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Differentiation of mouse primordial germ cells into female or male germ cells

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ABSTRACT Mouse primordial germ cells (PGCs) migrate from the base of the allantois to the genital ridge. They proliferate both during migration and after their arrival, until initiation of the sex-differentiation of fetal gonads. Then, PGCs enter into the prophase of the first meiotic division in the ovary to become oocytes, while those in the testis become mitotically arrested to become prospermatogonia. Growth regulation of mouse PGCs has been studied by culturing them on feeder cells. They show a limited period of proliferation *in vitro* and go into growth arrest, which is in good correlation with their developmental changes *in vivo*. However, in the presence of multiple growth signals, PGCs can restart rapid proliferation and transform into pluripotent embryonic germ (EG) cells. Observation of ectopic germ cells and studies of reaggregate cultures suggested that both male and female PGCs show cell-autonomous entry into meiosis and differentiation into oocytes if they were set apart from the male gonadal environments. Recently, we developed a two-dimensional dispersed culture system in which we can examine transition from the mitotic PGCs into the leptotene stage of the first meiotic division. Such entry into meiosis seems to be programmed in PGCs before reaching the genital ridges and unless it is inhibited by putative signals from the testicular somatic cells.

KEY WORDS: *primordial germ cells, oocytes, meiosis, sex-differentiation, EG cells.*

Introduction

Mouse primordial germ cells (PGCs) are first recognizable in the posterior region of the extraembryonic mesoderm by their surface alkaline phosphatase activity at around 7.25 days post coitum (dpc) (Chiquoine, 1954; Ginsburg *et al.,* 1990). They are localized at the base of allantois and surrounded by the extraembryonic mesoderm cells and the basal surface of the visceral endoderm layer. Prior to the first identification of PGCs, it is known that their precursor cells originate in the proximal part of the epiblast (Tam and Zhou, 1996) near the extraembryonic tissues. They are supposed to involute through the primitive streak and migrate posteriorly toward the allantois. It is still under investigation how epiblast cells are determined to become PGCs. A recent study indicates that BMP-4 from the extraembryonic region is required for the generation of PGCs from proximal epiblast (Lawson *et al.,* 1999). It may be possible that they are also influenced by a local signal from the surrounding extraembryonic mesoderm cells or visceral endoderm layer at the base of allantois.

From around 8.5 dpc, PGCs migrate into the embryonic mesoderm, through the hindgut endoderm and along the dorsal mesentery, and finally into the genital ridge at 10.5 - 11.5 dpc. PGCs continue to proliferate mitotically during the migration and after arrival at the genital ridge until 12.5 - 13.5 dpc. At 12.5 dpc, developing gonads show the first signs of sexual dimorphism with testicular cord formation in the males. PGCs at this stage take different fates according to the sex of embryos. They enter into the prophase of the first meiotic division in the ovary to become oocytes, while those in the male gonads are mitotically arrested to become prospermatogonia (Hilscher *et al.,* 1974). Previous studies have indicated that the sex-differentiation of the germ cells is directed by the sex of the gonadal somatic cells and not by that of the germ cells themselves (reviewed in McLaren, 1994).

Proliferation and migration of mouse PGCs have been studied both by culturing them *in vitro* and by using genetic mutants (reviewed in Wylie, 1999). Mouse PGCs can be grown for several days on suitable feeder cell layers in culture. They show a limited period of proliferation that correlates with their cessation of cell division *in vivo* (Donovan *et al.,* 1986; Ohkubo *et al.,* 1996). Steel factor (SLF), encoded by the Steel loci, was shown to be essential

Abbreviations used in this paper: dpc, days post coitum; EG, embryonic germ; LIF, leukemia inhibitory factor; PGC, primordial germ cells.

