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Expression and Possible Role of Phospholipase C β1 and γ1 in Mouse Oocyte Maturation and Preimplantation Embryo Development

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생쥐 난자의 성숙과 착상전 배발생에서의 Phospholipase C β1과 γ1의 발현 및 기능

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ABSTRACT : It has been well known that phospholipase C (PLC) plays an important role in the intracellular signaling in a variety of cell types. However, involvement of PLC in mouse oocyte maturation and preimplantation embryo development remains unknown. The present study examined the expression patterns of the mouse PLC β1 and γ1 during oocyte maturation and preimplantation embryo development by the competitive reverse transcription-polymerase chain reaction (RT-PCR method). PLC γ1 mRNA (0.1 fg) was readily detected in germinal vesicle (GV)-stage oocyte and its level was reduced as meiotic resumption proceeded. PLC-β1 mRNA (<0.1 fg) was detected at low level at GV-stage oocytes and scarcely detected at germinal vesicle breakdown (GVBD)-stage oocytes. After fertilization, both PLC β1 and γ1 mRNA levels began to increase at morula-stage embryos (0.2 fg) and were more prominent in blastocyst-stage embryos (1 fg). To elucidate the possible involvement of PLC via protein kinase C (PKC) pathway during oocyte maturation and preimplantation embryo development, the effects of sphingosine (PKC inhibitor), sn-diC8 (PKC activator) and U73122 (PLC inhibitor) were examined. Treatment of GV-stage oocytes with sphingosine (20 μM) facilitated the meiotic resumption by 10~20% over the control within 1 h as judged by GVBD, whereas U73122 failed to show any significant effect. U73122 (10 μM) effectively blocked the compaction of morula, while sn-diC8 (50 μM) resulted in facilitation of compaction of morula within 30 min. A blockade of PLC with U73122 (10 μM) was partially overcome by sn-diC8 (50 μM). In summary, the present study shows that the mouse PLC β1 and γ1 are expressed in a developmental stage-specific manner and PLC-PKC pathway may be involved in early preimplantation embryo development.

Key words: Phospholipase C, Competitive RT-PCR, Meiotic resumption of oocytes, Preimplantation embryo development.

요 약 : Phospholipase C (PLC)는 다양한 세포주에서 세포내 신호전달에 중요한 역할을 한다고 알려져 있으나, 생쥐 난자성숙 과정과 착상전 배아발생 과정에서 PLC의 역할과 발현은 아직 연구된 바 없다. 본 연구에서는 난자성숙과 착상전 배아발생 과정에서 생쥐의 PLC β1 과 γ1의 유전자 발현을 조사하기 위하여 한 개의 난자 혹은 배아에서 추출된 total RNA를 사용하여 경쟁적 RT-PCR 방법으로 mRNA를 정량하였다. PLC γ1의 유전자 발현은 germinal vesicle (GV) 난자에서 검출되었고, germinal vesicle breakdown (GVBD) 난자로 성숙하면서 감소하였다. 반면, PLC β1의 유전자는 전혀 발현하지 않았다. 수정후 PLC β1과 γ1의 유전자 발현은 상실기 배아에서 증가하기 시작하여 포배기 배아에서는 현저히 증가하였다. 난자성숙과 착상전 배아발생 과정에서 protein kinase C (PKC) 신호전달체계에 의한 PLC의 역할을 조사하기 위하여 PKC의 억제제인 sphingosine, PKC의 촉진제인 diC_8, 그리고 PLC의 억제제인 U73122의 효과를 조사하였다. Spihingosine은 처리후 1시간 이내에 대조군에 비해 20% 정도의 난자성숙을 촉진하였으나 U73122는 유효한 효과를 보이지 않았다. U73122는 상실기 배아의 compaction을 억제하였으나 diC_8에 의하여 부분적으로 극복되었다. 이상의 결과는 PLC β1과 γ1 유전자가 생쥐의 착상전 발생단계에서 특이적으로 발현하고 있으며, 난자성숙과 착상전 초기배아에서 PKC-PLC 신호전달체계가 관여하고 있으리라 사료된다.

INTRODUCTION

When fully-grown immature oocytes are removed from their follicular environment, spontaneous meiotic resumption occurs without stimulus of gonadotropin (Pincus &

Enzmann, 1935). The initial morphological manifestation involves germinal vesicle breakdown (GVBD), which is the first sign of the resumption of meiosis (Szybek et al., 1972; Sorensen et al., 1976). Embryogenesis begins following fertilization of the ovulated oocyte. Blastomeres of the mouse embryos are equivalent until 8-cell stage. At a certain point of the 8-cell stage, blastomeres are flattened, increase their contact with each other and develop distinct apical and basal membrane and cytoplasmic domain, which is termed compaction. After compaction, the embryo differentiates into blastocyst which consist of trophoectoderm and inner cell mass.

Intracellular signaling events are important in the developmental process, such as meiotic resumption of GV-stage oocyte and compaction of morula-stage embryo. *In vitro* spontaneous meiotic resumption is prevented by a membrane-permeable cAMP analog or a cyclic nucleotide phosphodiesterase (PDE) inhibitor (Cho et al., 1974; Dekel & Beers, 1978; Bornslaeger et al., 1984), or activators of diacylglycerol-dependent protein kinase (PKC) (Urner & Schorderet-Slatkine, 1984; Bornslaeger et al., 1986b; Lee et al., 1989). Previous studies also indicate that changes in protein phosphorylation by cAMP-dependent protein kinase (PKA) or PKC play an important role in the resumption of meiosis (Bornslaeger et al., 1986a, 1986b). PKC activation is involved in the regulation of embryogenesis, such as compaction (Winkel et. al., 1990).

