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# REST and its downstream molecule Mek5 regulate survival of primordial germ cells

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ABSTRACT

In mouse embryos, some primordial germ cells (PGCs) are eliminated by apoptosis, but the molecular pathways that lead to PGC survival versus apoptosis have not been fully characterized. Here, we found that REST (repressor element 1-silencing transcription factor), a transcription factor that binds a conserved regulatory element, NRSE/RE1, played a role in PGC survival. *REST* expression was higher in PGCs than in surrounding somatic cells. Moreover, in mouse embryos with a PGC-specific conditional *REST* mutation, the PGC population experienced more apoptosis and was significantly smaller than that in control embryos; these findings indicated that REST functioned in a cell-autonomous fashion that was critical for PGC survival. Several anti-apoptotic genes were among the previously identified *REST*-target gene candidates; moreover, some of these genes were downregulated in the *REST*-deficient PGCs. *Mek5*, which encodes a component in the a MAP kinase cascade, was one of these downregulated *REST*-target gene candidates, and a *Mek5* mutation, like the *REST* mutation, caused an increase in PGC apoptosis; these finding suggested that REST promoted PGC survival via regulation of the *Mek5* expression. Importantly, there were a normal number of PGCs in the *REST* mutants at birth, and both the male and female *REST*-mutant adults were fertile; these final observations revealed that the PGC population was very robust and could recover from a genetically induced reduction in cell number.
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## Introduction

Cells in various tissues undergo apoptotic cell death during embryogenesis, as well as in mature organisms in particular physiological conditions, and this apoptosis is often important for development and homeostasis. For example, removal of unnecessary and/or excess cells in embryos plays a critical role in the morphogenesis of embryos (Fuchs and Steller (2011)), and selective cell death of lymphocytes is essential for generating functional immune cells and for establishing immunotolerance (Lu and Finn (2008)). The molecular components and regulatory pathways that result in apoptosis have been studied extensively and are well characterized. Apoptotic signals enhance permeability of mitochondrial membrane (Youle and Strasser (2008); Martinou and Youle (2011)); consequently, cytochrome C and other apoptosis inducing molecules are released from mitochondria, and these molecules activate caspase family proteins that execute apoptosis. Bcl-2 family molecules regulate permeability of mitochondrial membrane and induce or suppress apoptosis (Youle and Strasser, 2008; Martinou and Youle, 2011). Several trophic factors and apoptosis-inducing factors such as TNF and FasL control the above-mentioned death-and-survival regulating molecular pathways (Lee et al., 1999; Lysiak (2004)).

Primordial germ cells (PGCs) are undifferentiated germ cells in embryos; PGCs emerge as a small number of cells in early gastrulating embryos in mouse, and migrate towards embryonic gonads (Anderson et al., 2000). During this migration, PGCs actively proliferate to increase in number (Tam and Snow, 1981), but a small fraction of these PGCs undergo apoptosis (Coucouvanis et al., 1993; Pesce et al., 1993). In particular, ectopic PGCs, which have failed to migrate correctly, are efficiently eliminated by apoptosis (Runyan et al., 2006); apoptosis of ectopic PCGs is a clinically important phenomenon because malignant pediatric tumors often arise from ectopic PGCs located

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at the midline of human embryos (Dehner (1983)). A number of intracellular molecules, as well as extracellular signals, control PGC survival. One of the most important extracellular signals for PGC survival is a growth factor, Steel factor (Dolci et al., 1991; Matsui et al., 1991; Pesce et al., 1993); downregulation of Steel factor causes apoptosis of migrating PGCs in the allantois and in the midline of embryos (Runyan et al., 2006). A small number of PGCs undergo apoptosis after colonizing the genital ridge; in particular, PGC subpopulations that can be recognized by expression of cell surface proteins tend to undergo apoptosis (Coucouvanis et al., 1993; Morita-Fujimura et al., 2009); these findings indicate that cell-intrinsic mechanisms, in addition to the extracellular trophic factors, are likely to be involved in selective apoptosis of PGCs.

Some molecules critical to the control of PGC death, survival, or both have been identified, but information on the molecular cascades that control PGC fate is still fragmented, and the details of these cascades remain largely obscure. For example, the molecules that link trophic factors (e.g., Steel factor (Runyan et al., 2006)) to the transcriptional network that controls the expression of signaling molecules regulating PGC survival are not known. Here, we show that REST (repressor element 1-silencing transcription factor), also known as the neuron-restrictive silencer factor (NRSF) (Chong et al., 1995; Schoenherr and Anderson, 1995), is a transcriptional regulator that promotes PGC survival. REST was initially identified as a key regulator of neurogenesis (Schoenherr et al., 1996), but it also plays roles in embryonic stem (ES) cells (Yamada et al., 2010). Previous studies showed that REST was a target of the regulatory circuitry of pluripotency in ES cells (Singh et al., 2008), but other reports indicated that REST functions were not critical for maintenance of pluripotency (Jørgensen et al., 2009; Buckley et al., 2009), and therefore functions of REST in ES cells had remained controversial. More recently, it was demonstrated that REST was not necessary for maintenance of pluripotency but promoted the early differentiation in ES cells (Yamada et al., 2010). Here, we found that REST induced the expression of the gene encoding a MAP kinase cascade molecule, Mek5, to support survival of PGCs in mouse embryos.

## Materials and methods

### *Mouse strains and staging of embryos*

The embryos used for immunohistochemistry, in situ hybridization, or isolation of PGCs were obtained from dams of the outbred strain ICR that had been mated to male BDF1 (B6D2F1) mice or to mil1-GFP transgenic male mice (Tanaka et al., 2004); the mil1-GFP transgenic mice had been maintained in a C57Bl/6 background. To isolate *REST* (+/+), *REST* (+/−), and *REST* (−/−) PGCs from embryos, we used embryos from intercrosses between heterozygote *REST* males and female that harbored the mil1-GFP transgene, which is specifically expressed in PGCs. Before each experiments, embryos were staged as described previously by Downs and Davies (1993): E7.0, E7.25, and E7.5 embryos corresponded to mid-streak to late-streak, early allantoic bud, and late allantoic bud stages, respectively. To obtain purified PGCs, tissue fragment containing PGCs were dissociated by trypsin and GFP-positive PGCs were manually isolated under a fluorescent microscope. The mice were kept and bred in the Animal Unit of the Institute of Development, Aging and Cancer (Tohoku University), an environmentally controlled and specific pathogen-free facility, according to the guidelines for experimental animals defined by the facility. Animal protocols were reviewed and approved by the Tohoku University Animal Studies Committee.

### *Targeted disruption of the REST locus*

To generate a *REST* targeting vector, exon 1 of *REST*, which includes the translational initiation site, was replaced with Pgk-Neo flanked by loxP sequences (Fig. 1E); the vector was constructed using the Red BAC (Bacterial artificial chromosome) recombineering system (Liu et al. (2003)). A BAC bearing the *REST* genomic locus (BMQ-75F23, 129S7 background) was purchased from Geneservice (Geneservice Limited, Cambridge, UK). The E14.1 ES cell line (129/Ola) was mixed with 40 mg of linear targeting vector and subjected to electroporation; selection was performed using 250 mg/ml G418 (GIBCO). ES cell colonies were isolated and then screened using PCR and primers specific for the 3′ arm of the predicted recombination product (Fig. 1E). Homologous recombination was confirmed using Southern blot analysis and the 5′, 3′ probes that are diagramed in Fig. 1E. We isolated three independent ES cell clones that each carried the predicted recombination product; for each ES clone, cells were injected into C57BL/6 blastocysts, and the chimeric blastocysts were transferred into the uteri of pseudo-pregnant ICR females. We obtained high-contribution chimeras, and germ line transmission of the recombinant locus (i.e., the new *REST* mutation) was determined by coat color in the next generation of mice. We backcrossed the chimeric mice or heterozygous mutant mice to C57BL/6 for three or four consecutive generations and created a population of mice heterozygous for the *REST* mutation.

### *Southern blot analysis to assess the recombination products*

Genomic DNA was extracted from ES cells using a DNA extraction kit (Qiagen). For Southern blot analysis of the 5′ end of the *REST* locus, genomic DNA was digested with XbaI; the genomic fragments were then separated in an 1% agarose gel and transferred to a nylon membrane. The 5′ probe (884 bp) was amplified using the *REST* 5′F (5′-GATGGTGGTGGTGACTCAGAG-3′) and *REST* 5′R as (5′-CCTCCATTGCCTCTATATCAG-3′) primers. The probe specifically labeled a 16.5 kbp band from the wild-type locus and a 10.8 kbp band from the recombinant locus (Fig.1E and F). Genomic DNA was also digested with SpeI for Southern blot analysis of the 3′ end of the *REST* locus. The 3′ probe (361 bp) was amplified with the *REST* 3′ F (5′-AACATTGAGGGAATAATTGC-3′) and REST 3′R (5′-ATTAGAACTATTCTTCAATC-3′) primers. The probe hybridized to an 8 kbp band from the wild-type locus and a 4.4 kbp band from the recombinant locus (Fig. 1E and F).