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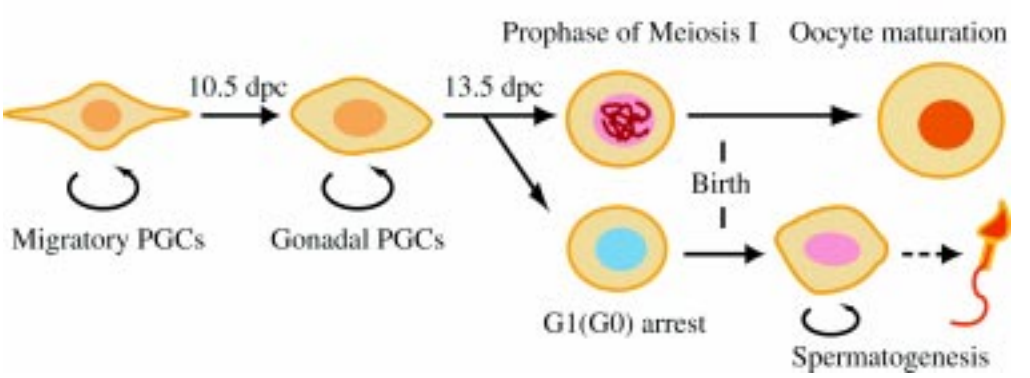

Fig. 1. Differentiation of PGCs into female or male germ cells. *PGCs proliferate during migration into fetal gonads. In the ovary, they enter into meiosis and become oocytes, which are arrested in the prophase of the first meiotic division. In the testis, PGCs are mitotically arrested in the G1/G0 phase and become prospermatogonia, which later restart mitosis as spermatogonia and produce meiotic spermatocytes postnatally.*

for the survival of PGCs *in vitro* as well as *in vivo* (Dolci *et al.,* 1991; Godin *et al.,* 1991). Leukemia inhibitory factor (LIF) (De Felici and Dolci, 1991; Matsui *et al.,* 1991) and oncostatin M (OSM) (Hara *et al.,* 1998), both of which belong to the interleukin-6 (IL-6) cytokine family, were shown to promote the growth and/or survival of PGCs *in vitro*. Signaling from their receptor subunit gp130 was essential for the derivation of pluripotential embryonic germ (EG) cells from PGCs *in vitro* (Koshimizu *et al.,* 1996).

Observation of ectopic germ cells in the mesonephroi or adrenal cortex (Zamboni and Upadhyay, 1983; Francavilla and Zamboni, 1985) suggested that both male and female PGCs show cell-autonomous entry into meiosis if they were set apart from the male gonadal environments. Studies of reaggregated cultures of embryonic gonads (McLaren and Southee, 1997) also demonstrated that both female and male PGCs at 10.5-11.5 dpc, shortly after arriving at genital ridges, enter into meiosis when they were surrounded by lung somatic cells. Compared with studies of growth regulation, however, relatively little is known about the regulative mechanisms that are involved in the differentiation of PGCs into oocytes or prospermatogonia in fetal gonads.

In this review, we describe development and differentiation of fetal germ cells by focusing on our studies related to the differentiation from PGCs into female or male germ cells (Fig. 1). Dr. Anne McLaren has been always the leading person in this field, and we would like to acknowledge that we have received numerous suggestions and inspiration from her.

Cell-autonomous growth regulation in PGCs

Our studies (Kawase *et al.,* 1994; Ohkubo *et al.,* 1996) and others (De Felici and McLaren, 1983; Donovan *et al.,* 1986; Matsui *et al.,* 1992) indicated that proliferation and growth arrest, as well as morphological changes of PGCs, are cell-autonomously programmed, and they are well correlated with their differentiation in embryos at corresponding stages. PGCs obtained from embryos at 8.5 dpc increase in number for 3 days and then start to decrease on day 4, while 11.5 dpc PGCs stop proliferation immediately *in vitro* (Fig. 2). Thus, PGCs *in vitro* seem to mimic the pattern of proliferation and growth arrest *in vivo*.