Although it is well known that phospholipase C (PLC) plays an important role in the intracellular signaling in a variety of cell types, the involvement of PLC in mouse oocyte maturation and early preimplantation embryo development remains unknown. Phosphoinositide-specific PLC catalyzes hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate diacylglycerol which is an activator of PKC, and inositol 1,4,5-triphosphate (IP_3) which induces the release of Ca^{2+} from internal stores (Berridge et al., 1984). PLC isozymes can be divided into β, γ, and δ types on the basis of the relative locations of the X and Y domains in the primary structure of the enzymes (Rhee et al., 1989; Rhee et al., 1992a). The γ-type isozymes have been shown to be phosphorylated by a number of protein tyrosine kinases which are associated with the SH2 domains of PLC γs (Rhee et al., 1992b). This phosphorylation is mediated by an intrinsic protein tyrosine kinase activity of the receptors for growth factors (Meisenhelder et al., 1989; Wahl et al., 1989; Mohmmadi et al., 1991; Rhee et al., 1992b). Meanwhile, activation of PLC β-like enzymes is mediated by the subunit of the Gq class of G protein (Smrcka et al., 1991; Waldo et al., 1991; Lee et al., 1992).

The present study attempts to examine the expression patterns of the PLC β1 and γ1 mRNA during mouse oocyte maturation and preimplantation embryo development. Moreover the effects of sphingosine (PKC inhibitor), sn-diC8 (PKC activator), and U73122 (PLC inhibitor) are examined to elucidate the possible involvement of PLC via PKC pathway during oocyte maturation and preimplantation embryo development.

MATERIALS AND METHODS

1. Oocyte collection and culture *in vitro*

Three-week-old female ICR mice were sacrificed by cervical dislocation and eight to ten ovaries were used in each experiment. Ovaries were placed in an embryological watch glass and punctured with a fine needle to release oocytes from their follicles under a dissecting microscope. Intact GV oocytes without cumulus cells were collected using a mouth-controlled micropipet. The puncture was performed in a standard egg culture medium (SECM) (Biggers et al., 1971) containing 3-isobutyl-1-methylxathine (IBMX; 0.1 mM, PDE inhibitor) in order to prevent the resumption of meiosis during oocyte collection. Denuded oocytes were cultured in a 50 μl drop of medium on a 35 mm plastic tissue culture dish flooded with mineral oil at 37℃ in a humidified atmosphere of 5% CO_2 in air.

2. Preimplantation embryo collection and culture *in vitro*

Six- to eight-week-old female ICR mice were superovulated by an intraperitoneal injection of 5 I.U. pregnant mare's serum gonadotropin (Sigma), followed by 5 I. U. human chorionic gonadotropin (Sigma) 48 h later, and subsequently mated with males of the same strain. In the next morning, mated mice were obtained by plug check.

The stage-specific embryos were collected by oviduct-flushing at specific time. The embryos were collected in preincubated M2 medium.

3. Cloning of native and mutant PLC β1 and γ1 cDNA

PLC β1 and γ1 PCR primers were synthesized based on the conserved sequence of the rat PLC β1 and γ1 cDNAs (Suh et al., 1988). The primer sets are designed 5'-TCAAGCCCGTGTGCGTGTCC-3' for the 5' primer, 5'-AGCCTCTAGCGCAGTTTCCA-3' for the 3' primer of PLC β1 and 5'-GCAACCCTGGCTTCTATGTAGAGGC-3' for the 5' primer, 5'-TTCTCGTCCAAGTGCTCCCTCTCCG-3' for the 3' primer of PLC γ1, respectively. Expected sizes of PCR products of the native PLC β1 and γ1 were 521 bp and 262 bp, respectively. To obtain native PLC β1 and γ1 cDNAs, total RNA isolated from blastocyst-stage embryo was employed to RT-PCR using each primer set. PCR products of the PLC β1 and γ1 cDNAs were subcloned into the Sma I sites of pGEM7Zf and pGEM4Z, respectively. The mutant PLC β1 was prepared by deleting 138 bp fragment with Sty I, followed by ligation. The mutant PLC γ1 was prepared by inserting 133 bp fragment (a size of Hae III marker). Expected size of PCR products of mutant PLC β1 and γ1 were 383 bp and 395 bp, respectively.

4. DNA sequencing of PCR products

DNA sequencing was carried out by the dideoxy chain termination method (Sanger et al., 1977) with the Sequenase kit (U.S. Biochemical Co.). The DNA sequence was determined for both strand by double-stranded sequencing method (Henikoff, 1984). Sequence data were then assembled and compared with sequence of rat PLC cDNA sequence (Suh et al., 1988).

5. Total RNA isolation

Total RNA was isolated by acid guanidinium phenol-chloroform method (Chomzynski & Sacchi, 1987) with some modifications. Various tissues were dissected and blood contamination was removed on Wattman filter paper. After samples were transferred into microfuge tubes, they were snap-freezed in liquid nitrogen and stored at −70℃ until use. Tissues were homogenized with tissue homogenizer in immunological tubes containing 650 μl solution D (4 M guanidinium isothiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% lauryl sarcosin, 0.1 M 2-mercaptoethanol) on ice. Subsequently, 0.1 volume of 2 M sodium acetate (pH 4.2), 1 volume of water-saturated phenol, and 0.25 volume of chloroform-isoamyl alcohol (49:1) were added to the homogenates. After vigorous vortexing the tubes, the mixture was incubated on ice for 15 min. Total RNA was centrifuged at 13,000 rpm for 30 min at 4℃ and the supernatant was precipitated in the presence of 1 volume of isopropanol. The pellet was washed with 70% ethanol, and reconstituted in 3rd distilled water. Concentration of total RNA was assessed by UV spectrophotometer at A260.

