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The *ter*/*ter* Gonadal Somatic Cells Cause Apoptosis in *ter*/*ter* Primordial Germ Cells (PGCs) with Normal Survivability and Proliferation Ability in the Mouse: Evidence from PGC-Somatic Cell "Exchange-Co-Culture"

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ABSTRACT—The *ter* (teratoma, chromosome 18) mutation causes a deficiency in primordial germ cells (PGCs) of *ter*/*ter* embryos from *ter* congenic mouse strains at around 8.0 days post coitum (dpc). Our previous studies indicated that *in vivo*, apoptosis of PGCs was caused by *ter*/*ter* gonadal somatic cells. To examine survival and proliferation in *ter*/*ter* PGCs and the deficiency caused by *ter*/*ter* gonadal somatic cells *in vitro*, we performed "exchange-co-culture" of PGCs and gonadal somatic cells by combining different *ter*-genotypes, on Sl/Sl4-m220 feeder cells. The number of PGCs after 3 days culture of 9.5 dpc *ter*/*ter* PGCs with +/+ 12.5 dpc gonadal somatic cells increased similar to that of +/*ter* or +/+ PGCs. The numbers of PGCs after 12 hr culture of +/+ and *ter*/*ter* 11.5 dpc PGCs with 11.5 dpc *ter*/*ter* gonadal somatic cells decreased significantly when compared to those cultured with +/+ somatic cells. PGCs preferred the WT1-positive gonadal somatic cells, Sertoli cells, to the feeder cells. Both TUNEL and BrdU assays showed that *ter*/*ter* somatic cells induced apoptosis but were independent of DNA synthesis in PGCs "exchange-co-cultured". Through these results, we demonstrated for the first time that *in vitro ter*/*ter* PGCs showed survival and proliferation activities in response to the gonadal somatic cells and that *ter*/*ter* gonadal somatic cells caused apoptosis in PGCs through cell-cell contact.

INTRODUCTION

Primordial germ cells (PGCs) are the embryonic founder cells of the male and female gametes. In the mouse, they are first recognized as a small cluster of cells with a high level of alkaline phosphatase (AP) activity in the extraembryonic mesoderm at 7.25 days post coitum (dpc) (Ginsburg *et al*., 1990). They move into the hindgut and dorsal mesentery at 9.5–10.5 dpc, and finally arrive at the genital ridges, the primordial gonads, at 11.5 dpc. After colonizing gonads at 12.5 dpc, they start to undergo mitotic arrest in the testis or meiosis in the ovary at around 13.5 dpc. The number of PGCs continues to increase during these stages, reaching approximately 25,000 at 13.5 dpc (Mintz and Russell, 1957; Tam and Snow, 1981). The mechanisms underlying the proliferation and survival of, and the growth factors related to, PGCs have yet to be elucidated.

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Several growth factors supporting the survival and/or proliferation of PGCs in mice have been identified through *in vivo* and *in vitro* analyses. Among them, stem cell factor (SCF) promotes the survival and represses the apoptosis of these cells *in vitro* (De Felici and Dolci, 1991; Dolci *et al*., 1991; Godin *et al*., 1991; Matsui *et al*., 1991; Pesce *et al*., 1993). SCF is encoded by the *Sl* (Steel) gene on chromosome 10 (Copeland *et al*., 1990), which is expressed in the somatic cells along the migration route of PGCs and in fetal gonads (Matsui *et al*., 1990). The receptor for SCF, c-Kit, is encoded by the *W* (Dominant white spotting) gene on chromosome 5 (Geissler *et al*., 1988) and expressed in the PGCs (Matsui *et al*., 1990). Both the *Sl* and *W* mutant genes cause polymorphous abnormalities in hematopoiesis and melanogenesis (Williams *et al*., 1992, Morrison-Graham and Takahashi, 1993). Thus, most of the growth factors are known to play important roles in supporting both the germ line and various somatic developments.

The *ter* (teratoma) mutation causes a deficiency in PGCs of both sexes of mice homozygous for the *ter* gene from the

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start of migration at 8.0 dpc, but not in the somatic cells (Noguchi and Noguchi, 1985; Sakurai *et al.*, 1995; Noguchi *et al.*, 1996). Based on differences in loci and functions, the *ter* gene, which is located near the *Grl1* (Glucocorticoid receptor 1) locus on chromosome 18 (Sakurai *et al.*, 1994), is thought to be unique and so important to our understanding of the restricted mechanism of germ line development. Noguchi and Noguchi (1985) found that the *ter* gene caused both germ-cell deficiency in the *ter*/*ter* male and female and a high incidence of congenital testicular teratomas (94% of *ter*/*ter* male mice, 17% of +/*ter*, 1.4% of +/+) in the 129/Sv-+/*ter* strain. This inbred strain is a subline of 129/SvJ, a strain susceptible to testicular teratomas (Stevens, 1973). Noguchi *et al.* (1996) established the *ter* congenic strains, C57BL/6J-+/*ter* (B6-+/*ter*), C3H/HeJ-+/*ter* and LTXBJ-+/*ter*, by backcrossing 129/Sv-+/*ter* mice to C57BL/6J (B6), C3H/HeJ and LTXBJ mice, respectively. These *ter*/*ter* mice and both +/+ and +/*ter* (hereafter, +/–) mice developed small gonads lacking germ cells and normal gonads, respectively, but did not suffer from testicular teratomas. This evidence indicated that the *ter* gene causes a single deficiency in PGCs of any genetic background and that the genes from the 129/Sv-+/*ter* strain are mainly responsible for testicular teratocarcinogenesis (Noguchi *et al.*, 1996). Thus, these *ter* congenic strains served as tools to analyze the function of the *ter* gene without the noise of testicular teratomas known to be originated from PGCs. Simple sequence length polymorphisms (SSLP) of the amplified PCR products of microsatellite DNA in the *Grl1* gene, which has been transferred with the *ter* gene from the 129/Sv-+/*ter* strain to the *ter* congenic strains, can be used to estimate the *ter* genotype of each individual in *ter* congenic strains (Sakurai *et al.*, 1994; 1995). This method enabled us to analyze the expression of the *ter* gene using embryos with predetermined *ter* genotypes, even though the *ter* gene has not been cloned yet. The number of PGCs in a B6-+/– embryo was about 30 at 7.5 dpc and 4,000 at 12.5 dpc. But that in a *ter*/*ter* littermate was reduced, being 30 at 8.0 dpc and less than 100 at up to 12.5 dpc (Sakurai *et al.*, 1995). The *ter* gene does not affect the appearance or migration of the PGCs in *ter*/*ter* embryos.