To obtain more detailed information about the proliferation pattern of PGCs, single PGCs from 8.5 dpc embryos were cultured separately on feeder cells (Ohkubo *et al.,* 1996). Counts of PGCs in each well showed weakly synchronized cell division for 4 days, followed by a decrease in number on day 5. It was noticed that PGCs in culture change their morphology according to their age and culture period. We divided the morphology of PGCs into three categories and counted each number separately in the clonal culture of 8.5 dpc PGCs (Fig. 3). The first type, «polarized» shows polarization and characteristics of motile cells. The second type, «spread» is well spread but shows no polarization. The last type, «round» is rounded up on the feeder cells. Ratios of the three types of PGCs started to change on day 3. The polarized type decreased, while the spread and round types increased. Such change simulates the developmental progress *in vivo* when PGCs arrive at the genital ridge and differentiate into nonmotile germ cells. It is worth to note that the three types of PGCs appeared in single PGC colonies very frequently on day 3 and 4. These results are consistent with the hypothesis that they are stochastically regulated events but not strictly determined by the age or number of cell division.

Growth factors for PGCs

Several studies have shown that the number of PGCs increases rapidly from 30-50 cells at 7.5 dpc toward around 3,000

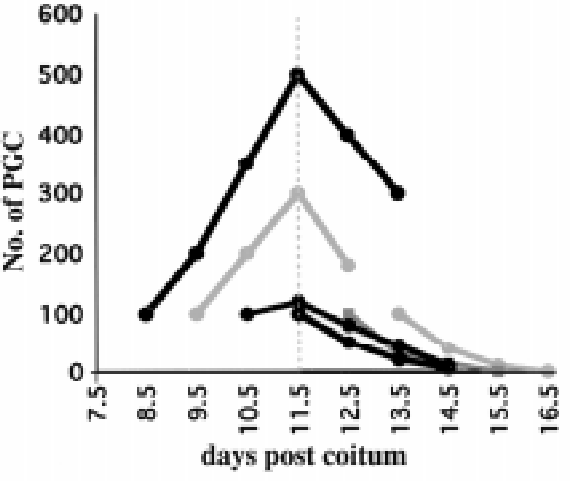

Fig. 2. Proliferative capacity of PGCs in culture. *Changes in PGC number when they were obtained from mouse embryos at various stages and cultured on feeder cells. PGCs at earlier stages proliferate for a few days before growth arrest, while those after arrival at fetal gonads stop their growth immediately in culture.*

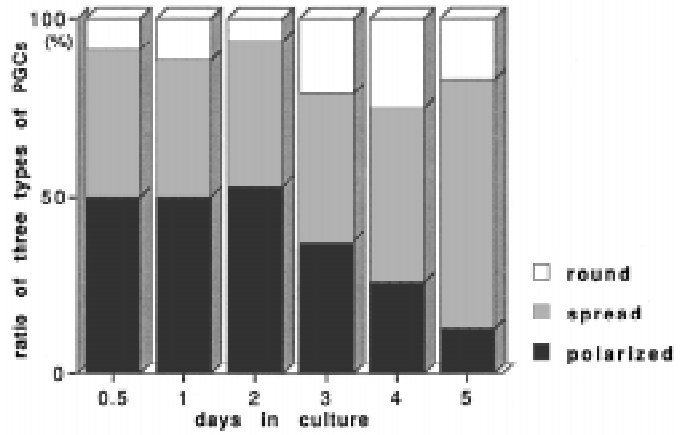

Fig. 3. Morphological change of PGCs in culture. *Changing ratios of the three types of PGCs in the clonal culture of PGCs obtained from 8.5 dpc embryos (Ohkubo et al., 1996).*

cells at 11.5 dpc (Snow *et al.,* 1981; Tam and Snow, 1981; Ginsburg *et al.,* 1990). They imply that PGCs proliferate at a cell cycle rate of 16 hours. Investigation of growth factors during this period has shown that SLF and LIF are both effective to support survival and proliferation of PGCs in culture (De Felici and Dolci, 1991; Dolci *et al.,* 1991; Godin *et al.,* 1991; Matsui *et al.,* 1991). Receptors for SLF, c-kit, are expressed on PGCs (Matsui *et al.,* 1990; Manova and Bachvarova, 1991). Even after addition of these growth factors to culture media, however, it is still not possible to obtain PGC proliferation at a rapid rate similar to that *in vivo*.