6. Northern blot hybridization analysis

For Northern blot hybridization, RNA samples (30 μg) were denatured in the presence of 50% formamide, 2.2 M formaldehyde, 20 mM MOPS (3-[N-morpholino] propanesulfonic acid), 4 mM sodium acetate, 0.5 mM EDTA at 65℃ for 15 min. Denatured RNA was fractionated in 1.0% agarose gel containing 2.2 M formaldehyde, and then transferred onto Nytran membrane (Schleicher & Schuell) by capillary action under 10x SSPE (1×SSPE: 0.18 M NaCl, 10 mM Na_2HPO_4, pH 7.7, 1 mM EDTA). After transfer, RNA was permanently attached onto the membrane by UV illumination for 1.5 min. Hybridization was performed in a polyethylene heat sealable bag containing 20 ml of hybridization buffer (5×SSPE, pH 7.4, 5×Denhardt's solution, 0.5% SDS, 0.2 mg/ml heat-denatured salmon sperm DNA, 50% formamide) and ^{32}P-labeled DNA probe at 42℃ for 24 h. The membrane was washed twice with 2X SSC (1X SSC: 0.12 M NaCl, and 15 mM sodium citrate) and 0.1% SDS at room temperature for 15 min, followed by second washing with 0.1X SSC and 0.1% SDS 42℃ for 10 mim. The membrane was exposed to X-ray film (Fuji) at −70℃ for 3 days.

7. Competitive RT-PCR for PLC β1 and γ1 in a single oocyte and embryo

A single oocyte or an embryo collected at each stage was

transferred into 0.5 ml- microfuge tube in 1μl media volume. The tube was quickly frozen with liquid nitrogen and stored at −70℃ until RNA preparation for RT-PCR. Each tube was homogenized with a sonicator (Branson Co.), placed on ice for 1 min and then RT mixture was directly added to each tube. The tube was then incubated at 37℃ for 1 h, followed by PCR. The plasmids containing native and deletion (β1) or insertion (γ1) mutant PLC cDNAs were linearized by digesting with Sal Ⅰ and Sty Ⅰ, respectively. The native and mutant PLC β1 and γ1 cRNAs were synthesized with SP6 RNA polymerase (Promega) for PLC β1 and T7 RNA polymerase (Promega) for PLC γ1, respectively. The concentration of synthesized native and mutant cRNAs was measured with UV spectrophotometer at A260 and compared with serially diluted yeast tRNA on 1.5% agarose gel after electrophoresis. Both native and mutant cRNA templates were co-reverse transcribed by 200 units of RNase H (−) MMLV reverse transcriptase (Promega) in 10 μl of the reaction mixture containing 5 mM of random hexamer, 5 units of RNase inhibitor (Promega), 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 5 mM $MgCl_2$ and 1 mM each of dNTPs. RT reaction was carried out at 37℃ for 1 h and at 95℃ for 10 min. Subsequently, PCR was performed with total RT solution in 50 μl of PCR reaction mixture containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 10 pmol of PLC β1 and γ1 primers and 1.25 units of Taq DNA polymerase (Perkin Elmer Cetus Co.).

8. Effect of sphingosine on oocyte maturation

To examine whether PLC is involved in oocyte maturation, sphingosine is used. Sphingosine was prepared as stock solution in ethanol (50 mM, RBI). Stock solution of IBMX (0.1 M, Sigma) was prepared in 0.3 N NaOH solution. Oocytes were cultures in the presence of IBMX (20 μM, 100 μM), sphingosine (20 μM), and IBMX (100 μM) + sphingosine (20 μM). GV and GVBD oocytes were scored at 1, 2, 3, and 4 h after culture in SECM medium under stereomicroscope (Wild M5) at 50-fold magnification. Student t-test and analysis of variance were used for analysis of data. Statistical significance was set $P<0.05$.

9. Effect of U73122 and sn-diC8 on embryo development

Two-cell and four-cell embryos were used to test the effects of U73122, and sn-diC8 on embryo development. U73122 (RBI), a PLC inhibitor and, sn-diC8 (Diacylglycerol analog, Sigma), a PKC activator were prepared as a stock solution in ethanol with concentrations of 2 mM and 10 mM, respectively. Embryos were cultured in sn-diC8 (50 μM), sphingosine (20 μM), sphingosine (10 μM) + sn-diC8 (50 μM), and sphingosine (20 μM) + sn-diC8 (50 μM). Development of embryos were observed at 0h, 0.5 h, 20 h after culture under a phase contrast microscope. Development was determined based on the shape of embryos at particular time of development.

RESULTS

1. Cloning of mouse PLC β1 and γ1 PCR products

To obtain mouse PLC β1 and γ1 cDNAs, total RNA isolated from blastocyst -stage embryos was reverse-transcribed and amplified using PCR procedure. Primers for amplification of PLC β1 and γ1 genes are shown in Fig. 1. The predicted sizes of PLC β1 and γ1 PCR products are 521 bp and 262 bp, respectively. PCR products of PLC β1 and γ1 were validated with restriction enzyme digestions (Fig. 2). PCR products of PLC β1 and γ1 were subcloned into pGEM-7Zf and pGEM-4Z vector, respectively. Cloned cDNAs were sequenced and showed 97.2% and 96.2% identity with cDNA sequences of the rat PLC β1 and γ1, respectively (Fig. 3). These results indicate that the PCR fragments were originated from the mouse PLC β1 and γ1 genes, respectively. PLC β1 and γ1 expression were examined in various tissues by Northern blot hybridization. PLC β1 mRNA levels were relatively high in kidney and brain, while PLC γ1 mRNA was constitutively expressed in all tissues examined (Fig. 4).

2. Competitive RT-PCR from a single oocyte or embryo

The total RNA isolated from a single oocyte or an embryo was employed to RT-PCR procedure. One of the advantages of this method is that we can reduce the RNA loss during isolation step. As shown in Figs. 5 and 6, com-