It is suggested, by immunohistochemical TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) staining, that the death of PGCs in *ter*/*ter* fetal testes is caused by apoptosis at G1 after the M phase of the cell cycle of *ter*/*ter* gonocytes (Noguchi *et al.* unpublished). However, the inductive mechanisms of the cell death caused by the *ter*/*ter* testicular somatic cells have not been clarified yet. The intrinsic response of the *ter*/*ter* PGCs themselves has not been analyzed *in vivo*, because the number of *ter*/*ter* PGCs at each stage was too small to produce "reconstituted testes".

Mixed-cultures of PGCs and somatic cells plated on feeder cells such as STO, Sl/Sl4-m220 or TM4 have demonstrated the effects of various growth factors including SCF, leukemia inhibitory factor (LIF) and basic fibroblast growth factor (bFGF) on the growth and survival of PGCs *in vitro* (Donovan *et al.*, 1986; Matsui *et al.*, 1991; De Felici and Dolci, 1991; Resnick *et al.*, 1992; Matsui *et al.*, 1992). It appeared that mixed-culture *in vitro* was very useful for analyzing the behavior of a small number of *ter*/*ter* PGCs and the interaction of PGCs with somatic cells.

To clarify the action of the *ter* gene leading to PGC deficiency, in this study, we examined *in vitro* whether *ter*/*ter* PGCs exhibit a survival and/or proliferative response and how the *ter*/*ter* testicular somatic cells cause the PGC death on Sl/Sl4-m220 feeder cells. In addition, feeder cells were used without growth factors to focus on the function of the *ter* gene. First, we examined the ability of *ter*/*ter* PGCs to survive and/or proliferate by comparing numbers of *ter*/*ter*, +/+ and +/*ter* 9.5 dpc PGCs co-cultured with +/+ 12.5 dpc testicular somatic cells. Second, we analyzed both the survival response of the *ter*/*ter* 11.5 dpc PGCs and the role of the *ter*/*ter* gonadal somatic cells by comparing the number of *ter*/*ter* or +/+ PGCs "exchange-co-cultured" with *ter*/*ter* or +/+ 11.5 dpc gonadal somatic cells.

Thus, we demonstrated for the first time that *ter*/*ter* PGCs respond normally in terms of both survival and proliferation *in vitro* when supported by +/+ gonadal somatic cells in co-culture. In addition, it was found that *ter*/*ter* gonadal somatic cells caused apoptosis in PGCs through cell-cell contact.

MATERIALS AND METHODS

Mice

We used 23 rd generation mice of the *ter* congenic strain LTXBJ-+/*ter* established by introduction of the *ter* gene of strain 129/Sv-+/*ter* into the genetic background of strain LTXBJ by repeated backcrosses of +/*ter* to LTXBJ (Noguchi *et al.*, 1996). The embryos at 9.5, 11.5 and 12.5 dpc were obtained from +/*ter* x +/*ter* crosses and genotypes (+/+, +/*ter* and *ter*/*ter*) were identified by SSLP as described below. Pregnancy was identified by the presence of a vaginal plug at noon (designated 0.5 dpc).

Determination of the *ter* genotype

According to Noguchi and Noguchi (1985), *ter* genotypes of the progenies were inferred by the progeny test. Briefly, the *ter* homozygotes were characterized by small testes and small ovaries and histologically by germ cell deficiency at 2 months of age. Normal size gonads inferred a +/– genotype. The heterozygotes were then detected by mating with "testers" known to be +/*ter*: if the offspring had small gonads, the mice were +/*ter*.

SSLP of PCR amplified microsatellite DNA at the *Grl1* locus

The *ter* genotype of the individual was estimated by SSLP of the PCR amplified microsatellite DNA marker *D18Mit17* (Research Genetics, Huntsville, AL, USA) at the *Grl1* locus located within 2 cM of the *ter* locus on chromosome 18 (Sakurai *et al.*, 1995). Briefly, template genomic DNA was extracted from tissue fragments of the adult or embryo. Amplification was performed for 1 min at 94°C, 2 min at 55°C and 2 min at 72°C for 30 cycles. As shown in Fig. 1, the PCR polymorphisms of *D18Mit17* separated on agarose gel showed a single band of 214 bp in LTXBJ and 190 bp in 129/Sv-+/*ter* as controls (lanes 1, 5). Noguchi *et al.* has confirmed the statistical exactitude in the estimation of the *ter* genotype in LTXBJ-+/*ter* strain by SSLP of this primer (unpublished). Thus, 214 bp/214 bp, 214 bp/190 bp and 190 bp/190 bp inferred +/+, +/*ter* and *ter*/*ter* in the LTXBJ-+/*ter* strain, respectively (lanes 2–4). All procedures were carried out within 6 hr of the PGC-somatic cell "separation culture" as described below.

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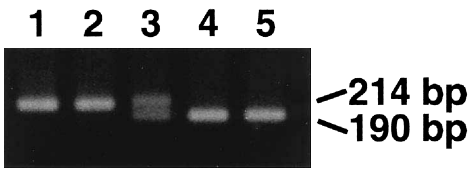

Fig. 1. SSLP of microsatellite DNA (*D18Mit17*) in strain LTXBJ-+/*ter*. Lanes 1 and 5: Amplified DNA of LTXBJ and 129/Sv-+/*ter* mice as controls showed homobands of 214/214 bp and 190/190 bp, respectively. Lanes 2-4: Amplified DNA of +/+, +/*ter* and *ter*/*ter* mice in LTXBJ-+/*ter*, identified by progeny tests, showed a homoband of 214/214 bp, hetero bands of 214/190 bp and a homoband of 190/190 bp, respectively.

Preparation of feeder cells

Sl/Sl4-m220 cells were prepared as the feeder layer for PGC culture according to Matsui *et al.* (1992). The confluent cells were treated with 5 μg/ml of mitomycin C (Sigma, St Louis, MO, USA) and were plated at a density of 2×10^5 cells/well onto 4-well plates (Nalge Nunc International, Rochester, NY, USA). These cells were cultured for 18 hr prior to use as feeder cells.

Separation of PGCs from somatic cells

The hindgut and dorsal mesentery (at 9.5 dpc), genital ridges with mesonephros (at 11.5 dpc), or testes without mesonephros (at 12.5 dpc) from each embryo were dissociated into single cells by trypsin-EDTA (Sigma, St Louis, MO, USA). To separate PGCs from somatic cells by their adhesiveness to culture dishes, single cells were incubated in embryonic stem cell medium (ESM) (Kawase *et al.*, 1994) supplemented with 10% fetal bovine serum (FBS) at 37°C for 6 hr ("separation culture"). Then the cells floating in the culture medium were collected as the "PGC fraction" and the cells adhering to the dishes were trypsinized again. Dissociated cells were designated as the "soma fraction". The ratio of separation of PGCs from somatic cells in each fraction was estimated from the number of PGCs detected by histochemical staining for AP activity as described below. About 70 and 30% of the cells in the "PGC fraction" from the hindgut mesentery and about 90 and 10% of those from the gonadal tissues were AP-positive and negative including blood cells, respectively. The "soma fraction" was composed entirely of AP-negative cells. The fractions with the same *ter* genotype, as determined by SSLP, were pooled for analysis. The viability of these single cells was approximately 100% by the standard dye exclusion test for trypan blue.