### *Genotyping PCR*

The genotype of individual embryos was determined using samples of yolk sac tissue and PCR amplification. Each tissue sample was lysed in 50 μl of Proteinase K solution (60 μg/ml) for 2 h at 55 °C. These crude tissue lysates (1 μl each) were used as template DNA. Amplification was performed using gene-specific primer sets (Table 1) over 30 PCR cycles that included denaturation for 30 s at 94 °C; annealing for 1 min at 65 °C; extension for 1 min at 72 °C. Products were resolved in a 1.5% agarose gel using electrophoresis and visualized using ethidium bromide staining. PCR-based genotyping of *Mek5* mutant embryos was performed as described by Wang et al. (2005).

### *Generation of the PGC-specific REST conditional knock-out embryo*

The TNAP-Cre transgenic mouse line was maintained by mating them with C57BL/6J mice. $REST^{2lox/2lox}$ females (Yamada et al., 2010) were mated with TNAP-Cre transgenic males. To generate embryos homozygous for the PGC-specific conditional

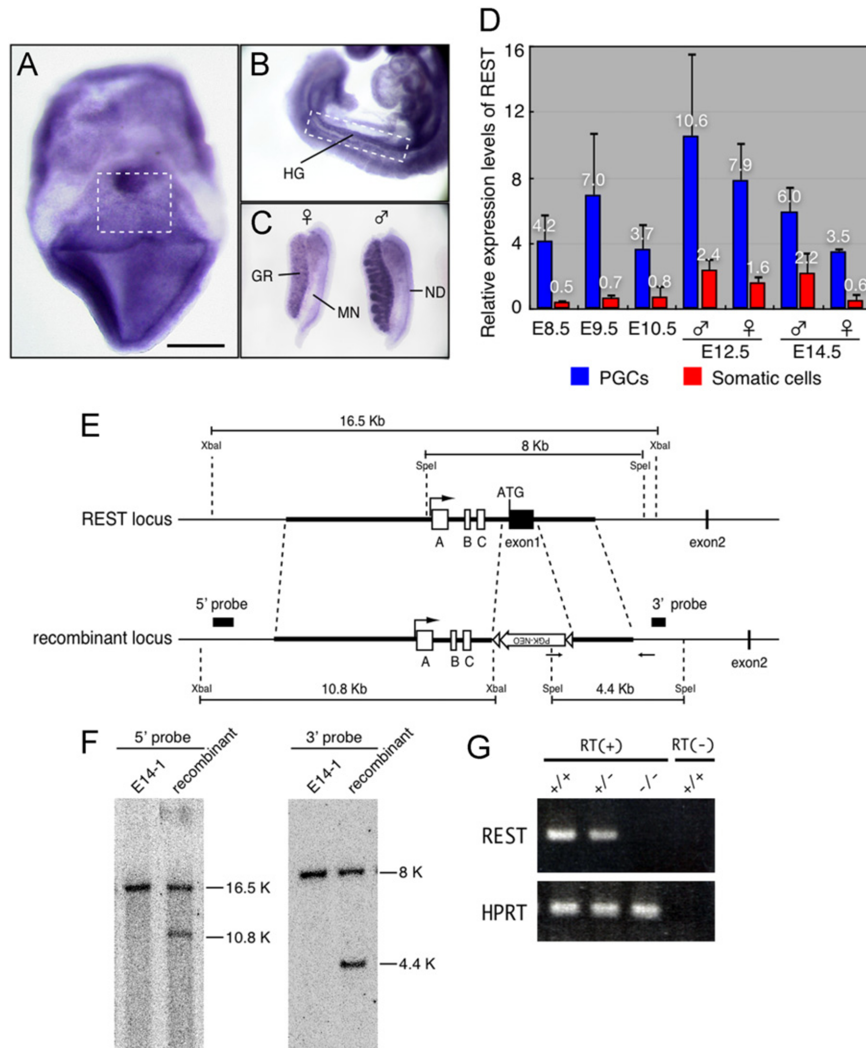


**Fig. 1.** Expression and targeted disruption of the *REST* gene. (A–C) In situ hybridization analysis of *REST* expression in germ cells. *REST* transcripts were preferentially detected in the nascent PGCs at E7.25, which corresponds to the early allantoic bud stage (A), in migrating PGCs at E9.5 (B), in gonadal PGCs at E12.5 (C). HG: hind-gut, GR: genital ridge, MN: mesonephros, ND: nephric duct. Scale bars: 2 mm (A), 0.6 mm (B), 1 mm (C). (D) Quantitative RT-PCR was used to assess expression of *REST* in purified GFP-positive PGCs and in the adjacent GFP-negative somatic cells from mil1-GFP transgenic embryos at different embryonic stages. The results of three independent experiments are presented. (E) Schematic representation of the wild-type and the mutated *REST* alleles. The genomic DNA regions used as the 5′ and 3′ homology arms in the *REST* targeting vector are indicated by thick lines. Closed boxes indicated the coding exons of *REST*. Coding exon 1 was replaced by the PGK-Neo cassettes (open arrow). The 5′ and 3′ probes (closed boxes) used for Southern blot analyses are shown above the recombinant *REST* allele. The pair of arrows indicates the primer set for detecting recombinant ES clones. (F) Southern blots of the parent ES cells and of a recombinant clone. Genomic DNA (10 μg) was digested with XbaI and SpeI. The 5′ external probe hybridized to 16.5-kb (wild-type) and 10.8-kb (targeted) XbaI fragments. The 3′ probe hybridized to 8-kb (wild-type) and 4.4-kb (targeted) SpeI fragments. (G) RT-PCR analysis of *REST* expression in cDNA from whole E9.5 embryos of three genotypes—(+/+) wild type, (+/−) heterozygote, (−/−) homozygote mutant. *HPRT* expression was analyzed and used a an internal control. RT: reverse transcriptase.

**Table 1**
Gene-specific primer sets for Genotyping PCR and Real-time PCR and ChIP.

| Primer sets | Forward primer (5′–3′) | Reverse primer (5′–3′) |
|---|---|---|
| TNAP-Cre | GGCTCTCCTCAAGCGTATTCAAC | CAAACGGACAGAAGCATTTTCCAG |
| REST-2lox | CCCTTATGGGTGCAAGTGTT | CCGGCTCTACTTCAGACCAG |
| REST | CCCAGGCCTATCTAGTGCTAGGA | GGCAGCTGAGGCTTCAAATACGG |
| Neo-gt1 | CGTAAAGCACGAGGAAGCGGTCAG | |
| Dad1 | CTCGTTCCTCTCTGGCTTCAT | GAAGTCAGCAAAGGCTCGCT |
| Raf1 | AATACTATCCGGGTTTTCTTGCC | GCGTGCTTTCTTACCTTTGTGT |
| Aven | GAGGAGAAAGAATGGGATGGTG | AGCAACATTAAGCCTGACTGC |
| Traf1 | AGGGTGGTGGAATTACAGCAA | GCAGTGTAGAAAGCTGGAGAG |
| Mek5 | GGGCCATCTCAACACACCAG | TGTCTCGATACCGTATGTCTTGT |
| Mek5-RE1 site | CACCAVGAAGATAGGTGCATT | GGAGGTTGAAGGAGGAGGATAG |
| Calb1 | GCTCCGCGCACTCTCAAA | GAGATGACTGCAGGTGGGATTC |
| Esrrb | CCAGAGAGAGTGTTTATGCAGGAC | CCCTTACAGTTATGAGCCTCACTC |

*REST*-mutation, the female progeny of *TNAP-Cre(+)/REST*$^{+/2lox}$ were mated with *REST*$^{2lox/2lox}$ males.

*In situ hybridization*

Embryos and embryonic gonads were obtained by mating ICR mice with B6D2F1 mice. Tissues were fixed in 4% paraformaldehyde. Whole-mount in situ hybridization was performed using an automated machine, InsituPro (INTAVIS Bioanalytical Instruments AG, Bergisch Gladbach, Germany), according to the manufacturer's instruction (Roche); anti-sense and sense probes were synthesized using a 1135 bp *REST* cDNA.