In search for other growth factors, we showed that tumor necrosis factor (TNF)-α stimulates proliferation of PGCs in culture (Kawase *et al.,* 1994). Such effect of TNF-α was evident for PGCs at earlier stages during migration (7.5-8.5 dpc), but it becomes ineffective at later stages (10.5-12.5 dpc). Then, we found that retinoic acid acts as a growth activator of mouse PGCs *in vitro* (Koshimizu *et al.,* 1995). Retinoic acid is known to induce differentiation of embryonic stem (ES) cells and thus inhibit proliferation of stem cells. However, unexpectedly, it increases growth of PGCs in culture and promotes derivation of EG cells from PGCs (Koshimizu *et al.,* 1996).

We also found that the conditioned medium made with Buffalo rat liver cells (BRL-CM) promotes proliferation of PGCs obtained from embryos at 7.5 to 11.5 dpc (Kawase *et al.,*1996).It is known that BRL cells secrete many growth factors and cytokines including soluble SLF (Zsebo et al.1990)and LIF (Smith et al.1988), both of which stimulate proliferation/survival of mouse PGCs. However, BRL-CM was much more effective than combination of soluble SLF and LIF to the culture medium.Moreover,a combination of BRL-CM,forskolin, and Sl/Sl4-m220 feeder cells that express the membrane-bound form of SLF stimulated rapid proliferation of PGCs at a rate similar to that *in vivo* (Kawase *et al.,* 1996).

Role of gp130 signaling in PGCs

Effects of LIF and LIF-related cytokines on PGC growth *in vitro* were examined in detail (Koshimizu *et al.,* 1996). Addition of LIF or OSM in culture prolonged survival of 10.5 dpc PGCs and slowed down the disappearance of PGCs isolated from 11.5 dpc genital ridges. In addition, the IL-6/sIL-6R complex retarded the depletion of PGCs effectively, confirming that these ligands acted on PGCs through gp130. The IL-6/sIL-6R complex induces homodimerization of gp130 and directly activates its downstrean signaling (Taga *et al.,* 1989; Yasukawa *et al.,* 1990; Yoshida *et al.,* 1994; Nichols *et al.,* 1994).

The functional contribution of gp130 to PGCs was further examined by using the neutralizing antibody against gp130 (Fig. 4). Its addition drastically inhibited the survival of PGCs isolated from fetal gonads. Effects on PGCs before arrival at genital ridges were less apparent. Thus, the functional contribution of gp130-mediated signaling seems to be more significant in the gonadal PGCs than the PGCs during migration.

De-regulation of growth arrest in PGCs and derivation of EG cells

EG cells were first reported to appear from the culture of PGCs in the presence of LIF and bFGF (Matsui *et al.,* 1992; Resnick *et al.,* 1992). We unexpectedly found that combination of LIF with forskolin or retinoic acid (RA) also causes the derivation of EG cells in the absence of bFGF (Koshimizu *et al.,* 1996). They were morphologically indistinguishable from EG cells produced by combination of LIF and bFGF.

The number of colonies in the presence of LIF plus forskolin or LIF plus RA was higher than that in LIF plus bFGF. In addition, a further increase of the colony number was observed in combination

