

PSF1 Is Essential for Early Embryogenesis in Mice

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Psf1 (partner of sld five 1) forms a novel heterotetramer complex, GINS (Go, Ichi, Nii, and San; five, one, two, and three, respectively, in Japanese), with Sld5, Psf2, and Psf3. The formation of this complex is essential for the initiation of DNA replication in yeast and *Xenopus laevis* egg extracts. Although all of the components are well conserved in higher eukaryotes, the biological function in vivo is largely unknown. We originally cloned the mouse ortholog of *PSF1* from a hematopoietic stem cell cDNA library and found that *PSF1* is expressed in blastocysts, adult bone marrow, and testis, in which the stem cell system is active. Here we used the gene-targeting technique to determine the physiological function of *PSF1* in vivo. Mice homozygous for a nonfunctional mutant of *PSF1* died in utero around the time of implantation. *PSF1*^{-/-} blastocysts failed to show outgrowth in culture and exhibited a cell proliferation defect. Our data clearly indicate that *PSF1* is required for early embryogenesis.

Eukaryotic chromosomal replication is tightly regulated to maintain the integrity of genomic information. In *Saccharomyces cerevisiae*, Orc (origin recognition complex) is bound to replication origins throughout the cell cycle (1, 4, 21). From late M to G₁ phase, MCM (minichromosome maintenance) protein is loaded onto the origin, marked by Orc, Cdc6, and Cdt1, and forms the prereplication complex (pre-RC) (2, 9). On activation and recruitment of additional factors, such as CDC45, the pre-RC is converted to the preinitiation complex, which is the complex essential for the transition to DNA replication (2, 23). In yeast, CDC45 is essential for the initiation and elongation of DNA replication (7, 20).

Recently, a novel multiprotein complex, GINS, was identified. The GINS complex contains Psf1 (partner of sld five 1), Psf2, Psf3, and Sld5 and forms a ring-like structure (8, 10, 19). During the S phase, the GINS complex is loaded onto chromatin after the formation of pre-RCs and then tightly associates with the replication origin. This binding is suppressed by p21 and geminin by inhibition of the loading of CDC45 onto chromatin (11) and the pre-RC formation by binding to Cdt1 (23). Moreover, the chromatin binding of GINS complex and that of CDC45 are mutually dependent processes, but they do not associate with each other. The association of PSF1 and Dpb11/Cut5 with the origins is also mutually dependent. All genes encoding GINS components are evolutionarily conserved and are essential for cell growth (22). However, the functions of the GINS complex in mammalian cells have not been reported.

In this study, we isolated the mouse ortholog of *PSF1* and generated *PSF1*-deficient mice by a gene-targeting technique. In mice, *PSF1* is specifically expressed in proliferating immature cells. Loss of *PSF1* causes embryonic lethality around the implantation stage. *PSF1*^{-/-} embryos revealed impaired pro-

liferation of the inner cell mass (ICM) and trophoblasts. Our data suggest that *PSF1* is essential for mouse embryogenesis.

MATERIALS AND METHODS

Mice. C57BL/6 mice were purchased from SLC. All animal studies were approved by the Animal Care Committee of Kanazawa University.

Construction and screening of a stem cell-specific cDNA library. Preparation of bone marrow (BM) cells and fluorescence-activated cell sorting analysis were performed as described previously (17, 18). The antibodies used in flow cytometric analysis for lineage marker (Lin) were phycoerythrin-conjugated Mac-1 (M1/70), Gr-1 (RB6-8C5), B220 (RA3-6B2), TER119, anti-CD4 (GK1.5), and anti-CD8 (53-6.72). Allophycocyanin-conjugated anti-c-kit (ACK2) and biotin-conjugated anti-Sca-1 were also applied. Biotinylated anti-Sca-1 was visualized with peridinin chlorophyll protein-streptavidin. These antibodies were purchased from BD Pharmingen. Lin⁺ cells and Lin⁻ c-kit⁺ Sca-1⁺ cells were purified from BM cells that had been obtained from an 8-week-old C57BL/6 mouse by cell sorting. The stained cells were analyzed with a FACSCalibur (Becton Dickinson) and sorted with an EPICS ALTRA (Beckman Coulter) instrument. Total RNAs were extracted from these cell populations and were subjected to cDNA synthesis and amplification using a SMART PCR synthesis kit (BD Clontech) according to the manufacturer's protocol. Subtractive cloning was performed using a PCR-Select cDNA subtraction kit (BD Clontech).