"Exchange-co-culture" of PGCs and somatic cells

The "PGC fraction" at 9.5 dpc, "soma fraction" at 12.5 dpc or both fractions at 11.5 dpc, in a concentration equivalent to one-half, one-quarter, or one-quarter of an embryo per well, respectively, were

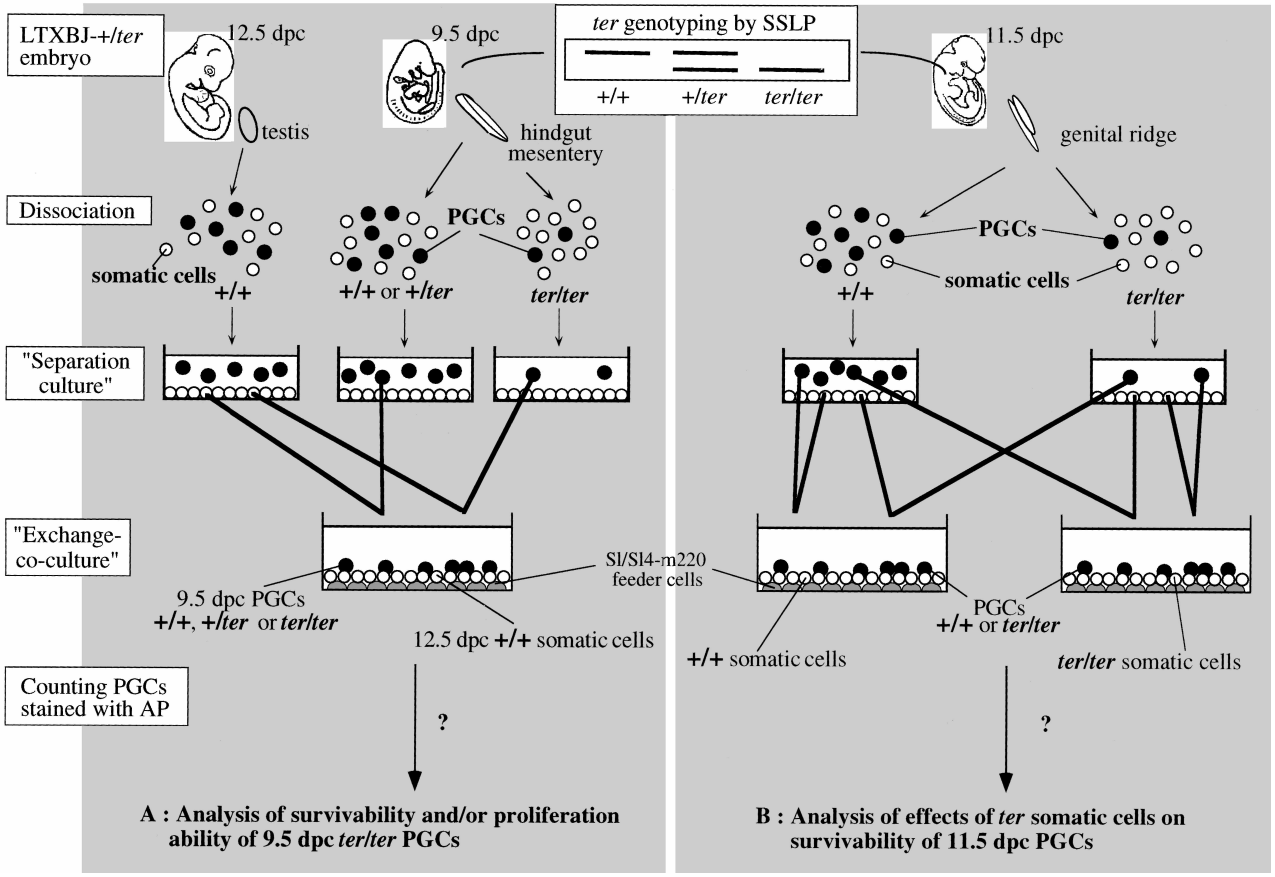

Fig. 2. Schematic representation of analyses of function of the ter gene using the "exchange-co-culture" of PGCs and somatic cells between ter genotypes *in vitro*. Experiment A: Analysis of the survivability and/or proliferative ability of 9.5 dpc *ter*/*ter* PGCs. PGCs were isolated by "separation culture" from the cell fraction of 9.5 dpc hindgut and mesentery and genotyped by SSLP. +/+, +/*ter* or *ter*/*ter* PGCs were co-cultured with +/+ somatic cells isolated from 12.5 dpc testes in wells containing Sl/Sl4-m220 feeder cells. The PGCs after 1 and 3 days of culture were identified by AP staining and their numbers were compared among *ter* genotypes. Experiment B: Analysis of effects of *ter* somatic cells on the survivability of 11.5 dpc PGCs. Both PGCs and somatic cells were isolated by "separation culture" from 11.5-dpc +/+ or *ter*/*ter* genital ridges. The genotyped PGCs and somatic cells were then "exchange-co-cultured" between genotypes. The PGCs were enumerated as in Experiment A.