*Alkaline phosphatase (AP) staining*

Embryos between E8.0 and E9.75 were dissected from the decidua, Reichert's membrane and yolk sac. Embryos were fixed in 4% paraformaldehyde in PBS for 2 h at 4 °C. Fixed embryos were washed three times in PBS and then treated with 70% ethanol for at least 1 h at 4 °C. Embryos were then washed three times with distilled water and then stained with a solution of 0.5 mg/ml of Naphtali AS-MX, 1 mg/ml of Fast Red TR (Sigma) (Lawson et al., 1999) for 10 min at room temperature. The embryos were then rinsed in PBS and cleared in 75% glycerol. AP-positive PGCs was manually counted under a stereomicroscope. Cultured PGCs were detected by staining for AP activity as described previously (Matsui et al., 1991).

*RNA preparation and cDNA synthesis*

Total RNA was purified from whole embryos at E9.5 or from isolated PGCs and surrounding somatic cells using an RNeasy Mini kit plus or RNeasy Micro kit (Qiagen). Total RNA was then used for cDNA synthesis with random primers (Promega).

*RT-PCR*

Synthesized cDNAs were used as RT-PCR templates. Amplification was performed over 30 cycles that included denaturation for 30 s at 94 °C; annealing for 30 s at 60 °C; extension for 45 min at 72 °C. Products were resolved in a 1.5% agarose gel via electrophoresis and visualized by ethidium bromide staining. The following PCR primer pairs were used for amplification of *REST*:

REST-F(ex1): 5′-AACTTCTGCAAGCACAATGG-3′
REST-R(ex3): 5′-CCGAGAAGTCATCACCAG-3′

Control PCR-amplification was performed using primers for *Hypoxanthine-guanine phosphoribosyltransferase* (*HPRT*):

HPRT-F: 5′-GGATTTGAAATTCCAGACAAG-3′
HPRT-R: 5′-GCATTTAAAAGGAACTGTTGAC-3′.

*Real-time PCR*

Real-time PCR was performed using gene-specific primer sets (Table 1) along with an ABI Prism 7000 system (Applied Biosystems) and Power SYBR Green PCR Master Mix, according to the manufacturer's instructions (Applied Biosystems). Quantitative expression analysis of *REST* was performed with Taqman probe for *REST* (Mm00803z68_m1) and Express qPCR super mix (Invitrogen).

*Assay for PGC proliferation*

PGC proliferation was assessed using the bromodeoxyuridine (BrdU) pulse-labeling method described by Seki et al. (2007) with minor modification. BrdU (Roche, 10 mg/kg body weight) was injected into the peritoneum of pregnant females that had been staged appropriately. Labeled embryos were isolated six hours after the dams were injected, and tissue fragments containing PGCs were dissected out and used to prepare frozen tissue sections. Detection of BrdU was performed as describe by Marusich et al. (1994) using anti-BrdU antibody (1:50; Becton Dickinson); PGCs were detected using anti-Stella/PGC7 antibody (1:10,000; Sato et al., 2002). Sections were counterstained with DAPI.

*Immunohistochemistry*

Immunohistochemical analyses of frozen tissue sections (10 μm) were performed as described previously (Yamaguchi et al., 2005). The antibodies used were: anti-Oct3/4 (1:100; Santa Cruz), anti-active Caspase3 (1:500; BD pharmingen), anti-BrdU (1:50; Upstate), anti-Stella/PGC7 (1:10,000; Sato et al., 2002), anti-Vasa (1:100; Toyooka et al., 2000). The appropriate species-specific fluorophore-labeled secondary antibodies (Molecular Probes) were applied at a 1:200 dilution. Nuclei were stained with DAPI (5 μg/ml). Staining was examined using a confocal laser scan microscope (Leica).

*Chromatin immunoprecipitation*

Chromatin immunoprecipitation (ChIP) was performed with samples of $1 \times 10^6$ dissociated genital ridge cells from E12.5 embryos or ES cells using the method described in the on-line protocol provided by NIPPON GENE CO., LTD. Briefly, REST antibodies (Millipore, 07-579) or rabbit IgG control antibodies (Cell Lab, 731642) were bound to protein G-coupled beads, Dynabeads (Invitrogen) at 4 °C for more than 4 h. The dissociated ES cells or genital ridge cells were subjected to protein–DNA crosslinking with 1% formaldehyde in 10% FBS/ Dulbecco's modified Eagle's medium (GIBCO) for 10 min at room temperature. Genomic DNA in chromatin complexes was sheared to 200–1000 bp with sonication (Branson Sonifier 250). Extraneous cellular debris was removed by centrifugation at 20,000 × *g* for 10 min at 4 °C, and the supernatant, which contained the chromatin, was incubated with antibodies-bound-beads overnight at 4 °C. The chromatin-bead complexes were washed; the chromatin was eluted and the protein–DNA crosslinking was reversed by incubation at 65 °C. DNA was purified from the eluates and used as template for PCR amplification with primers spanning REST binding site in the vicinity of *Mek5* (Table 1) using SYBR PCR Mastermix (Applied Biosystems). We also used primers spanning REST binding site in the vicinity of *Calb1*, a known REST target gene in ES cells, as a positive control, and primers spanning a transcription start site of *Esrrb* as a negative control (Table 1). *Esrrb* is expressed in ES cells but REST binding sites do not exist in the vicinity of this gene. The efficiencies of chromatin immunoprecipitation were quantified relative to a standard curve prepared using input chromatin. Rabbit IgG control antibodies served as negative controls.

*Dissection of embryos and dissociated PGCs culture*

Embryos were dissected away from deciduas, and fragments of hind-gut tissue were dissociated into single cells by gentle pipetting in 0.025% trypsin/0.75 mM EDTA. Dissociated hind-gut cells were cultured on a feeder layer of Sl/Sl4-m220 cells.

*Histological analysis*

For histological analysis, samples (testes or ovaries) were fixed in Bouin's solution and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin.

*Statistical analysis*

Statistical differences were assessed using the student's t-test. p values $<0.05$ or $<0.01$ were considered statistically significant. Error bars in graphs denote standard deviation of the mean (SEM).

## Results

### *Isolation of REST/NRSF as a candidate regulatory gene of PGC development*

To identify genes that are specifically expressed in primordial germ cells (PGCs) and have critical roles in the development of PGCs, we used PGC precursors and nascent PGCs in differential screens (Okamura et al., in press). In brief, GFP-positive PGCs and their precursor cells were isolated from mid-streak and late-streak stage embryos from ICR × mil1-GFP matings. A cDNA library from mixture of five independent nascent PGC cDNA was obtained and was identified by Southern blot analysis using the marker genes as probes such as Blimp1 and Stella. The library was differentially screened with the nascent PGC cDNA (also used for a library) and of their precursor cDNA probes. Among about 15,000 clones screened, 100 independent candidate clones were isolated. We identified *REST/NRSF* in one such screen; *REST* was preferentially expressed in PGCs throughout PGC development, although faint expression of *REST* was also evident in surrounding somatic cells (Fig. 1A–D).

### *Reduction of PGC number in the REST deficient embryos*

To examine the functions of REST in PGCs, we used homologous recombination in embryonic stem (ES) cells to generate mice with a knockout mutation at the *REST* locus. *REST* comprises three coding exons, and we designed a targeting vector to disrupt exon 1, which includes a translational initiation codon (Fig. 1E) and confirmed its correct recombination by Southern blot (Fig. 1F). Based on RT-PCR analysis, *REST* expression was not evident in *REST*-mutant embryos (Fig. 1G). As previously reported, *REST* deficient embryos were embryonic lethal by E11.5, and showed morphological abnormalities including forebrain malformation and widening of the mesencephalic flexure (Chen et al., 1998). We confirmed similar morphological abnormalities in the homozygous mutant embryos at E10.25 (Fig. S1).

We assessed whether the *REST* mutation affected the number of PGCs in embryos by comparing the number of PGCs in E9.5 embryos homozygous for the mutation with that in embryos that were heterozygous; PGCs in all embryos were identified by staining for alkaline phosphatase activity (Ginsburg et al., 1990). The number of migrating PGCs in homozygous mutant embryos was apparently smaller than that in heterozygous mutant embryos (Fig. 2A). A linear regression analysis indicated that the increase in the number of PGCs over the course of development was smaller in homozygous mutant embryos than in wild-type or heterozygous mutant embryos (Fig. 2B). For embryos of each of the three genotypes (homozygous mutant, heterozygous, or wild type), we counted the number of PGCs at the 5–10 somite stage (E8.5, nascent PGCs) and at the 20–25 somite stage (E9.5, migrating PGCs) (Fig. 2C). The number of nascent PGCs (5–10 somite stage) in homozygous mutant embryos was not significantly different from that in heterozygous embryos or wild-type embryos; in contrast, the number of migrating PGCs (20–25 somite stage) was significantly smaller in homozygous mutant embryos than in heterozygous embryos and in wild-type embryos (Fig. 2C).