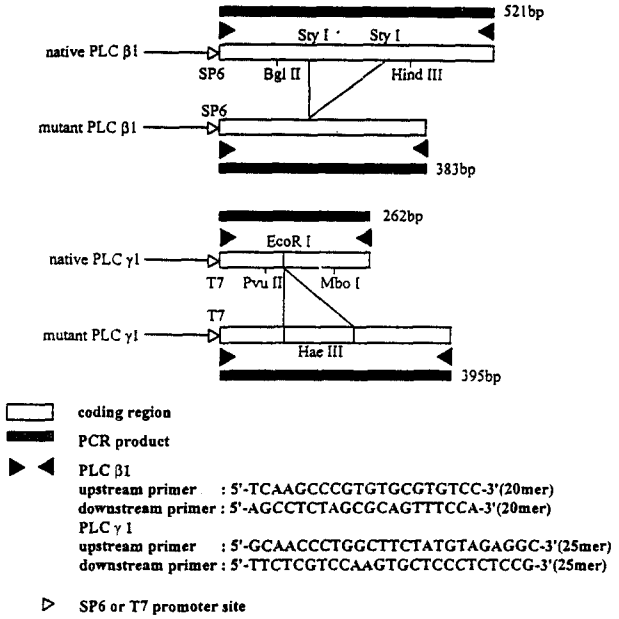


Fig. 1. Schematic diagram of cloning of mutant PLC β1 and γ1 cDNAs and competitive RT-PCR strategy for quantitation of PLC β1 and γ1 transcripts.
The map of native, deletion mutant (for PLC β1) or insertion mutant (for PLC γ1), and primers are shown. Expected size of PCR products of native PLC β1 and γ1 were 521 bp and 262 bp, respectively. The mutant PLC β1 was prepared by deleting 138bp fragment with Sty I followed by self-ligating. The mutant PLC γ1 was prepared by inserting 133bp fragment (a size of Hae Ⅲ marker). Expected size of PCR products of mutant PLC β1 and γ1 were 383 bp and 395 bp, respectively.

petitive RT-PCR method generated discrete PLC β1 and γ1 fragments with 521 bp and 262 bp in sizes, respectively, as predicted on the basis of the mouse PLC β1 and γ1 cDNA sequence. The deletion mutant of PLC β1 and insertion mutant of PLC γ1 showed 383 bp and 395 bp in sizes, respectively. To determine the competition range between biological RNA samples and mutant PLC β1 or γ1 cRNAs, biological RNA samples were co-reverse transcribed and subsequently co-amplified with a serial dilution of mutant cRNA. These titration experiments indicated that total RNA from a single oocyte or an embryo competed with 0.1 fg mutant PLC β1 cRNA. The RNA from a single oocyte and an embryo was competed with 0.1 fg and 1 fg of mutant PLC γ1 cRNA, respectively. Constant amounts of mutant PLC β1 cRNA (0.1 fg) and PLC γ1 cRNA (0.1 fg for oocyte and 1 fg for embryo) were co-amplified with a serial

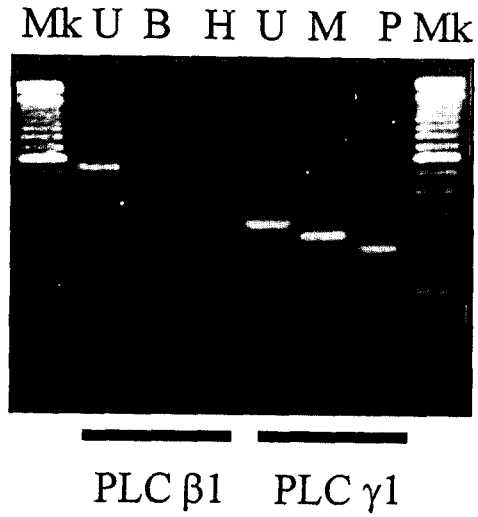


Fig. 2. Validation of PCR products by restriction analysis.
Preimplantation mouse embryos (20 e.a. per group) were collected at blastocyst stage and total RNA was purified by acid guanidinium phenol-chloroform extraction method. Total RNA was reverse transcribed and resulting cDNAs were subsequently amplified by PCR procedure. RT-PCR products (U) were digested with Bgl Ⅱ (B), Hind Ⅲ (H), Mbo Ⅰ (M), and Pvu Ⅱ (P), respectively.

dilution of native PLC β1 and γ1 cRNAs, respectively. Standard curves of PLC β1 and γ1 RT-PCR were then constructed (Figs. 5 and 6). PLC β1 mRNA levels at GV-stage oocytes were detected at low level (<0.1 fg) and scarcely detected at GVBD-stage oocytes (Fig. 5), while PLC γ1 mRNA levels (0.1 fg) were readily detected in GV-stage oocyte and decreased as meiotic resumption proceeded (Fig. 6). Both PLC β1 and γ1 mRNA levels began to increase at morula stage (0.2 fg) and were prominent in blastocyst stage (1 fg).

3. Effect of sphingosine and U73122 on oocyte maturation

To examine whether sphingosine, a PKC inhibitor, is able to overcome the blockade of meiotic resumption, IBMX-treated oocytes were exposed to sphingosine. Treatment of GV-stage oocytes with sphingosine (50 μM) facilitated meiotic resumption by 10~20% over the control within 1 h, as judged by GVBD. IBMX (100 μM) inhibited meiotic resumption completely. Combination of sphingosine(50 μM) with IBMX (100 μM) facilitated meiotic resumption by