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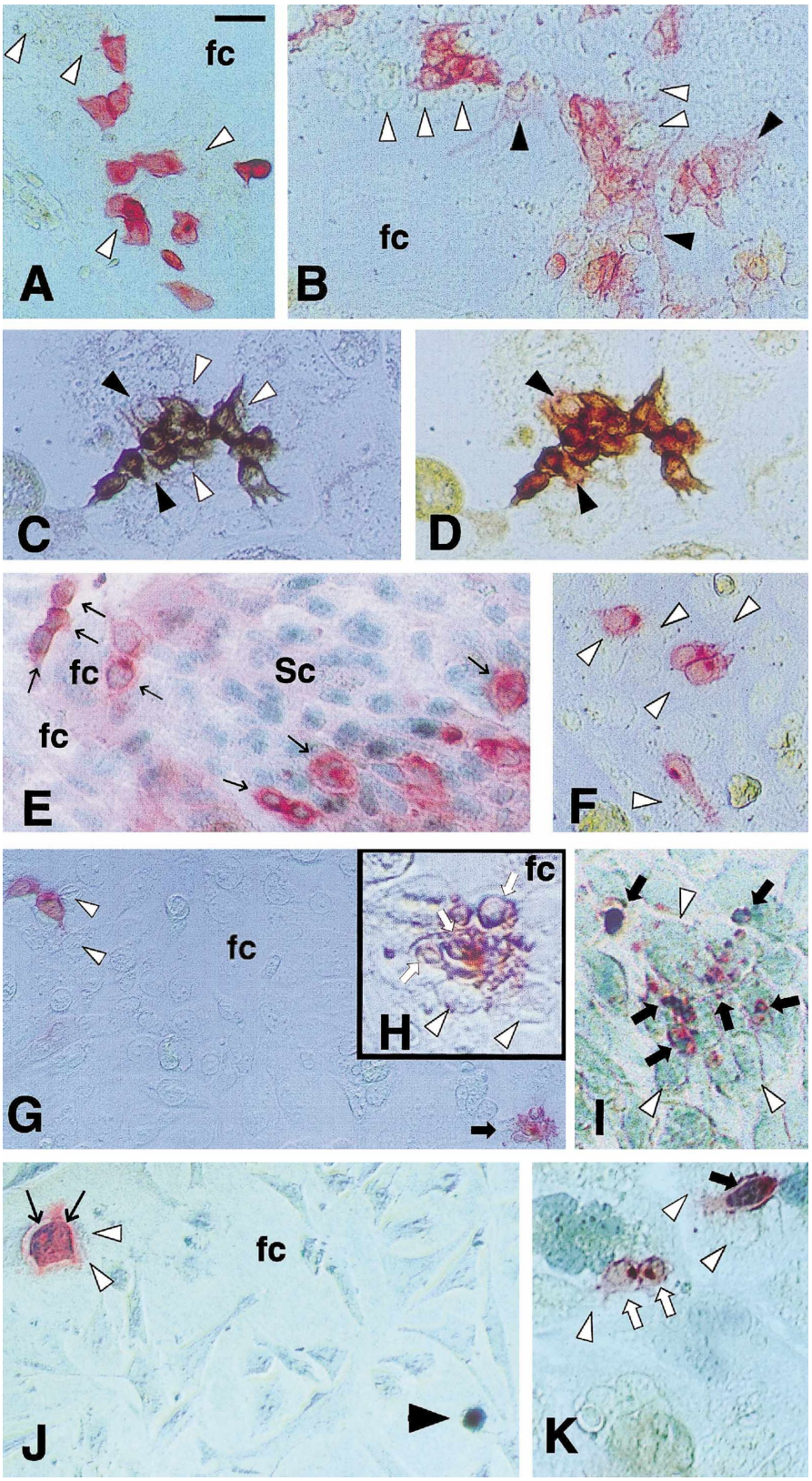

Fig. 3. Photomicrographs of PGCs and somatic cells "exchange-co-cultured" between *ter* genotypes. PGCs are detected as the red cells (A, B, E-K) with strong histochemical activity for AP other than red epithelial-like cells (black arrowhead) (B, D), the black cells stained positively with 4C9 antibody (C) and the brown cells AP/4C9 double stained (D). (A) 9.5 dpc +/+ PGCs co-cultured for 1 day with 12.5 dpc +/+ somatic cells (white arrowhead) on the feeder cells (fc). Note that the amoeboid shape of the PGCs is similar to that in 10–11 dpc embryos. (B) 9.5 dpc *ter*/*ter* PGCs co-cultured for 3 days with 12.5 dpc +/+ somatic cells (white arrowhead) on feeder cells (fc). Note that proliferating amoeboid or round type

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plated on the feeder cells in the 4-well plates. These cells were incubated in the ESM supplemented with 20% FBS at 37°C. The culture medium was changed every day.

Identification of PGCs by AP histochemical staining and anti-4C9 antibody staining

PGCs were detected by histochemical staining for AP activity (Matsui *et al.*, 1991). The cells in the plates were stained in a solution containing naphthol AS-MX phosphate (Sigma, St Louis, MO, USA) and Fast Red TR salt (Sigma, St Louis, MO, USA). The resultant red cells (AP-positive cells), other than AP-positive/epithelial-like cells, after 3 days of culture were counted as PGCs using an inverted microscope equipped with a Hoffman modulation contrast (Olympus, Tokyo, Japan). In addition, PGCs were detected by immunohistochemical staining with a rat monoclonal antibody against 4C9 antigen that was recognized in the cytoplasm of the mouse PGCs and gonocytes till around 15.5 dpc (Yoshinaga *et al.*, 1991; Kawase *et al.*, 1994). Briefly, fixed cells were incubated for 1 hr with anti-4C9 antibody diluted at 1:100 with 1% bovine serum albumin/PBS, subsequently with biotinylated anti rat IgG as secondary antibody (Vector, Burlingame, CA, USA) and finally with avidin/biotinylated horseradish peroxidase complex (Vector, Burlingame, CA, USA). The peroxidase substrate used was 3, 3'-diaminobenzidine (DAB) in Tris buffer containing H_2O_2 and $CoCl_2$. Resultant black cells were counted as PGCs with a 4C9-positive cytoplasm. In addition AP and 4C9 (AP/4C9) double staining was performed. Cells stained brown indicating AP/4C9-positivity were counted as PGCs, but epithelial AP-positive and 4C9-negative cells were not because differentiating peritubular cells and hindgut epithelial cells of these *ter* congenic mice are known to express activity of AP (Sakurai *et al.*, 1995; Noguchi *et al.*, 1996).

AP-positive or AP/4C9-positive PGCs were enumerated in at least six wells in 2–3 experiments. The values were analyzed using Student's t-test. p values < 0.05 were judged as significant.

Identification of Sertoli cells by WT1 antiserum staining

Fetal Sertoli cells and their precursors were detected by immunostaining with the anti-Wilms' tumor 1 (WT1) antiserum (Mundlos *et al.*, 1993, Kudoh *et al.*, 1995). The procedures were the same as those used in 4C9 staining except that anti rabbit IgG was used as secondary antibody. Somatic cells with black nuclei were identified as WT1-positive Sertoli cells and follicle cells.

TUNEL assay for apoptosis in PGCs *in vitro*

Cultured PGCs were fixed in 4% paraformaldehyde and first stained histochemically for AP activity and then stained with TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling), using the TACS *in situ* apoptosis detection kit (Trevigen, Gaithersburg, MD, USA) according to the manufacture's instructions. Apoptotic PGCs were detected as AP-positive fragmented cells with fragmented black nuclei. As control, feeder cells treated with DNase I were stained with TUNEL.

5-bromo-2-deoxyuridine (BrdU) labelling *in vitro*

PGCs in the S phase *in vitro* was identified by BrdU labelling using the cell proliferation kit (Amersham Pharmacia biotech, Buckinghamshire, UK) as previously described (Dolci *et al.*, 1991; Kawase *et al.*, 1994). Briefly, PGCs were cultured for last 1 hr of culture period with BrdU reagent that had been diluted 1,000-fold with ESM without thymidine and were fixed in 95% cold ethanol. The cells were histochemically stained for AP activity and then were visualized using anti-BrdU monoclonal antibody, peroxidase-conjugated anti-mouse IgG and DAB. The PGCs labelled with BrdU were exhibited as red cells with black nuclei.