### *Deficiency of REST functions causes enhancement of apoptotic cell death of PGCs*

We examined whether an increase in apoptosis, a decrease in proliferation, or both contributed to the reduction in the number of migrating PGCs in REST mutant embryos. First, we examined apoptosis among PGCs in mutant and wild-type embryos at E9.25 by monitoring active-Caspase3 (Fig. 3A–H). The ratio of apoptotic PGCs to viable PGCs was much higher in homozygous mutant

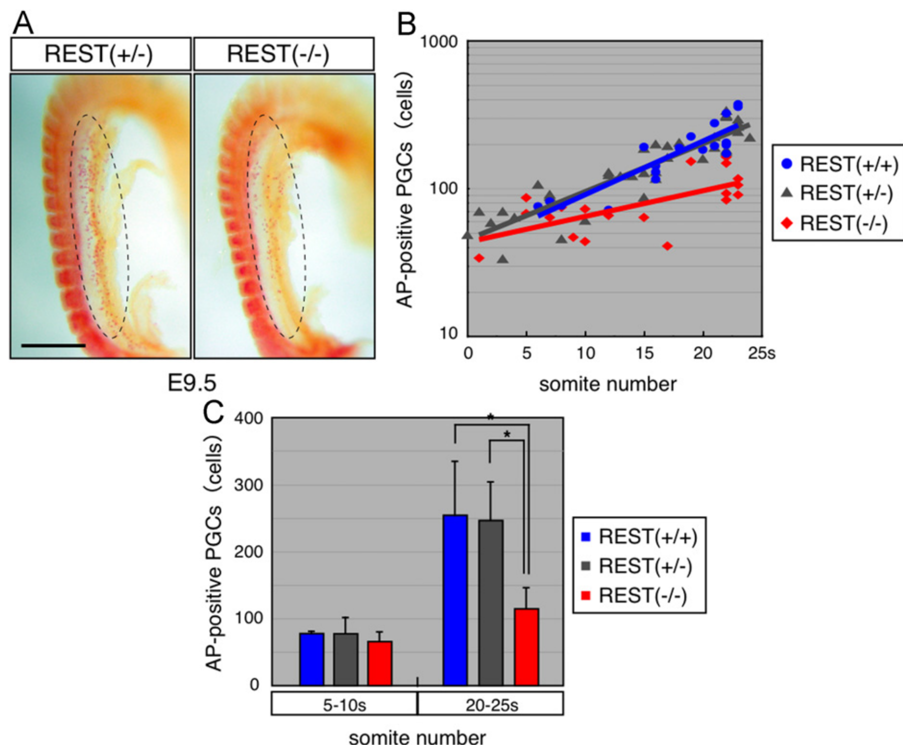


**Fig. 2.** The number of migrating PGCs was reduced in *REST*-mutant embryos. (A) Alkaline phosphatase (AP) staining of *REST* heterozygous and *REST* homozygous embryos at the 22 somite stage. Scale bar: 0.5 mm. (B) Linear regression analysis of PGC number; PGC number was determined via AP staining. (C) The number of AP-positive PGCs in the embryos between E8.0 and E9.5. PGCs in the hindgut were counted, and the average number of PGCs from mutant and wild-type embryos at the 5–10 somite stage (wild: $n=3$; hetero: $n=5$; homo: $n=7$) or from those at the 20–25 somite stages (wild: $n=9$; hetero: $n=15$; homo: $n=7$) is shown; $^{*}P<0.05$.

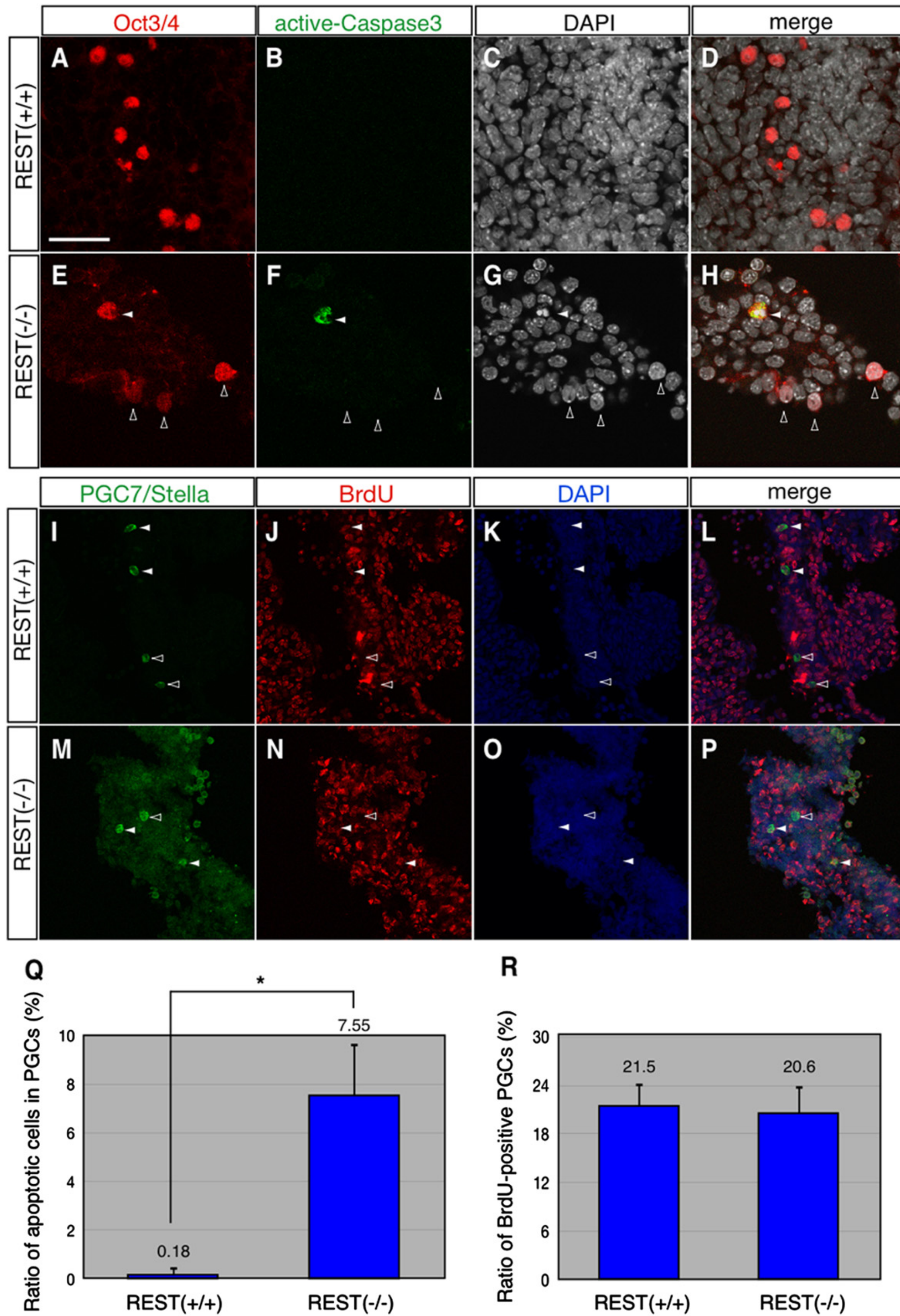


**Fig. 3.** In migrating PGCs, loss of REST function resulted in induction of apoptosis, but had no influence on proliferation. (A–H) Apoptotic cells in frozen sections of hindgut tissue from E9.25 embryos were detected by immunostaining for active caspase 3; wild-type (A–D) and *REST* homozygous mutant (E–H) embryos are shown. PGCs were identified by the expression of Oct3/4. Closed and open arrowheads indicated apoptotic and non-apoptotic PGCs, respectively. (I–P) BrdU-positive PGCs were detected using anti-BrdU and anti-PGC7/Stella antibodies in frozen sections of hindgut tissues from wild-type (I–L) and from *REST* homozygous mutant (M–P) E9.25 embryos. Closed and open arrowheads indicated BrdU-positive and -negative PGCs, respectively. DNA was labeled by DAPI counterstaining. Scale bar: 25 μm (A–H), 50 μm (I–P). (Q and R) The ratios of apoptotic to non-apoptotic PGCs (Q) and of BrdU-labeled and unlabeled PGCs (R) in hindguts of E9.25 embryos are shown. The data from four embryos of each genotype are shown; the percentages above the error bars represent average ratios. $^{*}P<0.05$.