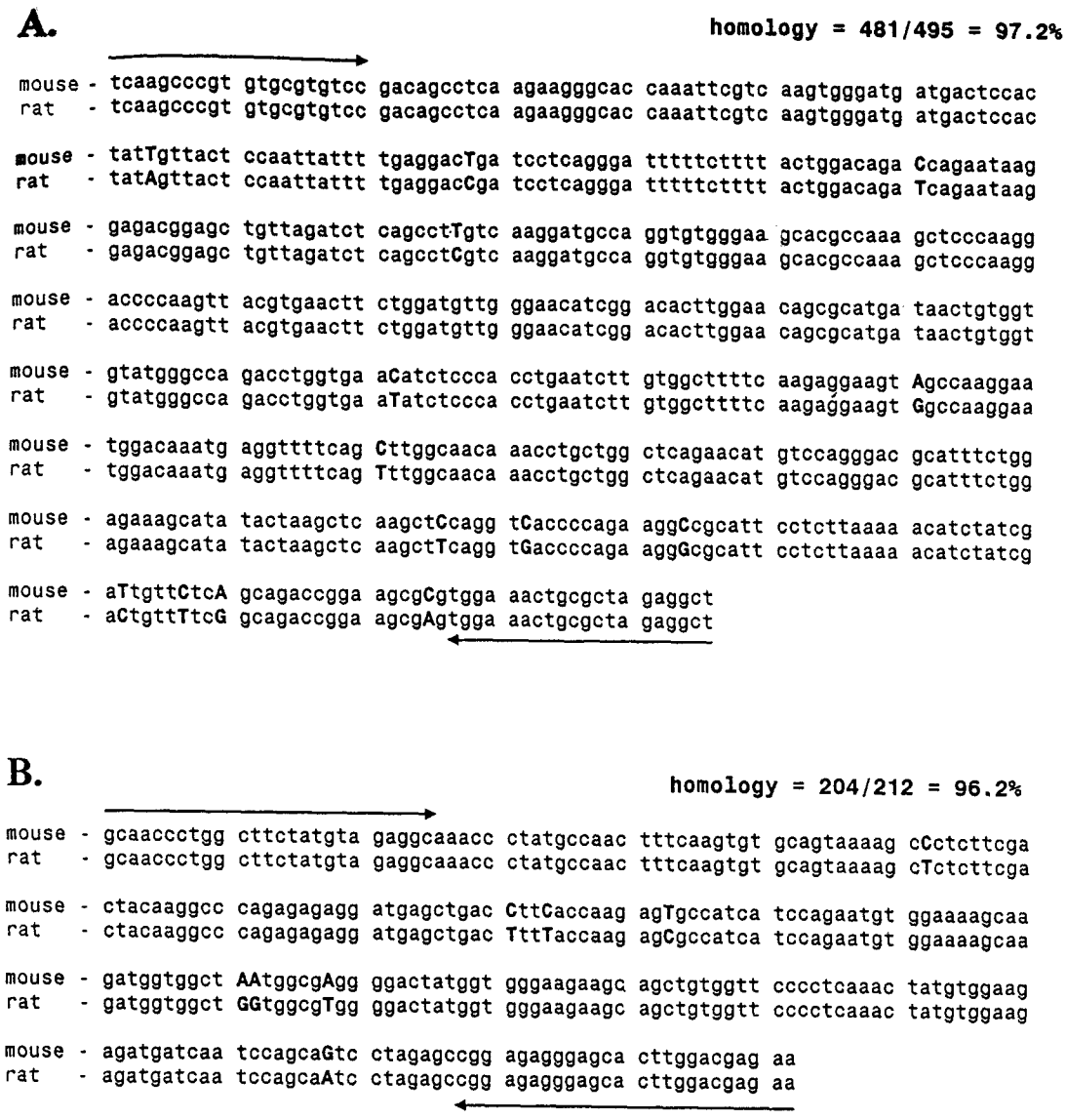


Fig. 3. Nucleotide sequences of RT-PCR products of mouse PLC β1 (A) and γ1 (B).
RT-PCR products were subcloned into pGEM7Zf and pGEM4Z vector to generate plasmid pGEM7Zf-mPLC-β1 and pGEM4Z-mPLC-γ1 and sequenced by dideoxy mediated chain-terminated (Sanger) method. DNA sequences were compared with the rat PLC β1 and PLC γ1. Bold capitals and arrows (⇄) indicate different nucleotides between mouse and rat and primer sequences, respectively.

20% over the IBMX (100 μM) within 1 h, as judged by GVBD (Fig. 7).

4. Effect of U73122 and sn-diC8 on early preimplantation embryo development

To examine whether PLC is involved in the early embryo development, especially the compaction at morula stage, U73122, a PLC blocker, was treated in stages of early embryo. Treatment of 2-cell stage embryos with U73122 showed no prominent effect on normal development up to 4-cell stage, but treatment of 4-cell stage embryos with U73122 blocked the normal development at 8-cell stage. sn-diC8 facilitated compaction of 8-cell stage embryo within 30 min when treated on 4-cell stage embryo. Compacted morula embryos were seen when sn-diC8 was treated with U73122 (Fig. 8).

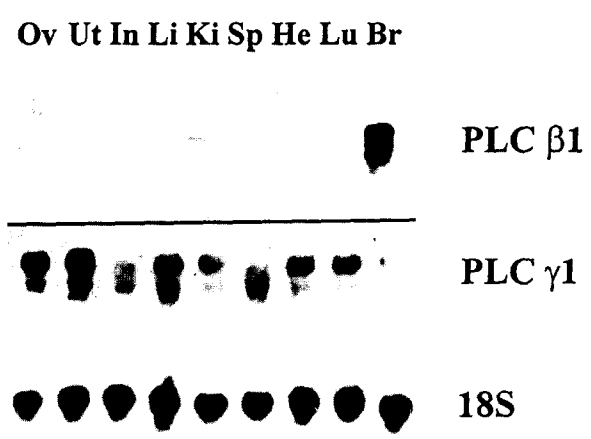


Fig. 4. Northern blot analysis of PLC β1 and γ1 in various tissues.
Total RNAs (30μg) isolated from various tissues were electrophoresed on 1.0 % agarose denaturing gel, blotted on the nylon membrane, and hybridized to ^{32}P-labeled PLC β1 and γ1 cDNA probes. The abbreviations: Ov; ovary, Ut; uterus, In; intestine, Li; liver, Ki; kidney, Sp; spleen, He; heart, Lu; lung, Br; Brain.