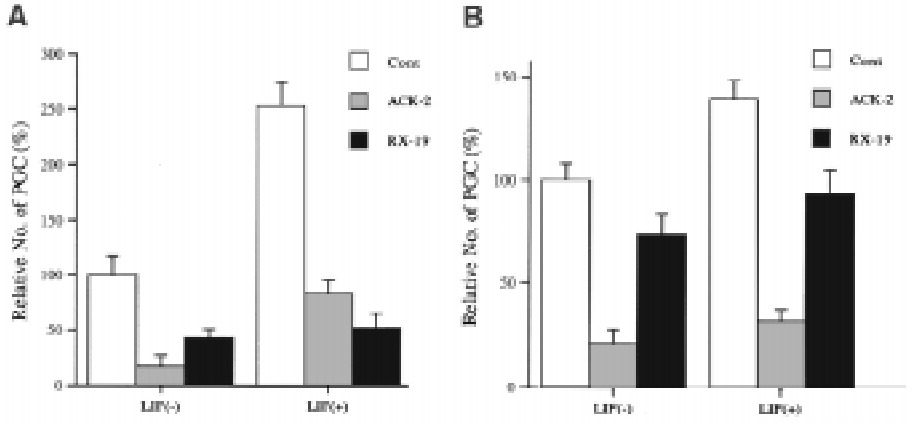

Fig. 4. Effects of anti-c-KIT or anti-gp-130 neutralizing antibodies on PGC growth in culture. *PGCs obtained from 11.5 dpc* **(A)** *or 8.5 dpc* **(B)** *embryos were cultured on feeder cells for 2 days in the absence (-) or presence (+) of 10^3 U/ml LIF (Koshimizu et al., 1996). In both groups, control rat immunoglobulin (Cont), anti-c-KIT monoclonal antibody (ACK-2), or anti-gp130 monoclonal antibody (RX-19) was added at 5 μg/ml. The number of PGCs cultured in the presence of control immunoglobulin alone (LIF(-), Cont) was designated as 100%. *, **; Significantly different from control at p<0.05 or 0.01, respectively.*

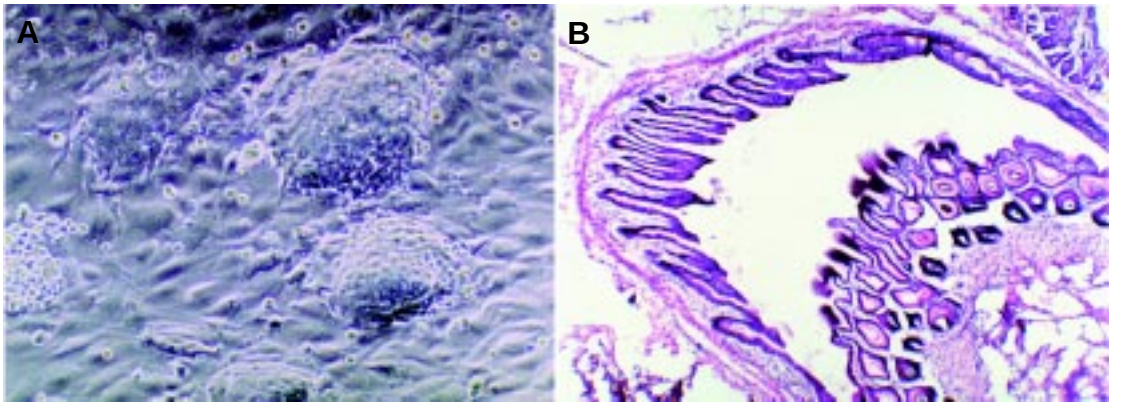

Fig. 5. EG cells derived from PGC culture. (A) *EG cell colonies obtained by culturing PGCs in the presence of LIF and forskolin or retinoic acid.* **(B)** *A histological section of the teratoma formed by transplantation of EG cells into the adult testis of W mutant mice.*

of three factors, namely, LIF, bFGF, and forskolin (or RA). These results indicated that bFGF and forskolin (or RA) activate independent signaling pathways and that they can work additively for the EG cell formation. However, ligands for gp130 was always indispensable for the appearance of EG cell colonies (Koshimizu *et al.,* 1996). We established several EG cell lines under this culture condition (Fig. 5). They produced cystic embryoid bodies and also differentiated into teratoma by transplantation into mouse testes (Chuma and Nakatsuji, unpublished results).