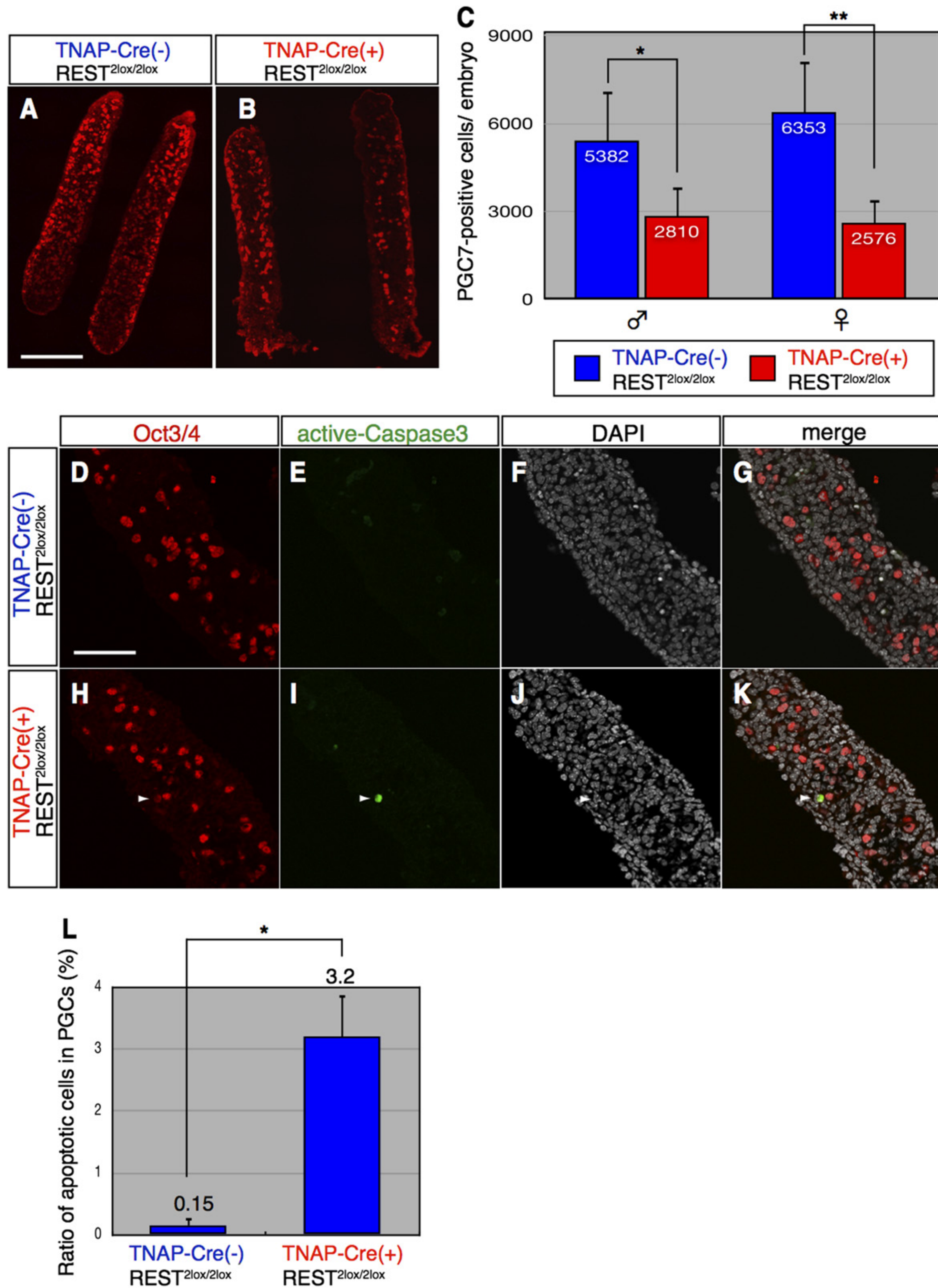


**Fig. 4.** Induction of apoptotic cell death due to PGC-specific loss of REST function. PGC number in E12.5 genital ridges from wild-type embryos (TNAP-Cre(−)) (A) or *REST*-mutant littermates (TNAP-Cre(+)) (B) was estimated by detecting Stella-positive PGCs by immunostaining. (C) The number of Stella-positive PGCs in E12.5 genital ridge per embryo is shown. PGCs in all serial frozen sections of genital ridges were counted, and the average number of PGCs in three embryos of each genotype was determined. (D–K) Apoptotic cells were detected by immunostaining for active caspase 3 in frozen sections of genital ridges from wild-type (D–G) or PGC-specific *REST*-conditional mutant (H–K) embryos at E11.5. Arrowheads indicate apoptotic PGCs identified by Oct3/4 expression. (L) The ratios of apoptotic to non-apoptotic cells in PGCs at E11.5. For each embryo, 500–1000 PGCs from both genital ridges were counted; eight tissues sections from each embryo were assessed; ratios of active caspase 3-positive to active caspase 3-negative PGCs were determined for three embryos of each genotype. The percentages above the error bars represent average ratios. Scale bar: 0.3 mm (A and B), 50 μm (D–K). $^{*}P < 0.05$.

embryos than in wild-type embryos (Fig. 3Q). Next, we examined PGC proliferation in mutant and wild-type embryos using BrdU labeling (Fig. 3I–P). The ratio of BrduU-labeled PGCs to unlabeled PGCs did not differ significantly between homozygous mutant and wild-type embryos at E9.25 (Fig. 3R). Taken together, the results indicated that reduced number of PGCs in REST homozygous mutant embryos was mainly caused by apoptotic cell death, but not by defective proliferation of PGCs.

### *Cell-autonomous function of REST promotes PGC survival*

The low-level of *REST* expression in the somatic cell surrounding PGCs (Fig. 1A–D) indicated that REST may promote PGC survival in non-cell-autonomous manner. To examine this possibility, we generated embryos that carried a PGC-specific conditional *REST*-KO mutation; female mice homozygous for a floxed *REST* allele (Yamada et al., 2010) were mated with males that carried a transgene encoding the cre recombinase driven by the promoter of the *TNAP* (*tissue non-specific alkaline phosphatase*) gene. This *TNAP-Cre* transgene is known to be specifically expressed in PGCs after at E9.0 (Lomeli et al., 2000). In the *REST*$^{2lox}$ allele, the floxed last exon of *REST* was followed by IRES-GFP, which enabled us to monitor the transcription of the modified allele, i.e., GFP expression should not be detected if the Cre-mediated recombination occurs (Yamada et al., 2010). By detecting the GFP expression, we confirmed that *REST* was deleted in more than 70% of PGCs in TNAP-Cre(+)/*REST*$^{2lox}$-heterozygous mutant embryos at E12.5, while GFP-negative cells were rarely observed among somatic cells in the same embryos (Fig. S2). In addition, 50–70% of the progeny obtained form TNAP-Cre(+)/*REST*$^{2lox/2lox}$ mice mated with wild-type C57Bl/6 had heterozygous *REST* deletion (Table S1). Those observations indicate that *REST* was efficiently and almost exclusively deleted in PGCs in TNAP-Cre(+)/*REST*$^{2lox}$-heterozygous and TNAP-Cre(+)/REST$^{2lox/2lox}$ homozygote mutant embryos.

At stage E12.5, PGC number in genital ridges of conditional homozygous mutant embryos and of *REST*$^{2lox/2lox}$ (control) litter mates was estimated, and PGC number was significantly smaller in the conditional mutant embryos than in control embryos (Fig. 4A–C). These findings indicated that loss of cell-autonomous functions of REST in PGCs caused a reduction in PGC number after E9.0. We next examined whether apoptotic cell death was also involved in this reduction in PGC number; the ratio of active caspase3-positive (apoptotic) PGCs to active Caspase3-negative (viable) PGCs was significantly higher in conditional *REST*-mutant embryos at E11.5 than in wild-type embryos (Fig. 4D–L). Taken together, these findings indicated that when individual PGCs lost *REST* function, they underwent apoptosis.