Immunohistochemistry and fluorescence-activated cell sorting analysis. Tissue fixation, preparation of tissue sections, and staining of sections with antibodies were performed as described previously (18). For immunohistochemistry, anti-PSF1 (see below) and antibromodeoxyuridine (anti-BrdU) antibodies (Zymed) were used. Cy3- or horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Jackson ImmunoResearch.

To obtain a specific antibody against PSF1, a rabbit was immunized with a synthetic peptide (CEQLIRQGVLEH) derived from the C-terminal region of PSF1. Antisera were affinity purified with the same peptide. Preimmunized rabbit immunoglobulins were used as a negative control to confirm specific staining.

RT-PCR analysis. Reverse transcription-PCR (RT-PCR) was performed as previously described (18). We used the following primer sets: 5'-TTA AGA AAT AGA CGC TGC ACG A-3' and 5'-TGC CAT CAT CAA CTT CAA ATT C-3' (for *PSF1*) and 5'-ACC ACA GTC CAT GCC ATC AC-3' and 5'-TCC ACC ACC CTG TTG CTG TA-3' (for glyceraldehyde-3-phosphate dehydrogenase [GAPDH]).

Gene targeting. We isolated genomic clones encoding mouse *PSF1* from the mouse 129Sv/J library (Stratagene) by using mouse *PSF1* cDNA as a probe. In the targeting construct, exon 5 was replaced with the β -galactosidase gene and a pGK-neomycin resistance gene (see Fig. 2). We linearized this construct with NotI and electroporated it into 129Sv/J E14.1 embryonic stem (ES) cells. We

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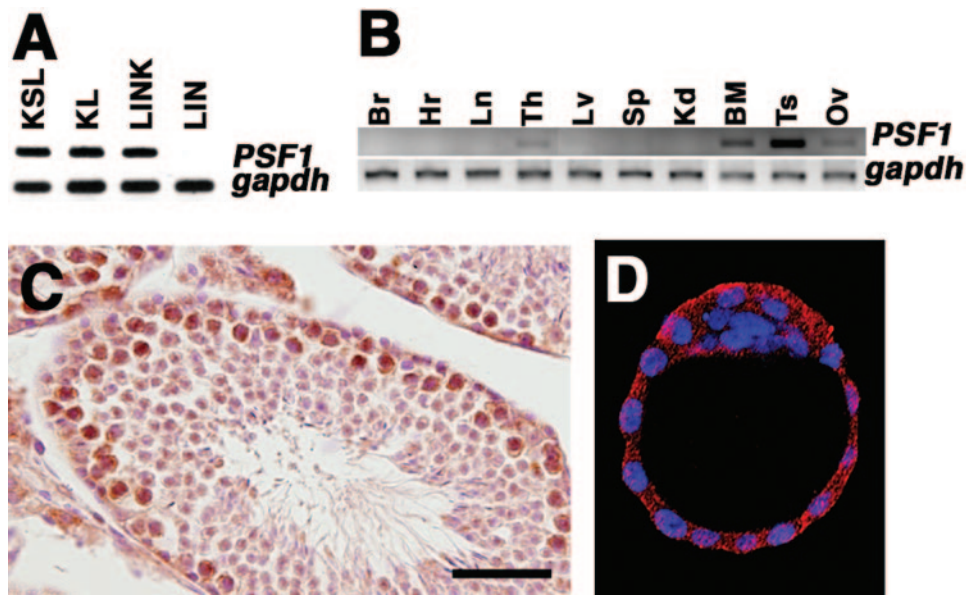