Thus, a combination of multiple signals for cell growth causes a small population of PGCs to escape from the growth arrest and re-initiate rapid proliferation. However, such EG cells seem to have lost many characteristics of germ cells and de-differentiate into pluripotent stem cells akin to the ES cells. Derivation of such EG cells is possible from the migratory PGCs and also from gonadal PGCs up to 12.5 dpc. It is interesting that EG cells derived from the gonadal germ cells showed the erased status of the genomic imprinting (Labosky *et al.,* 1994; Tada *et al.,* 1998). Thus, such EG cell lines possess both the important characteristics of the fetal germ cells and also the pluripotency to produce chimeras.

Cell-autonomous programming for transition into meiosis

Observation of ectopic germ cells (Zamboni and Upadhyay, 1983; Francavilla and Zamboni, 1985) and studies of reaggregated cultures of embryonic gonads (McLaren and Southee, 1997) suggested that both male and female PGCs show cell-autonomous entry into meiosis if they are set apart from the male gonadal environments. However, precise examination of the meiotic transition and identification of its regulative factors have been impeded by the lack of a dispersed culture system for fetal germ cells, in which the meiotic transition could be detected and analyzed in detail.

Female PGCs cease mitosis and enter into the prophase of the first meiotic division at around 13.5 dpc, progressing through the preleptotene, leptotene, zygotene, pachytene, diplotene stages and to diakinesis before arresting after birth (Borum, 1961; Speed, 1982). At the leptotene stage, each pair of sister chromatids forms a meiosis-specific longitudinal axial core to which the chromatin loops are attached. The Scp3 (Cor1/Sycp3) protein is one of the components of these axial cores (Heyting *et al.,* 1988; Dobson *et al.,* 1994; Klink *et al.,* 1997). Scp3 is a good marker to detect the meiotic transition, since its expression is highly specific for meiosis and it functions from the initial step of the first meiotic division (Yuan *et al.,* 2000). Another good marker for meiosis is Dmc1 that is a meiosis-specific mammalian RecA homologue (Yoshida *et al.,* 1998). We tried to devise a culture system of PGCs, in which patterns of meiotic transition and its regulative factors could be examined precisely (Chuma and Nakatsuji, 2001).

In the female gonads, expression of both *Scp3* and *Dmc1* were detected by RT-PCR from 12.5 dpc. Unexpectedly, male gonads also showed the expression of both genes at the same stages at lower levels. Using a rabbit polyclonal antibody that we raised against a synthetic polypeptide of the mouse Scp3 protein (Klink *et al.,* 1997), Scp3 was detected inside the nuclei of germ cells in the 12.5 dpc ovary. Then, formation of axial cores became visible as