RESULTS

Exchange-co-culture of +/+, +/*ter* or *ter*/*ter* 9.5 dpc PGCs with +/+ 12.5 dpc testicular somatic cells (see Fig. 2 A)

First, we examined whether *ter*/*ter* PGCs survive and proliferate like +/– PGCs *in vitro* by comparing the behavior of the 9.5 dpc +/+, +/*ter* and *ter/ter* PGCs that were "exchange-co-cultured" for 3 days with 12.5 dpc +/+ testicular somatic cells (Fig. 3).

After 1 day of culture, +/+, +/*ter* or *ter*/*ter* AP-positive PGCs somewhat amoeboid in shape dispersed on the AP-negative testicular somatic cells colonizing the feeder layer and remained in the migratory phase as did those *in vivo* (Fig. 3A). After 3 days of culture, some PGCs were round and showed AP-positive (Fig. 3B), 4C9-positive (Fig. 3C) or AP/4C9-positive staining (Fig. 3D) and most formed cohesive colonies. These characteristics resembled closely those of wild type PGCs in 11.5 dpc genital ridges and 12.5 dpc testes *in vivo* (Sakurai *et al.*, 1995; Noguchi *et al.*, 1996). On the other hand, colonies of AP-positive (Fig. 3B, D) but 4C9-negative (Fig. 3C) cells, which appeared to be epithelial in shape, were not observed until day 3 of culture. These cells appeared to be peritubular cells undergoing differentiation or hindgut cells contaminated the "PGC fraction" at 9.5 dpc. Thus, AP/4C9 double staining could distinguish PGCs from somatic cells co-cultured for 3 days, indicating that there were no differences in the behavior of PGCs among the *ter* genotypes.

Second, to clarify the inter-cellular interaction through

PGCs are colonized like PGCs in 12.5 dpc gonads and that epithelial-like cells (black arrowhead) are stained weakly with AP like peritubular cells in the fetal testes. In addition, PGCs are in contact with +/+ somatic cells rather than the feeder cells (fc) (A, B). (C) A colony of +/+ 4C9-positive PGCs in contact with 4C9-negative +/+ somatic cells (arrowhead) after 3 days of culture. (D) Same colony in (C) stained doubly with AP/4C9 antiserum. AP-positive/4C9-negative +/+ somatic cells (black arrowhead) are distinguished from PGCs. (E) 9.5 dpc +/+ PGCs and 12.5 dpc +/+ testicular somatic cells stained doubly with AP and WT1 antiserum after 3 days of co-culture. Sertoli cells are the cells with black nuclei (Sc) stained positively with WT1 antiserum. Note that AP-positive PGCs (arrow) locate on Sertoli cells rather than the feeder cells with larger WT1-negative nuclei (fc). (F) 11.5 dpc +/+ PGCs in contact with +/+ somatic cells (white arrowhead) after 24 hr of co-culture. Normal PGCs with round or amoeboid shape. (G) 11.5 dpc +/+ PGCs in contact with *ter*/*ter* somatic cells (white arrowhead) after 24 hr of co-culture. The fragmented AP-positive cells (black arrow) appear to be apoptotic PGCs. (H) A higher magnification of the apoptotic PGCs (black arrow) in (G). The various sized AP-positive blebs (white arrow) indicate "apoptotic bodies" of PGCs and are in contact with *ter*/*ter* somatic cells (white arrowhead). (I) 11.5 dpc +/+ PGCs and *ter*/*ter* somatic cells (white arrowhead) stained doubly with AP and TUNEL after 12 hr of co-culture. Note that fragmented AP-positive blebs (black arrow) contain fragmented TUNEL-positive black nuclei, indicating that these blebs are "apoptotic bodies" of PGCs. (J) 11.5 dpc +/+ PGCs and +/+ somatic cells (white arrowhead) stained doubly with AP and TUNEL after 12 hr of co-culture. Note that a cluster of normal AP-positive and TUNEL-negative PGCs (arorow) and a AP-negative and TUNEL-positive somatic cell (large arrowhead). (K) 11.5 dpc +/+ PGCs and *ter*/*ter* somatic cells (white arrowhead) stained doubly with AP and BrdU antibody after 24 hr of co-culture. Note that AP-positive PGCs with a black nucleus (black arrow) labeled with BrdU and with non-labeled nuclei (white arrow). Bar: 25 μm (A-E, G), 20 μm (F, I, J), 8 μm (H, K).

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co-culture of PGCs, testicular somatic cells and feeder cells, we examined the focal plane, the distribution and the expression of WT1 protein, a nuclear marker of Sertoli cells (Mundlos *et al.*, 1993; Kudoh *et al.*, 1995), in these cells. A difference in the focal plane and colonization pattern was observed. It appeared that most of the PGCs were in contact with the testicular somatic cells whose nuclei were smaller than those of the feeder cells (Fig. 3). Many somatic cells with WT1-positive nuclei (black nuclei) were identified as Sertoli cells after 3 days of culture (Fig. 3E). AP-positive PGCs with WT1-negative nuclei were often detected on or next to Sertoli cells with signals. Such WT1-positive signals were seen in the nuclei of neither the AP-positive epithelial cells nor the feeder cells. These observations show that PGCs associated closely with WT1-positive Sertoli cells rather than the feeder cells.

The PGCs were enumerated during the culture (Fig. 4). After 1-day culture, the mean number of +/+, +/*ter* and *ter*/*ter* PGCs per 0.5 embryo was about 30, 30 and 10, respectively, whereas after 3-day culture, the number was approximately 500 in each case. There was no significant difference in the number of PGCs among the three *ter* genotypes. Results showed that *ter*/*ter* 9.5 dpc PGCs had survived and proliferated in response to +/+ 12.5 dpc testicular somatic cells as well as had +/– PGCs. There were no AP-positive PGCs in the "soma fraction" isolated from +/+ 12.5 dpc testes. It was, however, questionable whether the hindgut somatic cells in the PGC fraction had enhanced the survival and/or proliferative rate of PGCs. When the +/+ 9.5 dpc PGC fraction was seeded on the feeder cells in the absence of the 12.5 dpc testicular somatic fraction, the number of PGCs after 3 days of culture remained 120, about one forth of that of the PGCs co-cultured with 12.5 dpc somatic cells (Fig. 4).

Thus, *ter*/*ter* 9.5 dpc PGCs survived and proliferated similar to the PGCs in the +/– littermates, and the testicular somatic cells supported them through cell-cell contact.