FIG. 1. *PSF1* expression in adult tissues. (A and B) RT-PCR of fractionated adult BM cells (A) and adult tissues (B). KSL, Lin^- Sca-1 $^+$ c-kit $^+$ cells; KL, Lin^- Sca-1 $^-$ c-kit $^+$; LINK, Lin^+ c-kit $^+$; LIN, Lin^+ . Br, brain; Hr, heart; Ln, lung; Th, thymus; Lv, liver; Sp, spleen; Kd, kidney; Ts, testis; Ov, ovary. (C and D) Immunostaining of sections of the testis (C) and blastocysts (E3.5) (D) with anti-*PSF1* antibody labeled with HRP (C) or Cy3 (D). Sections were counterstained with hematoxylin or 4',6'-diamidino-2-phenylindole (DAPI). Color staining: HRP, brown; Cy3, red; DAPI, blue. Bar, 50 μm .

selected G418-resistant clones and screened them by PCR and Southern blot analysis for the correct recombination (see Fig. 2). Chimera mice were generated by the aggregation method (12). We mated chimera males with C57BL/6J females and screened the offspring by Southern blotting and PCR analyses for mice bearing the *PSF1* $^{+/-}$ genotype.

Northern blot. Total RNA was extracted from testis and liver of 8-week-old mice. Thirty-microgram aliquots of total RNA were separated on 1% formaldehyde-agarose gels and transferred onto nylon membrane filters by capillary transfer using 20 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer. Filters were hybridized overnight at 68 $^{\circ}\text{C}$ in DIG Easy Hyb (Roche) with a digoxigenin-labeled *PSF1* cRNA probe. Hybridized probe was detected with alkaline phosphatase-conjugated antidigoxigenin antibodies using the DIG luminescent detection kit (Roche), following the manufacturer's instructions. GAPDH mRNA was used as an internal standard to ensure that equal amounts of RNA were loaded in each lane.

In vitro culture. Embryos (E3.5) were flushed out from the uteri of pregnant mice and individually cultured in standard ES medium with leukemia inhibitory factor (Invitrogen) for 10 days. DNA synthesis was assessed by BrdU (10 μM ; Sigma) incorporation in cultured blastocysts from days 4 to 5; on day 5 of culturing, the blastocysts were fixed with 4% paraformaldehyde in phosphate-buffered saline, stained with anti-BrdU antibody (1 $\mu\text{g}/\text{ml}$; Zymed), and counterstained with hematoxylin. The TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling) assay for detection of apoptotic cells was performed using the In Situ Cell Death Detection kit (Roche) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Cloning of mouse *PSF1*. Although most terminally differentiated somatic cells are not able to proliferate, stem cells and immature progenitor cells are constitutively in cycle to produce mature cells. To elucidate the molecular mechanism regulating mammalian cell division, we constructed a subtraction library from the BM-derived Lin^- c-kit $^+$ Sca-1 $^+$ hematopoietic stem cell (KSL cells; stem/progenitor cells) fraction as a tester and the Lin^+ mature hematopoietic cells as the driver in order to isolate genes encoding proteins that are involved in DNA replication and specifically expressed in immature cells. Among

521 clones that were abundantly expressed in KSL cells, one gene named *#e11* was expressed in KSL cells and their progenitor cells (Lin^- c-kit $^+$ Sca-1 $^-$) but not in Lin^+ mature hematopoietic cells, as confirmed by RT-PCR (Fig. 1A). This gene corresponded to a hypothetical gene in GenBank (accession no. AK013116) and was closely related to *Psf1* in a budding yeast (partner of *sld5-1*), which was shown to encode a protein involved in DNA replication in yeast and in an in vitro model using *Xenopus laevis* egg extracts (8, 10). We identified the binding partner of mouse *#e11* to be *sld5* by the two-hybrid system (M. Ueno and N. Takakura, unpublished data). Therefore, we considered this *#e11* gene to be a mouse ortholog and named it *PSF1*.