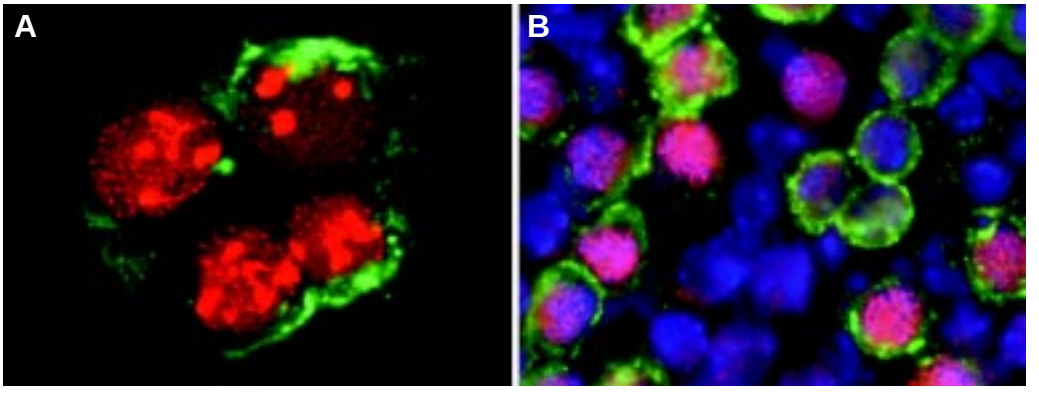

Fig. 6. Entry into meiosis by fetal germ cells *in vitro*. (A) *Meiotic germ cells in dispersed culture, stained with the anti-Scp3 (red) and anti-SSEA-1 (green) antibody. Female genital ridges at 11.5 dpc had been dissociated and cultured for 3 days on feeder cells. In such culture, PGCs expressed the Scp3 protein and later began to form the axial core structures.* **(B)** *Meiotic germ cells at the zygotene-pachytene stage in the fragment culture, stained with the antibody raised against synaptonemal complex proteins (red, kindly provided by Dr. Moens), anti-SSEA-1 antibody (green) and with Hoechst 33258 dye (blue). Fragments of the 11.5 dpc female genital ridge had been cultured for 4 days.*

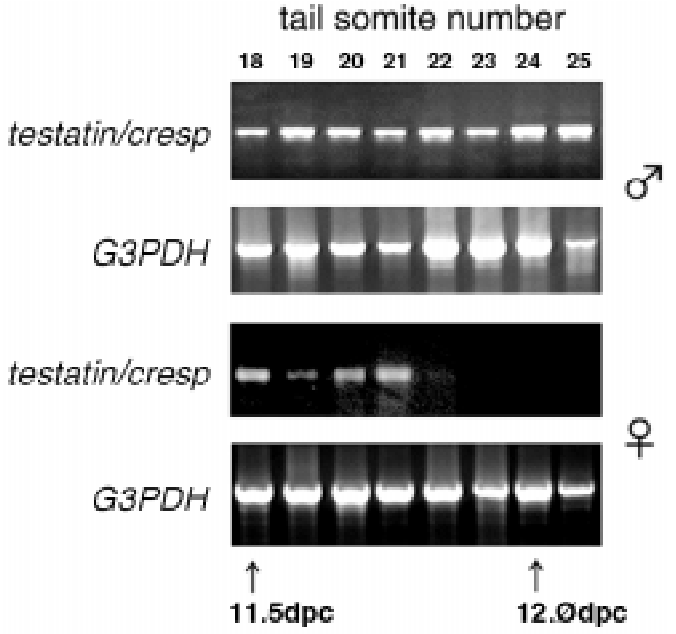

Fig. 7. RT-PCR analysis of testatin/cresp. *Stages of the development of embryos from 11.5 to 12.5 dpc were determined by counting the tail somites. Expression in the female gonad decreased to an undetectable level in the middle of these stages (Kanno et al., 1999). An embryo with 18 tail somites corresponds to the 11.5 dpc, and 24 somites to 12.0 dpc.*

fine fibrous structures inside nuclei at 13.5 dpc. In the male, no signal was observed at 12.5 dpc, but fetal testes at 13.5 - 15.5 dpc contained germ cells that showed weak Scp3 staining, as also reported by Di Carlo *et al.* (2000).

We next examined the expression of both genes by female PGCs in dispersed culture. Female urogenital ridges at 10.5 dpc were dissociated and cultured on Sl/Sl4 m220 feeder cells. RT-PCR products from both *Scp3* and *Dmc1* genes increased after 3 days of culture. Immunostaining confirmed that female PGCs obtained from urogenital ridges or genital ridges at 10.5-11.5 dpc started to express the Scp3 protein and later began to form axial cores (Fig. 6A), indicating that female PGCs entered into the leptotene stage of the first meiotic division after dissociation and cultivation on feeder cells. In addition, not only the PGCs isolated from the fetal gonad, migratory PGCs obtained from mesenteries at 10.5 dpc also expressed Scp3 and some of them formed axial cores after cultivation for several days. This observation supports the hypothesis that PGCs acquire meiotic competence before reaching the gonadal environment. (Chuma and Nakatsuji, 2001).