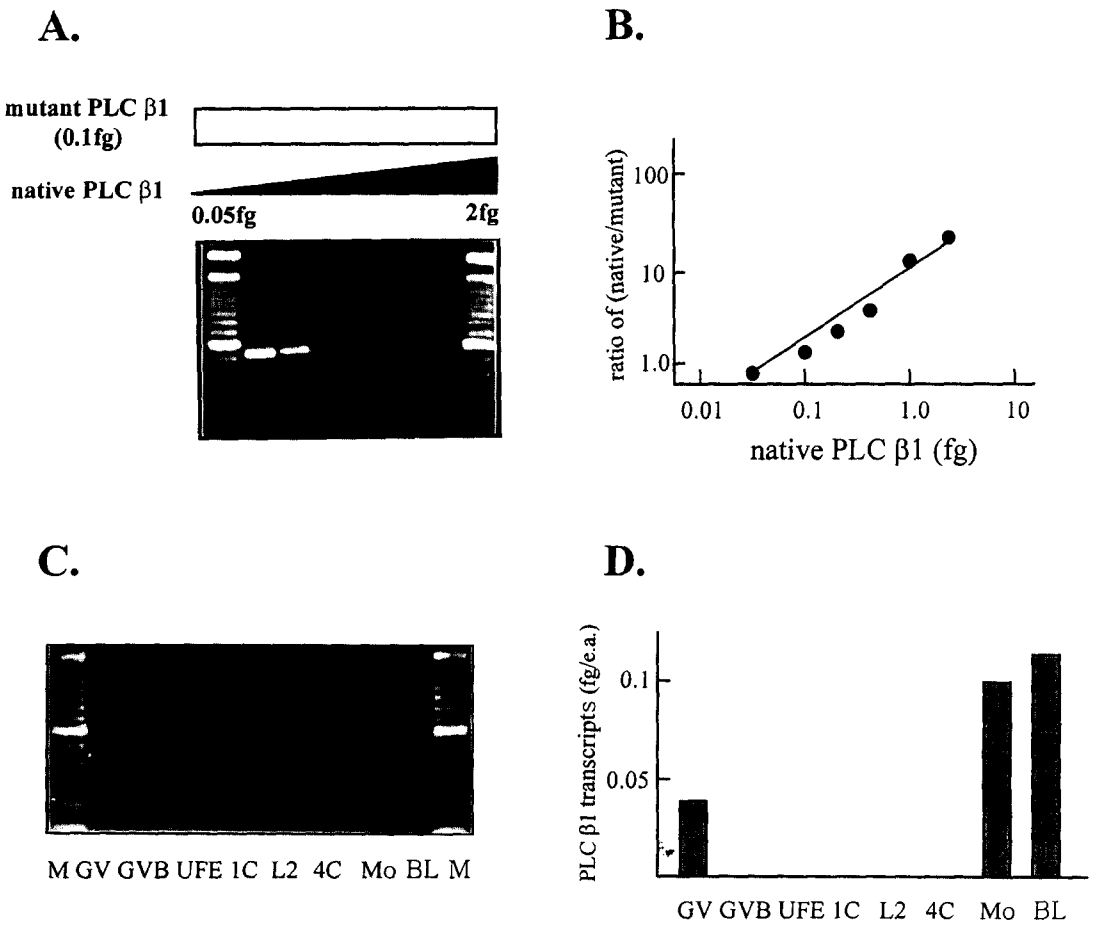


Fig. 5. Titration and standard curve of competitive RT-PCR for PLC β1 and developmental changes of PLC β1 transcripts in oocyte and embryo.
A constant amount of mutant PLC β1 was co-amplified with various concentrations of native PLC β1 cRNA. 0.1 fg mutant PLC β1 cRNA was co-amplified with 2, 1, 0.5, 0.25, 0.1, 0.05, and 0.025 fg native PLC β1 cRNA (A). The plot of ratio of native to mutant reveals a linear relationship (B). PCR products were separated on 1.5% agarose gel (C). Developmental changes of PLC β1 transcripts were calculated from standard curve (D).

DISCUSSION

The present study provided evidence that the expression of the PLC β1 and γ1 genes was stage-specific during oocyte maturation and preimplantation embryo develop-