***PSF1* is predominantly expressed in highly proliferative organs, especially in the immature cell population.** We analyzed *PSF1* expression in several adult tissues. *PSF1* expression was predominantly observed in hematopoietic tissues such as the adult BM and thymus on RT-PCR (Fig. 1B). Moreover, *PSF1* expression was observed in reproductive tissues, i.e., the testis and ovary, which have an active stem cell system. In other adult tissues (brain, heart, lung, liver, spleen, and kidney), *PSF1* expression was not detectable. These data suggested that *PSF1* is expressed specifically in tissues with higher rates of proliferation.

To determine the spatial distribution of *PSF1* protein in the adult testis, we generated antibody against *PSF1* peptide. Immunohistochemistry on mouse testis sections showed that *PSF1* protein is present in immature cells, i.e., spermatogonia (Fig. 1C). *PSF1* is expressed in other immature cell populations including blastocysts (Fig. 1D), adult thymic progenitor cells, and yolk sac-containing hematopoietic progenitor cells (data not shown). These data suggested that *PSF1* is expressed specifically in the immature cell population.

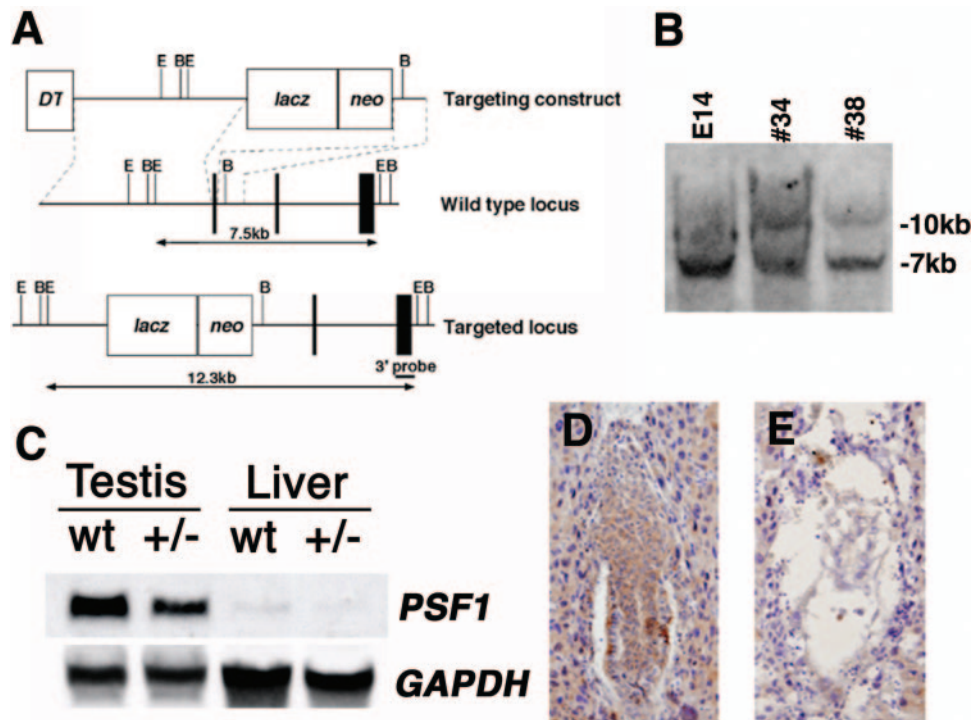


FIG. 2. Targeted disruption of mouse *PSF1*. Generation of *PSF1*-deficient mice. (A) Exons are represented by vertical bars and introns by intervening horizontal lines. Exon 5 of *PSF1* was replaced by homologous recombination with a β -galactosidase gene (*lacZ*) and a neomycin resistance gene driven by the *Pgk1* promoter (*neo*). E, EcoRI; B, BamHI. This increased the size of the EcoRI restriction fragment. (B) Southern blot analysis of wild-type (E14) and targeted (#34 and #38) ES cells demonstrated homologous recombination in *PSF1*. (C) Northern blot analysis of 30 μ g of total RNA isolated from wild-type (wt) and heterozygous *PSF1* mutants hybridized with a *PSF1* cRNA probe. Heterozygous *PSF1* mutants had decreased *PSF1* mRNA in the testis (top). *GAPDH* mRNA signals were used as an internal standard (bottom). (D and E) *PSF1* expression was analyzed by immunohistochemical staining using anti-*PSF1* antibody in *PSF1*^{+/+} (D) and *PSF1*^{-/-} (E) embryos at E6.5. Original magnification, $\times 100$.