Additionally, we tested whether *REST* function in PGCs was cell autonomous by co-culturing hind-gut cells isolated from Oct3/4-GFP transgenic embryos (Yoshimizu et al., 1999) with hind-guts cells from *REST*-mutant embryos or control embryos (Fig. 5A). PGCs from *REST*-deficient and from their normal littermates were distinguished from PGCs from Oct3/4-GFP embryos because they were AP-positive but GFP-negative cells (Fig. 5B and C). In the presence of normal somatic cells from the hind-guts of Oct3/4-GFP embryos, the percentage of homozygous *REST* mutant PGCs ($n=4$) among all PGCs declined significantly between 12 and 60 h in culture (Fig. 5D); in contrast, the percentage of heterozygous mutant PGCs ($n=6$) and of wild-type PGCs ($n=6$) did not change significantly between 12 and 60 h of culture (Fig. 5D). Because the expression of REST in somatic cells

**Table 2**
Candidates of REST regulated apoptotic genes.

| | | | | |
|---|---|---|---|---|
| Adm | Casp9 | Dyrk1b | Mapk8ip1 | Rtn4r |
| Alg2 | Cdkn1a | Efnb3 | Mek5 | Sfrp2 |
| Apaf1 | Cradd | Elmo1 | Morc1 | Smad2 |
| Api5 | Ctnnbl1 | Enc1 | Msx2 | Stat5b |
| Aven | Cttn | Gas6 | Nr4a1 | Stk17b |
| Bcl2 | Dad1 | Gast | Pdcd6ip | Tada3l |
| Bik | Dapk2 | Gspt1 | Pla2g6 | Tnfrsf8 |
| Bnip2 | Diablo | Ihpk2 | Pmaip1 | Tnfsf10 |
| Bnip3 | Dnase1l3 | Madd | Prkce | Traf1 |
| Casp8ap2 | Dpf2 | Map3k7 | Raf1 | Trp73 |
| | | | | Zc3h12a |

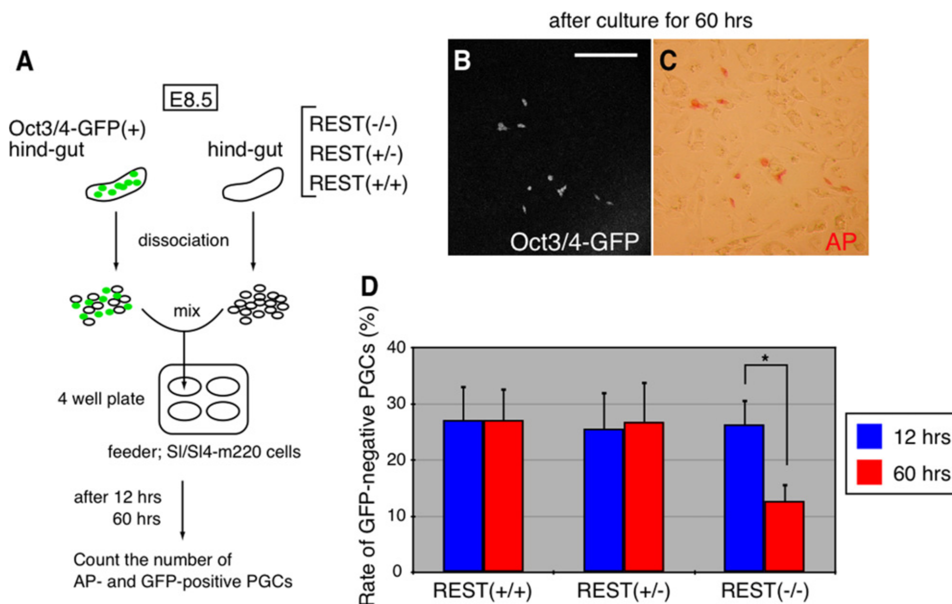


**Fig. 5.** Co-culture experiments to assess cell-autonomous function of REST in PGCs. (A) Scheme of the co-culturing setup used to examine the cell-autonomous function of REST in PGC. The dissociated *REST*-homozygous, *REST*-heterozygous or wild-type hindgut cells were cultured with hind-gut cells from Oct3/4-GFP transgenic embryos; all hindgut cell were taken from E8.5 embryos. (B and C) After culturing the cells for 60 h, the cultured PGCs derived from Oct3/4-GFP transgenic embryos were detected as Oct3/4-GFP-positive (B), and AP-positive cells (C). Scale bar: 160 μm (B and C). (D) The percentage of Oct3/4-GFP-negative PGCs, which should be derived from *REST* mutant embryos, among all AP-positive cells, which should include all PGCs, after culturing for 12- or 60-h are shown. *$P<0.05$.

surrounding PGCs was low in wild type embryos (Fig. 1D), it is unlikely that the REST-deficient somatic cells have negative effect on PGC to increase in number in the culture. These findings further supported that *REST* functioned in a cell-autonomous manner to maintain PGC number.

*Identification of candidate anti-apoptotic genes regulated by REST*

Previous reports indicated that REST directly associates with regulatory elements of target genes to regulate their expression (Schoenherr et al., 1996); therefore, we sought to identify possible