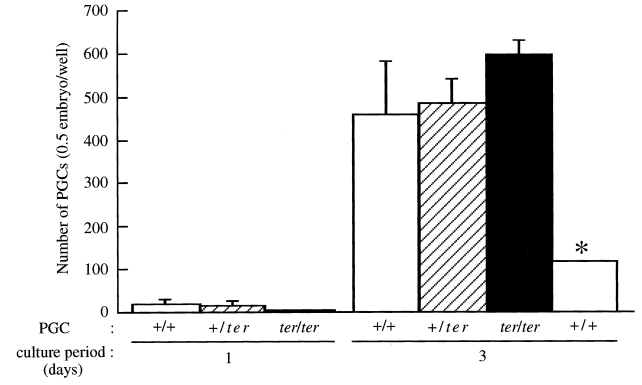

Fig. 4. Estimation of the number of 9.5 dpc +/+, +/*ter* or *ter*/*ter* PGCs after "exchange-co-culture" for 3 days with 12.5 dpc +/+ testicular somatic cells on a feeder layer. Each value represents the average plus the standard deviation. Note that there are no differences in the number of PGCs among *ter* genotypes.
*: PGCs were cultured for 3 days without 12.5 dpc somatic cells on the feeder layer as a control.

Exchange-co-culture of PGCs and somatic cells isolated from +/+ and *ter*/*ter* 11.5 dpc genital ridges (see Fig. 2 B)

Second, to clarify whether *ter*/*ter* 11.5 dpc PGCs can survive *in vitro* and whether *ter*/*ter* somatic cells cause PGCs to die *in vitro*, "exchange-co-culture" for 12 and 24 hr of the PGCs and somatic cells isolated from +/+ or *ter*/*ter* 11.5 dpc genital ridges between *ter* genotypes was carried out, focusing on the restricted effect of cell-cell interaction between the PGCs and the gonadal somatic cells.

Most of the +/+ or *ter*/*ter* PGCs co-cultured with +/+ somatic cells became round in shape after 12 and 24 hr, indicating a loss of the ability to migrate in these PGCs (Fig. 3F). On the other hand, the morphology of +/+ and *ter*/*ter* PGCs co-cultured with *ter*/*ter* somatic cells showed two features, normal non-apoptotic characteristics and typical "apoptotic bodies", *in vitro* (Fig. 3G, H). Then, whether these fragmented AP-positive cytoplasmic blebs were "apoptotic bodies" was examined by TUNEL assay for the apotic cells (Gavrieli Y *et al.*, 1992). Results showed that AP-positive PGCs fragmented on *ter*/*ter* somatic cells contained various-sized blebs stained positively with TUNEL assay (Fig. 3I), whereas morphologically normal PGCs were AP-positive and TUNEL-negative on both *ter*/*ter* and +/+ somtic cells (Fig. 3J). This indicated that these AP-positive "apoptotic bodies" on co-culture of PGCs and *ter*/*ter* somatic cells were apoptotic PGCs. Successively, whether *ter*/*ter* somatic cells affect the proliferation of PGCs was examined by BrdU incorporation assay for 11.5 dpc PGCs co-cultured for 24 hr with *ter*/*ter* or +/+ somatic cells. Results showed that normal PGCs labeled with BrdU were similarly detected in co-cultures with either *ter*/*ter* or +/+ somatic cells (Fig. 3K), indicating that *ter*/*ter* somatic cells did not inhibit DNA synthesis in the S phase of the cell cycle in PGCs.

The normal PGCs were enumerated (Fig. 5). When +/+ PGCs were "exchanged-co-cultured" with +/+ or *ter*/*ter* somatic cells, their number per 0.25 embryo was 44.1±14.4 or 16.0±5.0 after 12 hr, and 20.2±6.5 or 8.7±7.3 after 24 hr, respectively (Fig. 5A). On the other hand, when *ter*/*ter* PGCs were "exchange-co-cultured" with +/+ or *ter*/*ter* somatic cells, their number was 25.5±4.2 or 1.5±0.7 after 12 hr, and 14.0±1.2 or 0.5±0.7 after 24 hr, respectively (Fig. 5B). The number after 24 hr culture in either case declined more than that after 12 hr culture. In addition, there was a significant difference in the number of +/+ PGCs or *ter*/*ter* PGCs between *ter* genotypes of the somatic cells. As fewer *ter*/*ter* PGCs than +/+ PGCs were seeded, we normalized the number of surviving PGCs cultured with +/+ somatic cells after 24 hr to that after 12 hr between the *ter* genotypes of PGCs (Fig. 5C). When the number of PGCs after 12 hr culture was set as 100% in each case, it was found that about 50% of both +/+ and *ter*/*ter* PGCs survived after 24 hr.

Through the experiments, surviving or apoptotic PGCs were found to be in contact with *ter*/*ter* somatic cells rather than the feeder dells (Fig. 3G–I) as well as did normal PGCs with +/+ somatic cells (Fig. 3F, J). Then, to identify the cell type of the somatic cells in contact with PGCs more precisely, +/+ somatic cells co-cultured for 12 hours with either 11.5 dpc

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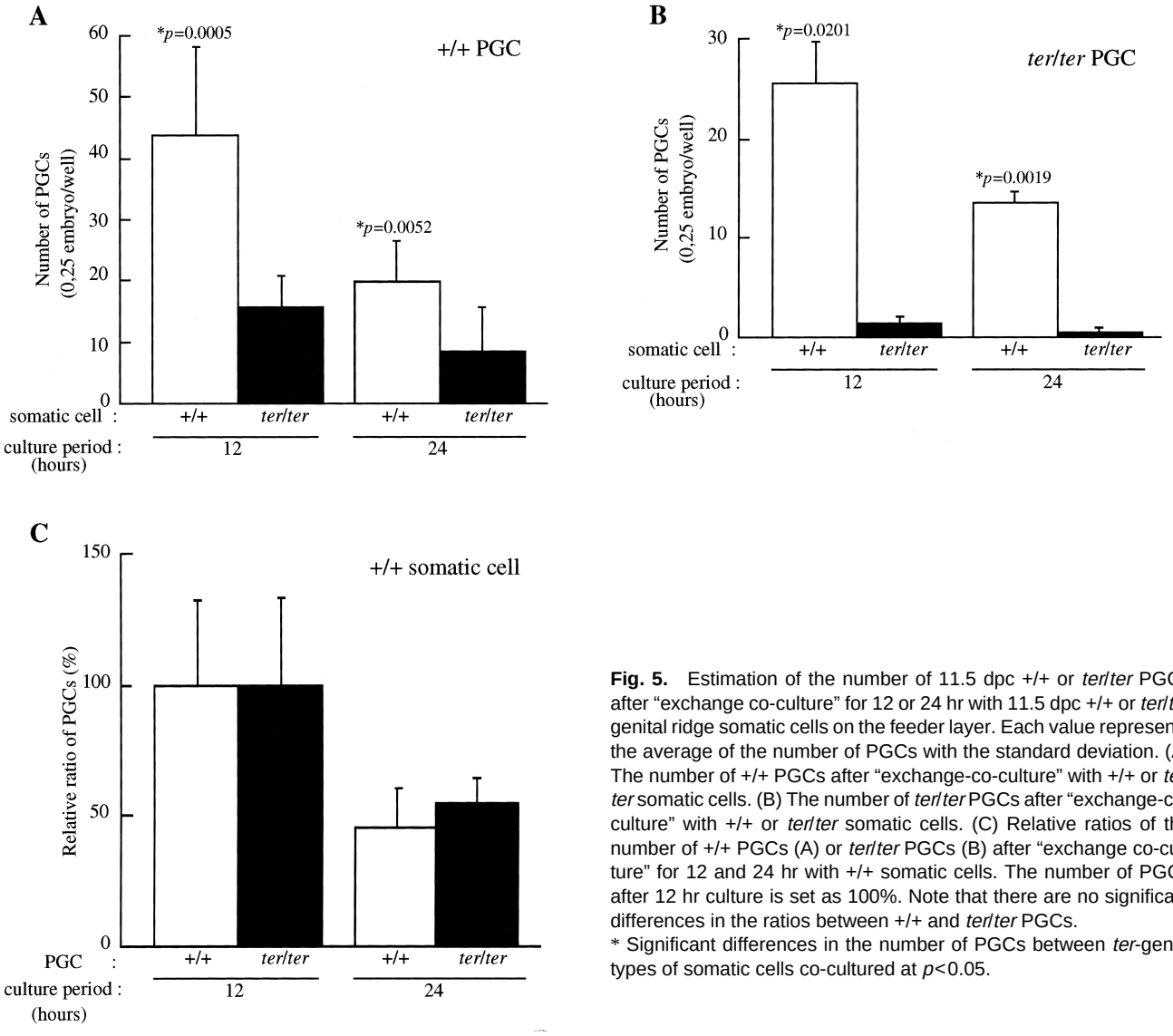