Targeted disruption of the mouse *PSF1* gene. To analyze the function of *PSF1*, we generated mice lacking a functional *PSF1* gene (Fig. 2). The mouse *PSF1* gene contains seven putative coding exons. The targeting vector was designed by deleting exon 5 and inserting a *lacZ*-*neo* cassette. Among the 95 independent ES cell colonies examined, we found two homologous recombinants. Correct targeting was confirmed in these ES clones by Southern blot analyses with a 3' probe (Fig. 2B). One ES clone was aggregated with C57BL/6 blastocysts to generate a chimera, which subsequently produced germ line transmission. The *PSF1*^{+/-} line was established by backcrosses with C57BL/6 mice. To confirm the loss of *PSF1* transcript in mutant mice by gene disruption, we performed Northern blot analysis. As expected, *PSF1* mRNA was reduced in *PSF1*^{+/-} testis (Fig. 2C). *PSF1*^{+/-} mice were born at Mendelian frequency, and there were no apparent differences between *PSF1*^{+/-} mice and wild-type mice.

***PSF1* is required for cell proliferation, and loss of *PSF1* leads to early embryonic lethality.** We analyzed the *PSF1* gene in neonates resulting from *PSF1*^{+/-} intercrosses and did not obtain any homozygous offspring (Table 1). In normal E6.5 embryos, a cylinder-like two-layered cellular structure was observed (Fig. 2D and 3A). However, *PSF1*-deficient embryos, which could be identified at this stage by the absence of *PSF1* immunoreactivity (Fig. 2E), lacked such cylinder-like structures (Fig. 2E and 3B). These data suggested that the *PSF1*^{-/-}

embryos failed to develop past E5.5 and showed disorganized embryonic and extraembryonic structures.

The cellular proliferation and differentiation of mutant embryos were investigated in *in vitro* cultures of blastocysts. On light microscopy, there were no differences among individual blastocysts (data not shown). In *PSF1*^{+/+} and *PSF1*^{+/-} embryos (total $n = 104$), trophoblasts started to spread over the culture dish after hatching and supported robust ICM outgrowths after 2 days. While the trophoblasts from *PSF1*^{-/-} blastocysts ($n = 32$) also attached and spread over the dish,

TABLE 1. Progeny from *PSF1* heterozygotes

Age	No. of offspring with genotype:			No. resolved	No. total
	+/+	+/-	-/-		
Neonate	15	36	0	0	51
E11.5	9	14	0	3	26
E9.5	7	16	0	6	29
E7.5	12	16	0	4	32
E6.5	ND ^a	ND	ND	8	26
E3.5	4	13	5	NA ^b	22

^a ND, not determined. The genotype of 16 E6.5 embryos was not determined.

^b NA, not available. This table shows the number of offspring obtained by mating *PSF1* heterozygotes that were studied.

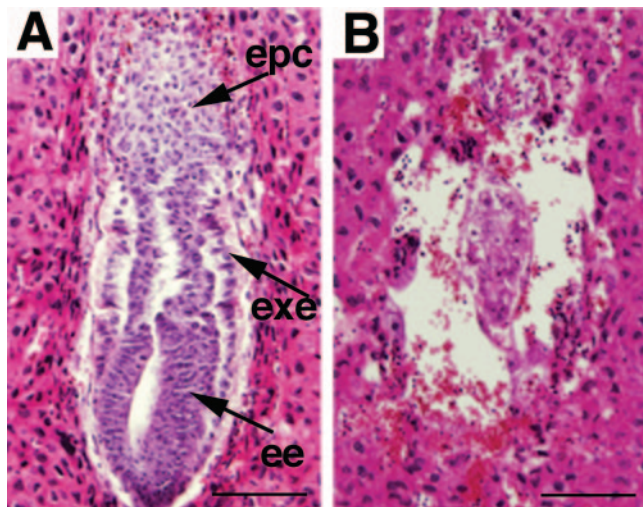


FIG. 3. Histological analysis of *PSF1*^{-/-} embryos. Hematoxylin- and eosin-stained, longitudinal sections of E6.5 embryos in decidua. (A) Wild-type embryo; (B) mutant *PSF1*^{-/-} embryo. epc, ectoplacental cone; exe, extraembryonic ectoderm; ee, embryonic endoderm. Bars, 100 μ m.

