both groups. The hatched bar and black bar represent the rates of EGFP-positive embryos in the pCAG-EGFP injected and pCAG-EGFP and pCAG-RB co-injected groups, respectively. The values with asterisks were significantly different from the embryos without expressing RB.

stage embryos but are degraded during the two-cell stage of mouse embryos, and E2F-dependent transcription is a prerequisite for the entrance into the S-phase after the four-cell stage. Exogenous pRB might suppress the embryo from entering the S-phase by inhibiting of E2F activation. The expression of pRB in normal mouse blastocysts in the present study suggests that the preimplantation-embryo-specific cell cycle changes into the somatic cell cycle, which has a long G1-phase, at this developmental stage.

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