Fig. 5. Estimation of the number of 11.5 dpc +/+ or *ter/ter* PGCs after "exchange co-culture" for 12 or 24 hr with 11.5 dpc +/+ or *ter/ter* genital ridge somatic cells on the feeder layer. Each value represents the average of the number of PGCs with the standard deviation. (A) The number of +/+ PGCs after "exchange-co-culture" with +/+ or *ter/ter* somatic cells. (B) The number of *ter/ter* PGCs after "exchange-co-culture" with +/+ or *ter/ter* somatic cells. (C) Relative ratios of the number of +/+ PGCs (A) or *ter/ter* PGCs (B) after "exchange co-culture" for 12 and 24 hr with +/+ somatic cells. The number of PGCs after 12 hr culture is set as 100%. Note that there are no significant differences in the ratios between +/+ and *ter/ter* PGCs.
* Significant differences in the number of PGCs between *ter*-genotypes of somatic cells co-cultured at $p<0.05$.

Table 1. Localization of 11.5 dpc +/+ PGC co-cultured on +/+ somatic cells and feeder cells.

	on Sertoli cells*	on the feeder cells**
Number of PGCs	263±43 (85%)	44.5±0.7 (15%)

There was significant difference in the number of PGCs on Sertoli cells and that on the feeder cells ($p=0.01$).
*; Sertoli cells are cells with nuclei stained positively with WT1 antiserum.
**; Cels are containing feeder cells and somatic cells with no WT1-signals.

+/+ or *ter/ter* PGCs were WT1-stained. It appeared that both +/+ and *ter/ter* PGCs were in contact with WT1-positive Sertoli cells rather than WT1-negative feeder cells and non-Sertoli cells as well as were 9.5 dpc PGCs (Fig. 3E). The normal PGCs were enumerated and the number of PGCs on WT1-positive cells vs that on WT1-negative cells was 264 (85%) vs 45 (15%) per one embryo (Table 1). PGCs appeared to prefer Sertoli cells rather than feeder cells and non-Sertoli cells.

Thus, *ter/ter* or +/+ 11.5 dpc PGCs responded similarly to +/+ gonadal somatic cells. In addition, *ter/ter* 11.5 dpc gonadal somatic cells induced apoptosis in *ter/ter* or +/+ PGCs probably via cell-cell contact within 12 hr, whereas +/+ somatic cells supported the survival of PGCs.

DISCUSSION

In the present study, it was concluded that *ter/ter* PGCs are normal in their ability to survive and proliferate. In addi-

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tion, it was shown that *ter*/*ter* and wild type gonadal somatic cells caused apoptosis in PGCs and supported PGC survival, respectively, by cell-cell interaction on co-culture *in vitro*.

Here, we analyzed the role of the *ter* gene in the PGC deficiency by "exchange-co-culture" of PGCs and somatic cells from fetal gonads between *ter* genotypes. Both *ter*/*ter* males and females in the *ter* congenic strains suffered from a similar PGC deficiency in the initial stages of the PGC migration (Sakurai *et al.*, 1995), but the deficiency was milder in the ovary than in the testis (Noguchi *et al.*, 1996). Therefore, we eliminated the noise derived from this sexual difference by using embryos at 2 earlier stages, 9.5 and 11.5 dpc. We used feeder cells known to produce membrane-associated SCF without any other growth factors (Matsui *et al.*, 1992; Ohkubo *et al.*, 1996). The 9.5 dpc *ter*/*ter*, +/+ or +/*ter* PGCs, which were co-cultured with +/+ 12.5 dpc testicular somatic cells for 3 days, showed similar behavior in terms of migration, proliferation and colonization to the wild type PGCs *in vivo*. Thus, we concluded that *ter*/*ter* PGCs in the proliferative stage such as at 9.5 dpc survive and proliferate normally and can respond to the somatic cells supporting them. Secondly, we confirmed the viability of *ter*/*ter* PGCs and showed that PGC death is induced by *ter*/*ter* somatic cells by the exchange-co-culture of PGCs and somatic cells isolated from both +/+ and *ter*/*ter* genital ridges at 11.5 dpc between *ter* genotypes. The data obtained *in vitro* revealed that the *ter* mutation is expressed in the somatic cells of male and female gonads, not the PGCs, in the *ter*/*ter* embryos.

How the *ter*/*ter* somatic cells affect the survival of PGCs was partly clarified in this study. First, wild type 9.5 dpc PGCs in contact with only feeder cells proliferated at one-forth the rate of PGCs co-cultured with +/+ 12.5 dpc testicular somatic cells on feeder cells. Concerning the relationship between the PGCs and somatic cells in co-culture, 9.5 dpc PGCs appeared to be associated or in contact with +/+ 12.5 dpc WT1-positive Sertoli cells rather than the feeder cells. Both 11.5 dpc +/+ and *ter*/*ter* PGCs survived on WT1-positive +/+ Sertoli cells rather than the feeder cells. On the other hand, some of the +/+ or *ter*/*ter* PGCs cultured on *ter*/*ter* somatic cells produced AP-positive and TUNEL-positive "apoptotic bodies", a typical characteristic of apoptosis (Arends and Wyllie, 1991; Fesus *et al.*, 1991). This cell death was induced within 12 hr of co-culture, whereas the culture medium was changed every day during 3 days of culture. Thus, it is likely that gonadal somatic cells support the survival and/or proliferation of PGCs predominantly via cell-cell interaction. In addition, the results suggested that the cell membrane-associated factor of the wild type gonadal somatic cells is an unknown growth factor supporting PGC survival and/or proliferation. The *ter*/*ter* somatic cells causing the apoptosis might disrupt such a membrane-associated growth factor. On the other hand, it is also likely that a soluble form of the supportive factor and its default type are secreted from +/– and *ter*/*ter* fetal gonadal somatic cells, respectively. We are now examining conditioned medium from these somatic cells.