PSF1^{-/-} ICM cells failed to form outgrowths (see Fig. 4). These data suggested that the lethality of *PSF1*^{-/-} embryos in utero was caused at least by the death of ICM cells.

To further delineate the proliferation defect of the *PSF1*^{-/-} blastocysts, we carried out bromodeoxyuridine incorporation

assays during blastocyst outgrowth (Fig. 4I and J). Vigorous DNA synthesis was observed in *PSF1*^{+/-} ICM cells by immunostaining with anti-BrdU antibody (Fig. 4I). However, the presumed ICM cells from the mutants ceased to proliferate, while DNA synthesis was still observed in trophoblasts (Fig. 4J). Moreover, in the ICM of *PSF1*^{-/-} blastocyst cultures, TUNEL-positive apoptotic cells appeared on day 5 (Fig. 4K and L). Therefore, the *PSF1*^{-/-} ICM cells were unable to proliferate and underwent apoptosis in culture.

While BrdU⁺ cells were found in *PSF1*^{-/-} trophoblasts after 5 days of culturing (Fig. 4J), *PSF1*^{-/-} trophoblasts stopped proliferation after 8 days of culturing (Fig. 4O). BrdU was not incorporated in *PSF1*^{-/-} trophoblasts beyond 8 days (data not shown), and the number of trophoblasts declined (Fig. 4P). By contrast, *PSF1*^{+/-} embryo trophoblasts continuously proliferated after 10 days of culturing (Fig. 4M and N). These data indicated that *PSF1* is essential for both ICM and trophoblast proliferation.

In this study, we examined the functions of PSF1 in vivo by gene targeting technology. Our results revealed impaired proliferation of the ICM and trophoblasts in *PSF1*^{-/-} embryos. Recently, it was reported that Psf1 and CDC45 are involved cooperatively in the initiation of DNA replication in yeast (10, 19) and that both molecules are prerequisite for DNA replication in yeast (7, 20). In mice, the phenotype of *CDC45*-deficient embryos after uterine implantation (24) is quite similar to that of *PSF1*-null embryos. Mice deficient in the *CDC45* ortholog of yeast show a defect in cell proliferation in blasto-