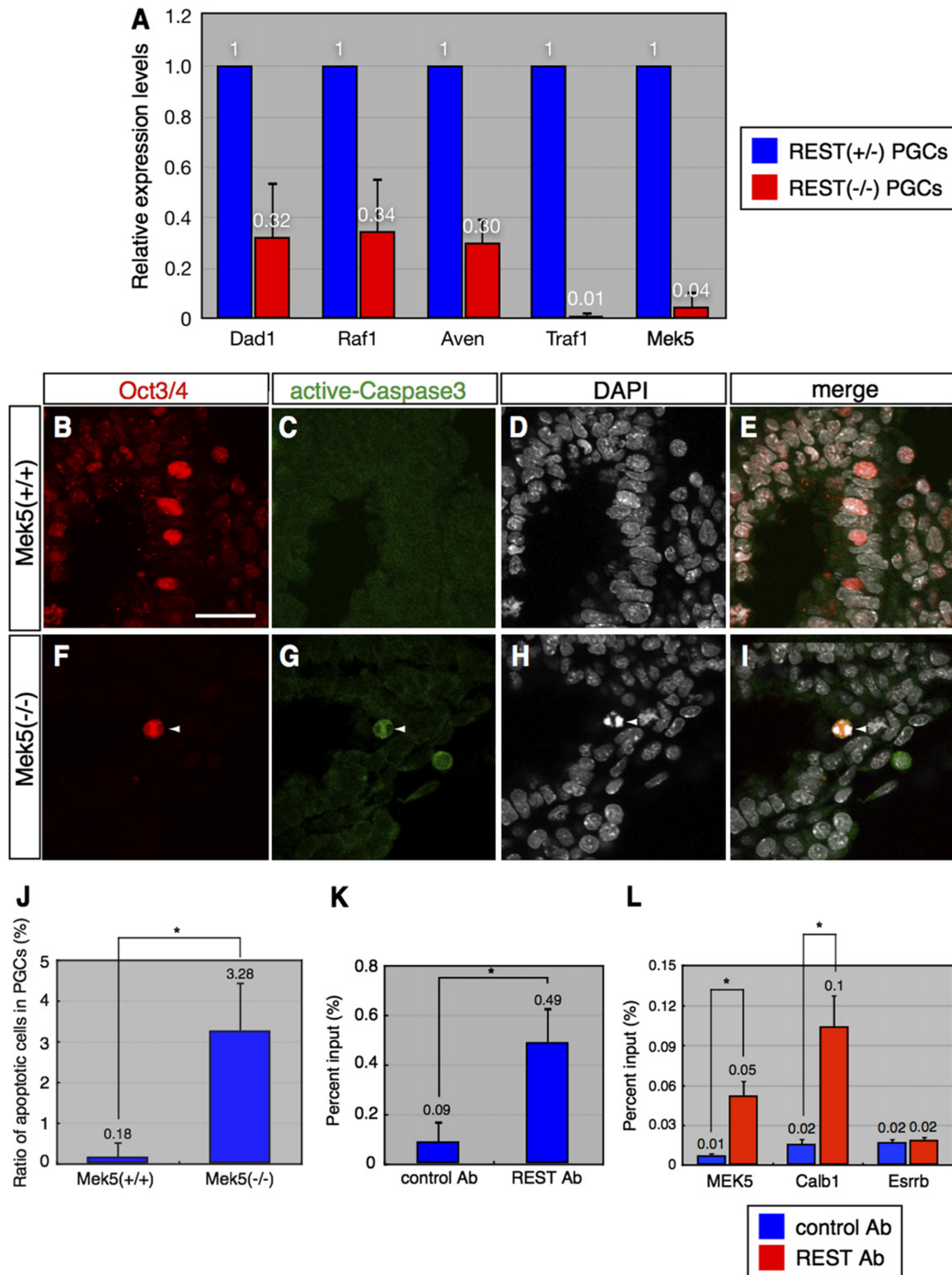


**Fig. 6.** Mek5 functions downstream of REST to promote PGC survival. (A) The expression of anti-apoptotic REST-target genes was examined in PGCs from *REST*-heterozygous and *REST*-homozygous mutant embryos at E9.25. The results of three independent experiments are presented. (B–I) Apoptotic cells were detected by immunostaining for active caspase 3 in frozen sections of hindgut from wild-type (B–E) or from *Mek5* homozygous mutant (F–I) embryos at E9.25. PGCs were identified by the expression of Oct3/4. Arrowheads indicated apoptotic PGCs. DNA was labeled by DAPI counterstaining (D, E, H and I). Scale bar: 25 μm. (J) The ratios of apoptotic to non-apoptotic PGCs are shown. Data from four embryos of each genotype are shown. $^{*}P<0.05$. (K) ChIP analysis for REST binding at the NRSE/RE1 site within 3′ flanking region located 16.5 kbp downstream of *Mek5* (Johnson et al., 2008) in genital ridge cells of E12.5 embryos. (L) ChIP analysis for REST binding site in at the RE1 site of *Mek5* and the vicinity of *Calb1* and for transcription start site of *Esrrb* in ES cells as positive and negative controls, respectively. The results for three independent experiments are shown; percentages above the error bars represents average ratios. $^{*}P<0.05$.

direct targets of REST in PGCs that control in the survival of PGCs. Johnson et al. (2008) identified REST binding in 2500 genes distributed throughout the genome of ES cells; we focused on 51 apoptosis-associated genes in this collection of 2500 genes (Table 2). To assess the effects of REST on these 51 genes, we used quantitative PCR to measure the expression of each gene in PGCs that were homozygous or heterozygous for the *REST* mutation. Specifically, we isolated PGCs as GFP-positive cells from *REST*-heterozygous or *REST*-homozygous embryos that carried the mil1-GFP transgene (Tanaka et al., 2004). Among the 51 apoptosis-associated candidate genes, five anti-apoptotic genes—*Dad1* (Nishii et al., 1999), *Raf1* (Mikula et al., 2001), *Aven* (Chau et al., 2000), *Traf1* (Arron et al., 2002), and *Mek5* (Wang et al., 2005)—were expressed at significantly lower levels in *REST*-homozygous PGCs than in *REST*-heterozygous PGCs (Fig. 6A), though REST often functions as a repressor of transcription. These results indicated that PGCs survival was likely maintained by the functions of those REST-target genes.

### *Mek5 functions downstream of REST to promote PGC survival*

Among the five candidate REST targets, we focused on *Mek5* in further analyses. We used alkaline phosphatase staining to assess PGC number in *Mek5* mutant and wild-type embryos (Wang et al., 2005) at E9.25; we found that there were significantly fewer PGCs in embryos homozygous for the *Mek5* mutation than in heterozygous or wild-type embryos (Fig. 7). Next we monitor active caspase3 in mutant and wild-type E9.25 embryos to assess the effect of the *Mek5* mutation on apoptosis in PGCs; we found that the ratio of apoptotic to viable PGCs was apparently higher in homozygous *Mek5* mutant embryos than that in normal (heterozygous or wild-type) littermates (Fig. 6B–J). Based on a genome-wide ChIP-ChIP analysis, REST binds to a putative regulatory site downstream of the last *Mek5* exon in ES cells (Johnson et al., 2008). We confirmed this result in a standard ChIP analysis that involved embryonic gonad cells at E12.5 (Fig. 6K and L); our findings suggested that REST regulated the expression of *Mek5*.

### *Viable REST-deficient germ cells undergo normal gametogenesis.*

To examine the consequences of a REST deficiency in PGCs, we examined vasa-expressing germ cells (Toyooka et al., 2000) in embryonic testes and ovaries of PGC-specific *REST*-conditional mutant mice at E18.5; germ cell number in the mutant embryos was not significantly different from that in normal littermates (Fig. 8A–E). Male and female mice (8-weeks old, mutant and wild type) were used to investigated hematoxylin and eosin stained testes and ovaries. Average testes size and average ovary size did not differ significantly between the *REST*-conditional mutant and wild-type mice (data not shown). Additionally the gonads of mutant and wild-type mice were similar in histological appearance, and morphologically normal sperm and oocytes were maturing in the *REST*-conditional mutant mice (Fig. 8F–I). We assessed the fertility of the *REST*-conditional mutant mice by mating them with B6 mice; the fertility of the mutant male and female mice was indistinguishable from that of wild-type mice (Table S1). These observations indicated that spermatogenesis and oogenesis proceed normally in the *REST*-conditional mutant mice.

## Discussion

REST is a well-known regulator of transcription, and a number of previous studies have demonstrated that REST plays critical roles on neural cell development by repression of neural cell-specific genes in non-neural cells (Chong et al., 1995; Schoenherr and Anderson, 1995; Chen et al., 1998; Ooi and Wood, 2007). REST interacts with various epigenetic factors, and it represses gene expression in many cases (Ballas et al., 2005; Ooi and Wood, 2007), but REST is occasionally involved in activation of gene expression (Bessis et al., 1997; Majumder (2006); Abramovitz et al., 2008; Yamasaki et al., 2011). Here, we found that REST promoted the survival of PGCs in mouse embryos (Figs. 2–5) and that this novel function of REST was mediated, at least in part, by induction of *Mek5* (Figs. 6 and 7). Of 2500 previously identified

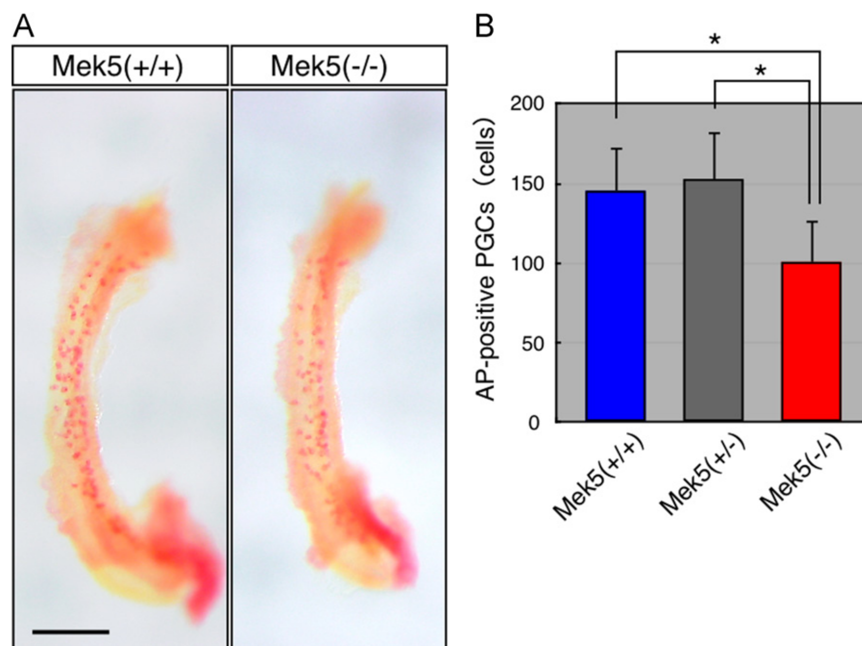


**Fig. 7.** The number of migrating PGCs was reduced in *Mek5*-mutant embryos. (A) Alkaline phosphatase (AP) staining of wild-type and *Mek5*-homozygous embryos at the 15 somite stage. Scale bar: 300 μm. (B) The number of AP-positive PGCs in E9.25 embryos. PGCs in hindgut were counted, and the average number of PGC in embryos of each genotype is shown. Wild-type ($n=5$), *Mek5*-heterozygous ($n=9$), homozygous ($n=5$) embryos. $^{*}P<0.05$.