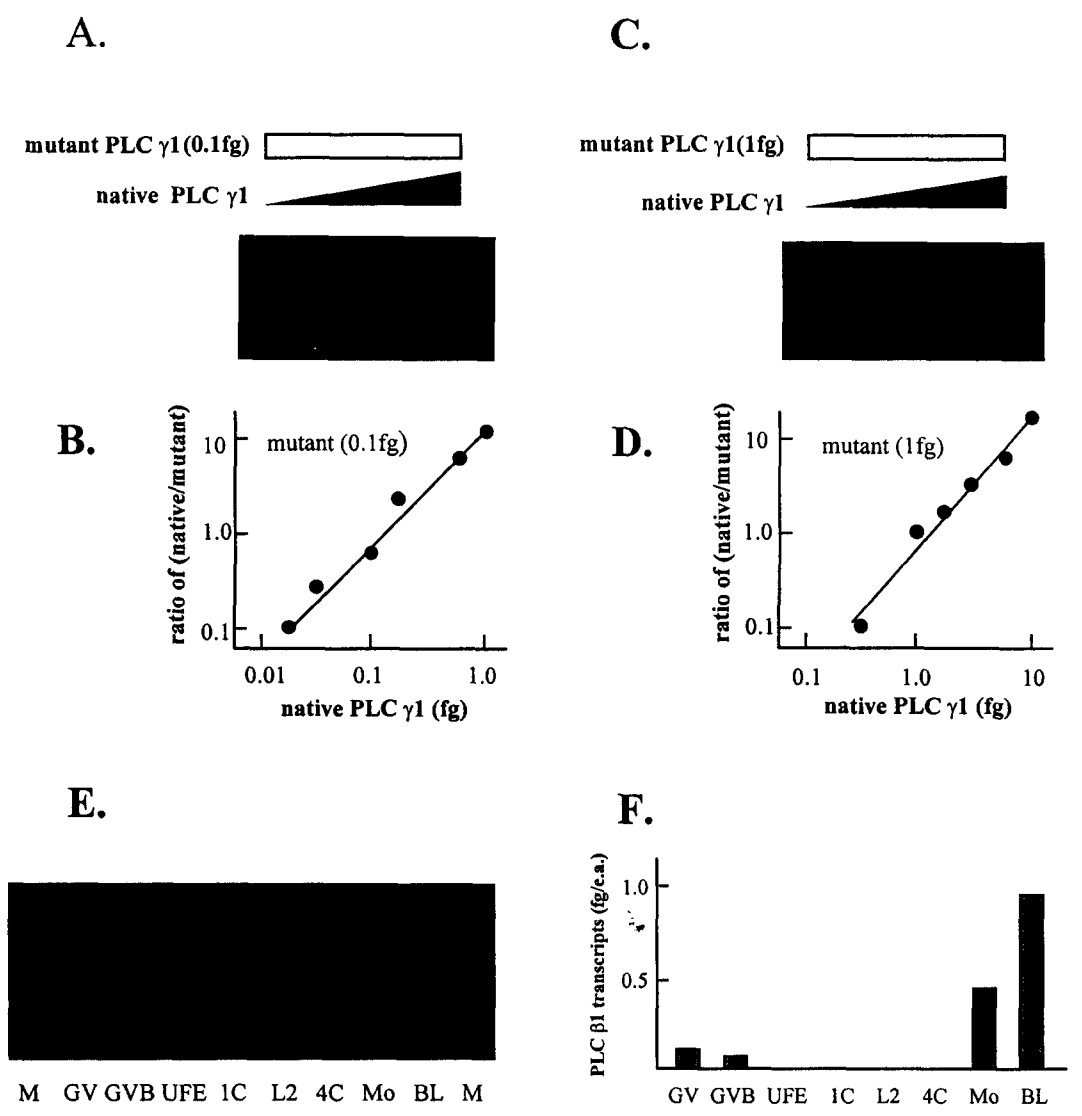


Fig. 6. Titration and standard curve of competitive RT-PCR for PLC γ1 and developmental changes of PLC γ1 transcripts in oocyte and embryo.

A constant amount of mutant PLC γ1 was co-amplified with various concentrations of native PLC γ1 cRNA. 0.1 fg mutant PLC γ1 cRNA was co-amplified with 1, 0.5, 0.25, 0.1, 0.05, and 0.025 fg native PLC γ1 cRNA (A) and 1 fg mutant PLC γ1 cRNA was co-amplified with 10, 7.5, 5, 2.5, 1, and 0. 5 fg native PLC γ1 cRNA (C). The plots of ratio of native to mutant reveal a linear relationship (B, D). PCR products were separated on 1.5% agarose gel (E). Developmental changes of PLC γ1 transcripts were calculated from standard curve (F).

Fig. 8. Effects of sphingosine (PKC inhibitor), sn-diC8 (PKC activator) and U73122 (PLC inhibitor) on preimplantation embryo development.

Four-cell embryos were collected by flushing oviducts with M2 media and incubated in 50 μl drops of M2 media under mineral oil in an atmosphere of 5% CO_2 at 37℃. Embryos were observed at indicated time. Arrow indicates partially compacted morula. A. Control (0 hr) B. Control (0.5 hr) C. Control (20 hr) D. sn-diC8 (0.5 hr, 50 μM) E. sn-diC8 (20 hr, 50 μM) F. spingosine (20 hr, 5 μM) G. U73122 (20 hr, 10 μM) H. U73122 (10 μM)+diC8 (50 μM) (20 hr)

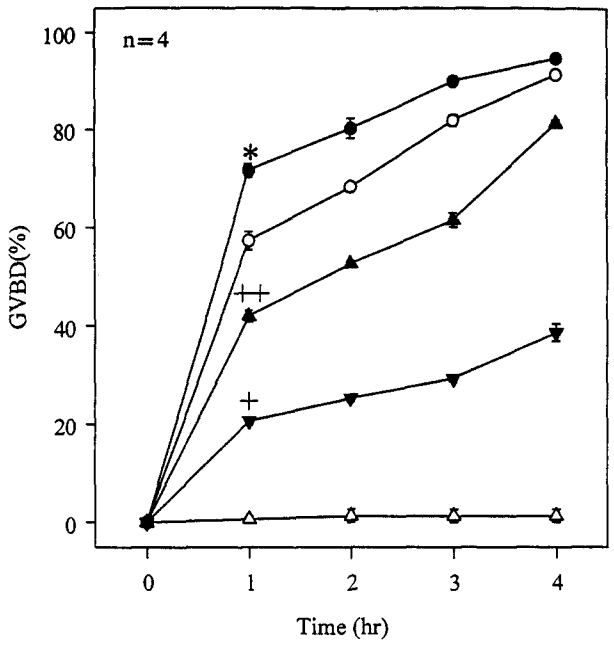
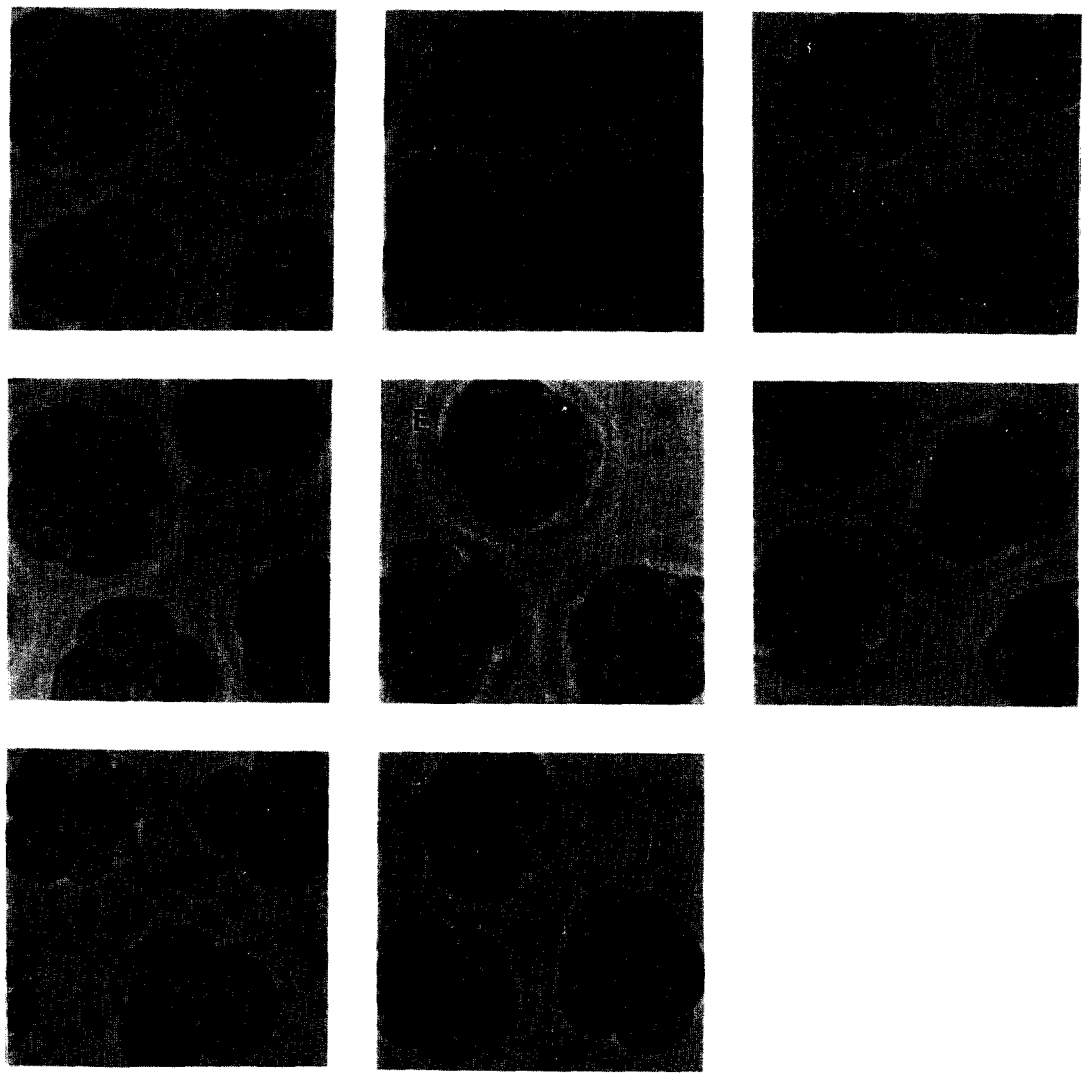