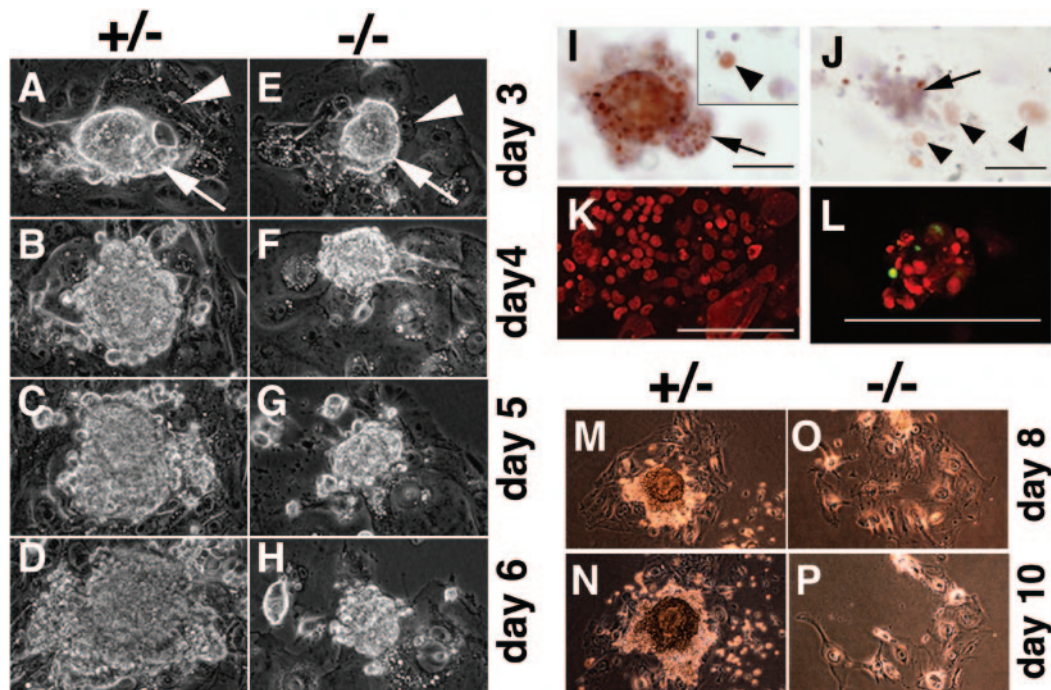


FIG. 4. Defective growth of *PSF1*^{-/-} blastocysts in vitro. Blastocysts (E3.5) were recovered from heterozygous intercrosses, cultured individually for a period of 6 days as indicated, and genotyped by PCR. Cultures of representative +/+ (A to D) and -/- (E to H) blastocysts are shown. Arrow, ICM; arrowhead, trophoblast. (I and J) DNA synthesis in cultured blastocysts (BrdU incorporation). Immunostaining with anti-BrdU antibody was performed (brown). (I) *PSF1*^{+/-} blastocyst; (J) *PSF1*^{-/-} blastocyst. Inset in panel I shows a different field in this culture plate. Arrow, ICM; arrowhead, labeled trophoblast. (K and L) Apoptosis of cells in cultured ICM from blastocysts (TUNEL assay; green, apoptotic cells). (K) *PSF1*^{+/-} blastocyst; (L) *PSF1*^{-/-} blastocyst. Nuclei were counterstained with propidium iodide (red). Bars, 100 μ m. (M to P) Blastocysts were cultured for 8 days (M and O) and 10 days (N and P). (M and N) *PSF1*^{+/-} blastocyst; (O and P) *PSF1*^{-/-} blastocyst.

cyst culture (24). Therefore, the molecular functions of PSF1 and CDC45 for DNA replication may be conserved in mammalian cells.

Although *Psfl* is indispensable for cell proliferation in yeast (19), no obvious morphological abnormality was found in *PSF1*^{-/-} embryos before implantation (data not shown). This observation raised the possibility of the existence of maternal *PSF1* transcript stores and could account for the proliferation of *PSF1*^{-/-} embryos through the early developmental stages. To ascertain this, we performed immunostaining on unfertilized eggs with anti-PSF1 antibody which revealed potent expression of PSF1 (data not shown). Because zygotic gene transcription initiates at the two-cell stage and paternal protein contributions are thought to be negligible (16), we conclude that the timing of *PSF1*^{-/-} lethality is due to the loss and/or dilution of maternal *PSF1* transcripts around implantation stage.

Most of our knowledge on DNA replication has accumulated from studies on lower eukaryotes. However, it can be argued that DNA replication of higher eukaryotes is more complex. For example, DNA replication is linked to gene transcription in higher eukaryotes including *Drosophila melanogaster* (15), *Xenopus* (5), and mice (6) but not in yeast (14). Although origin recognition complex (Orc) binds to chromatin at replication origins throughout the cell cycle in yeast (1, 4, 21), a part of Orc2 localizes at the centrosomes and heterochromatin during the M phase and participates in chromosome segregation in human cells (13). Mcm10, which is a conserved protein and is involved in initiation of DNA replication in yeast, is required for chromosome condensation in fly cells (3). Interestingly, in nematodes, loss of PSF1 causes abnormality of chromatin segregation (M. Ueno and N. Takakura, unpublished data). Thus, PSF1 may have pivotal roles in other biological processes. Further analysis on the function of PSF1 will shed light on the complex mechanism of DNA replication in mammalian cells.

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