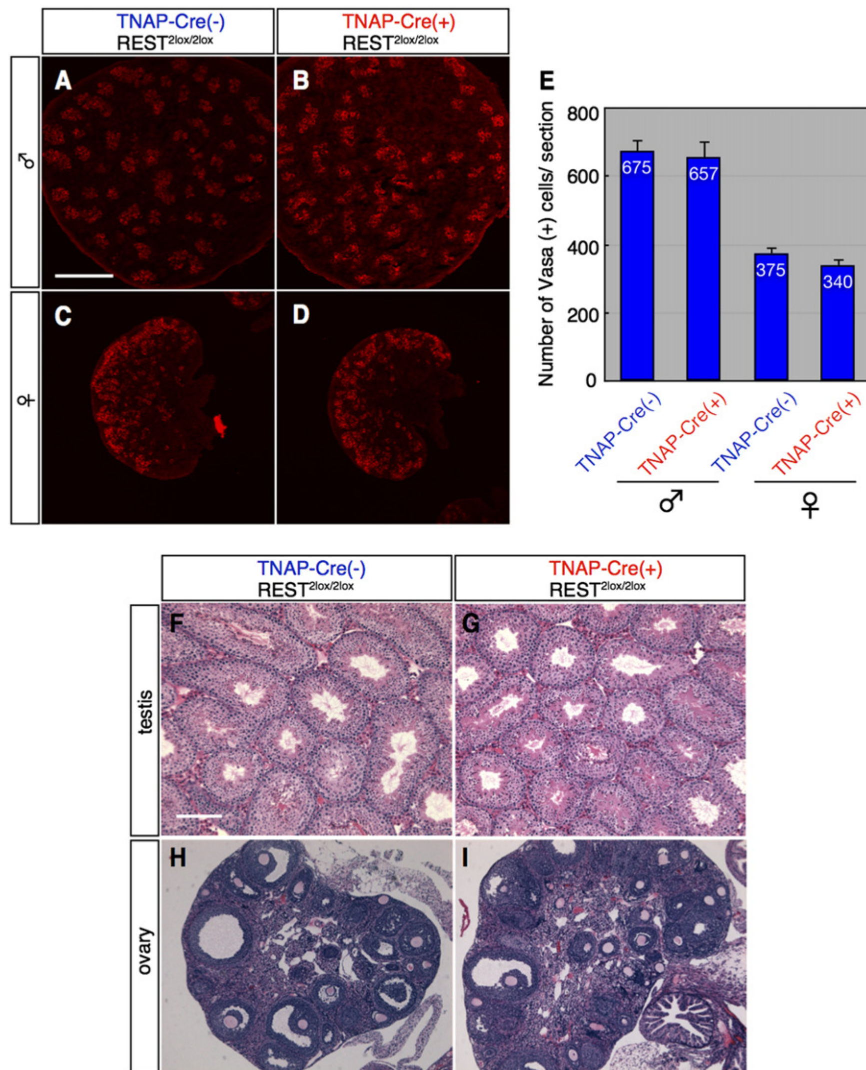


**Fig. 8.** Normal gametogenesis occurred in *REST*-deficient germ cells. (A–D) Gonads of both sex from wild-type (A and C) or PGC-specific *REST* conditional mutants embryos at E18 (B and D) were immunostained for Vasa protein. (E) The average number of Vasa-positive PGCs in E18 gonads per section is shown for each class of embryos. PGCs in the 5 serial frozen sections from the middle part of individual gonads were detected using immunostaining and counted; the number of PGCs in three embryos of each genotype was determined. (F–I) Hematoxylin and eosin-stained sections from wild-type (F and H) and PGC-specific *REST* conditional mutant (G and I) mice sacrificed 2 month after birth. Male gonads (A, B, F and G) and female gonads (C, D, H and I). Scale bar: 200 μm (A–D) and 100 μm (F–I).

putative REST-target genes (Johnson et al., 2008), five anti-apoptotic genes, including *Mek5*, were downregulated in the REST-deficient PGCs (Fig. 6A). We confirmed that REST was indeed associated with the conserved REST binding sequence in region that flanked the *Mek5* gene (Fig. 6K). We then found evidence indicating that *Mek5*-deficient PGCs also tended to undergo apoptosis (Figs. 6–7). The results suggested that REST directly activated the expression of *Mek5* in PGCs, which in turn supported survival of PGCs (Fig. 9). However, it is also likely that REST indirectly causes increased expression of *Mek5* via repression of genes that negatively affect *Mek5* expression.

Survival of PGCs is supported by trophic factors such as Steel factor (Dolci et al., 1991; Matsui et al., 1991; Pesce et al., 1993), and it is likely that intracellular signaling pathways activated by the trophic factors induce the expression of REST. However, the mechanisms leading to REST induction are poorly understood, though it is known that the expression of *REST* is induced by various extracellular stimuli in different cell types (Willert et al., 2002; Nishihara et al., 2003). In ES cells, Oct3/4, as well as Nanog, activates the expression of *REST* (Boyer et al., 2005; Loh et al., 2006; Kim et al., 2008), and embryos with a PGC-specific conditional mutation in *Oct3/4* or *Nanog* showed increased PGC apoptosis (Kehler et al., 2004; Yamaguchi et al., 2009). Therefore, we supposed that Oct3/4 or Nanog also induced the expression of *REST* to enhance survival in PGCs; consequently, we examined the expression of *REST* in *Oct3/4* or *Nanog*-deficient PGCs (data not shown) (Kehler et al., 2004; Yamaguchi et al., 2009), but our evidence did not indicate that *REST* expression was downregulated in *Oct3/4*-deficient or *Nanog*-deficient PGCs.

The MAP kinase cascade is activated by signals from trophic factors, and this cascade enhances cell survival (Wada and Penninger, 2004). MEK5 is an activator of two MAP kinases, ERK5 and MKK4, and it inhibits caspase 3 activity to prevent apoptosis (Drew et al., 2012). Our finding indicated that the expression of *Mek5* was induced by REST and was involved in PGC survival (Figs. 6 and 7), and these finding further indicated that regulation of the expression of components of the MAP kinase cascade, in addition to the activation of these components by trophic factors, was critical for proper survival of PGCs in embryos.

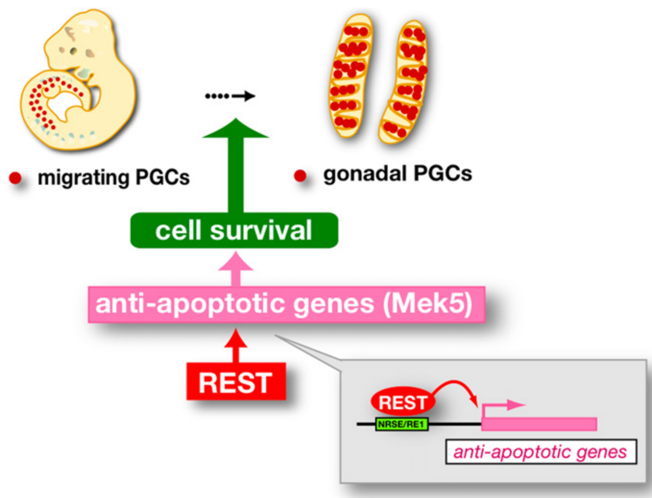


**Fig. 9.** REST controls survival of PGCs, and this control is mediated by the activation of anti-apoptotic genes in mouse embryos. *REST* was preferentially expressed in PGCs from the stage of their formation until they colonized the embryonic gonads; REST functioned in a cell-autonomous manner to inhibit apoptotic cell death of PGCs. The effect of REST seems to be mediated, at least in part, by upregulating expression of five anti-apoptotic genes, each of which was previously identified as putative REST-target. *Mek5*, one of these five anti-apoptotic genes, was demonstrated to play critical role on PGC survival.

Although the number of PGCs was significantly decreased in *REST*-deficient embryos by the mid-gestational stages, PGC number in these embryos was restored by E18.5 (Fig. 8); moreover, fertility of both male and female homozygous mutant mice was normal. Taken together, these observations indicated that REST function was not essential for germ cell survival during the later half of gestation; this conclusion is consistent with the finding that *REST* expression decreased after E12.5 (Fig. 1D). Because maintenance of a healthy population of germ cell should be crucial for normal fertility and consequent flourish of species, germ cells should have strong robustness for maintenance of their number. Our results indicated that PGCs could express this robustness to restore their numbers after PGC number suffered to be decreased due to a transient increase in apoptosis in REST-deficient embryos probably by stimulating proliferation and/or by being more resistant to apoptosis at later developmental stages.

## Conclusion

Here, we demonstrated that REST (1) supported the survival of developing PGCs in mouse embryos during the mid-gestational stages, (2) functioned in a cell-autonomous manner, and (3) induced *Mek5* expression to promote PGC survival. Our result suggested that a novel transcriptional network that included REST and downstream REST-target genes, which encoded anti-apoptotic proteins, functioned to ensure proper survival of PGCs. Although *REST*-deficient germ cells could generate functional gametes, our results indicated that germ cells in embryos could recover their numbers even after the population had decrease due to a genetic abnormality; this robustness of the germ cell population may have an important meaning for maintenance of fertility in these organisms.

## Acknowledgments

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2012.09.013.

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