Next, the phase of the cell cycle in which PGCs are targeted by the *ter*/*ter* somatic cells was discussed. Present data showed that BrdU-labeled PGCs were similarly observed on the somatic cells, irrespective of *ter* genotype and that *ter*/*ter* somatic cells caused apoptosis in PGCs. This means that *ter*/*ter* somatic cells permit the PGCs to come into contact with them to enter S phase but not survive. This is consistent with evidence obtained *in vivo* that the *ter*/*ter* fetal testes contained very small numbers of mitotic figures in the M phase but no gonocytes in mitotic arrest at G1 (Noguchi *et al.*, 1996). When +/+ PGCs in the G1 phase of mitotic arrest and *ter*/*ter* fetal testicular somatic cells were reaggregated and then grafted, these +/+ gonocytes ceased undergoing mitotic arrest and their mitotic figures were detected in the *ter*/*ter* tubules in reconstituted testes differentiated from the grafts. But, other spermatogenic cells in advanced stages including the next G1 phase have not been identified (Noguchi *et al.*, unpublished). Thus, the evidence obtained *in vivo* and *in vitro* suggested that *ter*/*ter* somatic cells cause apoptotic cell death in PGCs at the M/G1 transition or the next G1 phase but not in the S phase or M phase. Based on these data it seems that an unknown factor from the wild type gonadal somatic cells promotes survival in 9.5 dpc PGCs that in turn proliferate. Disruption of this unknown factor seems to affect the survival but not the proliferation of PGCs and to result in germ cell apoptosis in *ter*/*ter* gonads.

In order to characterize the *ter* gene, we would like to summarize several factors related to PGC development. One of the PGC growth factors, SCF, exists in both membrane-associated and soluble forms generated by alternative splicing (Flanagan *et al.*, 1991). In 13 dpc mouse testes, membrane-associated SCF is predominant and freshly isolated Sertoli cells mainly express this type in the first 24 hr of culture. A switch to soluble SCF, however, occurs after 48 hr (Mauduit *et al.*, 1999). These findings suggest that PGC development is affected by a disruption in the interaction of the PGCs with the somatic cells surrounding them *in vivo*. SCF and LIF, which support survival and prevent apoptosis, do not influence the S phase of PGCs (Matsui *et al.*, 1992; Pesce *et al.*, 1993). Recently, it was reported that c-Kit/SCF-mediated apoptosis in PGCs and germ cells is controlled by a p53-mediated pathway (Lee, 1998; Jordan *et al.*, 1999). The tumor suppressor protein p53 is stabilized by various stresses to cells, for example, UV radiation, growth factor deprivation and serum withdrawal, and caused cell cycle arrest near the G1/S boundary and apoptosis (Mercer *et al.*, 1990; Yonish-Rouach *et al.*, 1991; Shaw *et al.*, 1992). p53-mediated apoptosis is inhibited by adenovirus type 2 E1B, a protein with a molecular weight of 19,000 (E1B19K) (Debbas and White, 1993), and promoted by Bcl-2-associated X protein (bax) (Miyashita and Reed, 1995). The transient expression of E1B19K on PGCs significantly promotes cell survival *in vitro* and prevents apoptosis (Watanabe *et al.*, 1997). SCF decreases bax expression in PGCs *in vitro* (De Felici *et al.*, 1999). The relation between the *ter* gene and the apoptosis-mediated factor, E1B19K, bcl-2, bax or p53, should be clarified in the near future.

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The present study indicated that the *ter* gene was expressed in gonadal somatic cells but not PGCs similar to the *Sl* gene (Motro *et al*., 1991; Matsui *et al*., 1990). Both *Sl* and *W* mutations caused anemia, loss of pigmentation and PGC deficiency (Williams *et al*., 1992), but the *ter* mutation resulted only in PGC deficiency (Noguchi *et al*., 1996). The *Sl*-*W* system started to function at 8.5 dpc (Buehr *et al*., 1993), whereas the *ter* gene was first expressed at 8.0 dpc (Sakurai *et al*., 1995). In the present study, SCF from the feeder cells *in vitro* did not overcome the disruption to *ter*/*ter* somatic cells co-cultured with PGCs. LIF (chromosome 11) and oncostatin M (chromosome 11) are thought to promote the survival of PGCs by stimulating the proliferation of somatic cells in an autocrine manner (Bottorff and Stone, 1992; Cheng *et al*., 1994; Koshimizu *et al*., 1996; Hara *et al*., 1998). In this study, there were no differences in the number of cultured gonadal somatic cells among *ter* genotypes, suggesting that the product of the *ter* gene does not act on somatic cells in an autocrine manner. Furthermore, PGC proliferation was also stimulated by tumor necrosis factor-α (TNF-α), forskolin and other factors *in vitro* (De Felici *et al*., 1993; Kawase *et al*., 1994). It has been reported that growth arrest-specific gene 6 (Gas6, chromosome 8) and Neuregulinβ (NRGβ) are also expressed in genital ridges, and, in addition, support PGC growth or survival in culture (Colombo *et al*., 1992; Matsubara *et al*., 1996; Toyoda-Ohno *et al*., 1999). Gas6 and NRGβ have, however, multiple functions in response to various cells (Manfioletti *et al*., 1993; Meyer and Birchmeier, 1995). These facts and the different loci suggest that the functions of the products of the genes at the *ter* locus are completely different from those of these factors.

Concerning the receptors for the growth factor ligands, 9.5 and 11.5 dpc PGCs *in vitro*, irrespective of *ter* genotype, showed similar responses to *ter*/*ter* or +/+ somatic cells in co-culture. This means that *ter*/*ter* and +/– PGCs must have the same type of receptor for the unknown membrane-associated factor of the gonadal somatic cells and that this receptor must not recognize the faulty factor of *ter*/*ter* somatic cell membrane. PGCs have receptors for SCF, LIF, bFGF, Gas6 and NRGβ (Cheng *et al*., 1994; Matsubara *et al*., 1996; Resnick *et al*., 1998; Toyoda-Ohno *et al*., 1999). None of these receptors seems to be correlated with the *ter* gene.

The present findings should help to clarify the mechanism underlying survival and proliferation in PGCs and the role of somatic cells therein.

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