Fig. 7. Effects of sphingosine (PKC inhibitor) and IBMX (phosphodiesterase inhibitor) on mouse oocyte maturation.

Germinal vesicle-stage oocytes were collected by puncturing 3 week-old ICR female mice in SECM and incubated in 50 ml drops of SECM under mineral oil in an atmosphere of 5% CO_2 at 37℃. Germinal vesicle breakdown-stage oocytes were counted at indicated time. Values are mean S.E. (n=4). *, $p<0.05$ vs. control group, +, $p<0.01$ vs. IBMX (100 μM) and ++, $p<0.001$ vs. IBMX (100 μM). Symbols represent experimental group. ○, control; ●, sphingosine (50 μM); △, IBMX (100 μM); ▲, IBMX (10 μM) + sphingosine (50 μM); ▼, IBMX (100 μM) + sphingosine (50 μM).



ment. To quantitate the exact amount of transcripts in a single oocyte and embryo, competitive RT-PCR protocol was employed. PLC γ1 mRNA was detected in a GV-stage oocyte (0.1 fg/oocyte) and its level was reduced when meiotic resumption proceeded, while PLC β1 mRNA (<0.1 fg) at GV-stage oocyte was detected at a low level and hardly detected at GVBD-stage oocyte. After fertilization, both PLC β1 and γ1 mRNA levels began to increase at morula stage embryo (0.2 fg) and were more prominent in blastocyst-stage embryo (1 fg). These results are consistent with the notion that maternal-to-zygotic transition occurs at 2-cell stage and is characterized by a precipitous decline in maternal mRNA contents (Pal et al., 1993) and activation of transcription of the embryonic genome at 4-cell, 8-cell or morula stage (Kidder, 1993).

Treatment of GV-stage oocyte with sphingosine (50 μM) facilitated meiotic resumption by 10-20% over the control within 1 h, as judged by GVBD. A blockade of GVBD with IBMX was overcome by sphingosine by 20% within 1 h, indicating that suppression of PKC activity may facilitate GVBD. However, U73122 itself failed to show any significant effect on meiotic resumption. U73122 (10 μM) effectively blocked the compaction of morula and application of sn-diC8, a PKC activator, resulted in facilitation of compaction of morula within 30 min. A blockade of PLC with U73122 was partially overcome by sn-diC8, indicating that PLC may be involved in compaction of morula-stage embryo. Compaction of the blastomere in mouse embryo marks the beginning of differentiation in the preimplantation embryo. A previous report has shown that activation of PKC triggers premature compaction in 4-cell stage mouse embryo (Winkel et al., 1990). Evidence that PKC is involved in embryonic events, coupled with the fact that PKC acts within the plane of the plasma membrane, suggested that PKC might play an important role in the initiation of compaction in the mouse embryo (Winkel et al., 1990). Although it remains to be explored at the protein level, the present study indicates that PLC γ1 expression may be important for oocyte maturation and embryo development during which EGF plays a critical role by activating PLC γ type isozymes. It is of interest to note that predominant site of TGF α, a predominant ligand for EGF receptor, is localized to the theca component of follicles (Kudlow et al., 1987; Lobb et al., 1989), and plays an inhibitory role in oocyte maturation. TGF α may modulate the development of ovarian follicles by inhibiting the process of apoptosis (Tilly et al., 1992). PLC γ1 is activated by phosphorylation of specific tyrosine residues (Meisenhelder et al., 1989; Wahl et al., 1989). This phosphorylation is mediated by intrinsic protein tyrosine kinase activity of growth factor receptors (Meisenhelder et al., 1989; Wahl et al., 1989; Mohmmadi et al., 1991; Rhee et al., 1992). These growth factors have been shown to enhance *in vitro* oocyte maturation. Little is, however, known about how EGF enhances oocyte maturation. Hubbard and Romani (1991) suggest that EGF might trigger follicle-enclosed oocyte maturation via PKC.

Taken together, the present study shows that PLC β1 and γ1 are expressed in oocytes and early embryos in a developmental stage-specific manner suggesting that the PLC-PKC pathway may be involved in preimplantation embryo development.

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