We seldom observed progression of meiosis to the zygotene-pachytene stages in this dispersed culture condition. Since germ cells at these progressed stages were frequently observed in fragmented or reaggregated cultures of female genital ridges (Fig. 6B), further progression of meiosis probably require three-dimensional structures with surrounding somatic and supportive cells.

Previous studies have shown that male PGCs also enter into meiosis if they had been isolated at 10.5 or 11.5 dpc and cultured in reaggregates in the absence of male gonadal environments (McLaren and Southee, 1997). Consistent results were obtained that male germ cells at 10.5 or 11.5 dpc formed axial cores when dissociated and cultured on feeder cells for several days, but those from the 12.5 or 13.5 dpc testes showed only weak expression of the Scp3 protein. Thus, an irreversible determination for the sex-differentiation of fetal germ cells seems to occur at around 12.5 dpc. The unexpected expression of these genes in male gonads *in vivo* also raises the possibility that male PGCs may start to prepare for meiosis until around 12.5 dpc but the male somatic cells produce intercellular signals such as putative meiosis-preventing substances (MPS) (Dolci and De Felici, 1990) to stop meiosis.

Signaling mechanisms involved in the sex differentiation of fetal germ cells

At 12.5 dpc, genital ridges show the first signs of sexual dimorphism with testicular cord formation in the males. During this period, several genes, such as *Sry, Sox9, Dax-1, WT1* and *Ad4BP/SF-1,* were shown to play important roles in the differentiation of somatic cells (reviewed in Swain and Lovell-Badge, 1999). However, there is little information about the signaling molecules involved in the somatic-germ cell interaction that directs the sex-differentiation of germ cells.

We attempted to isolate genes involved in the sex-differentiation of fetal germ cells by using subtraction and differential screening between male and female fetal gonads at 13.5 dpc (Tamura and Nakatsuji, unpublished results). One of novel genes found in the screening showed specific expression in the testis from the early stages of sex-differentiation (Kanno *et al.,* 1999). It was related to type II cysteine proteinase inhibitors, the cystatin family, and it had the highest similarity with the *CRES (cystatin-related epididymal specific)* gene (Barrett *et al.,* 1986; Cornwall *et al.,* 1992). After we named it *cresp*, a paper reporting the same gene named *testatin*, was also published by another laboratory (Töhönen *et al.,* 1998).

The cystatins are present in most tissues and biological fluids (Abrahamson *et al.,* 1986; Tavera *et al.,* 1990). In the testis, the cysteine protease cathepsin L (Elicson-Lawrence *et al.,* 1991) and its inhibitor cystatin C (Tavera *et al.,* 1990) are secreted from Sertoli cells. Expression of the *testatin/cresp* mRNA was almost confined exclusively to the male gonad and the expression increased immediately after the initiation of testis differentiation at 11.5 - 12.5 dpc, while it decreased to an undetectable level in the female during the same period (Kanno *et al.,* 1999) (Fig. 7). Töhönen et al. (1998) reported the expression of *testatin/cresp* in Sertoli cells and its precursors from 11.5 dpc, and they suggested that this gene is located downstream of *Sry* or *Sox9* during early events of the sex-differentiation in testicular somatic cells. In contrast, our results of

TABLE 1

CHANGES IN THE NUMBER OF PGCs AFTER TRANSFECTION OF *SV40LT, E1B 19K, BCL-2* OR *BCL-X_L* IN CULTURE
(Watanabe *et al.,* 1997)

Expression vector	Relative changes in PGC number (%)[#]				
pSV-LT	100.5	±	7.6	(n=15)	P=0.49
pEFBOS-E1B19k	184.9	±	17.2	(n=18)	P<0.001
pCAGGS-hbcl-2	202.3	±	25.0	(n= 4)	P<0.001
pCAGGS-c bcl-x_L	195.1	±	24.0	(n=11)	P<0.001

[#]The number of PGCs in the *pBSSK*-transfected well was used as the control (100%). Statistical significance of difference was evaluated by the Student t-test. Genital ridges at 11.5 dpc were dissociated and cultured on feeder cells.

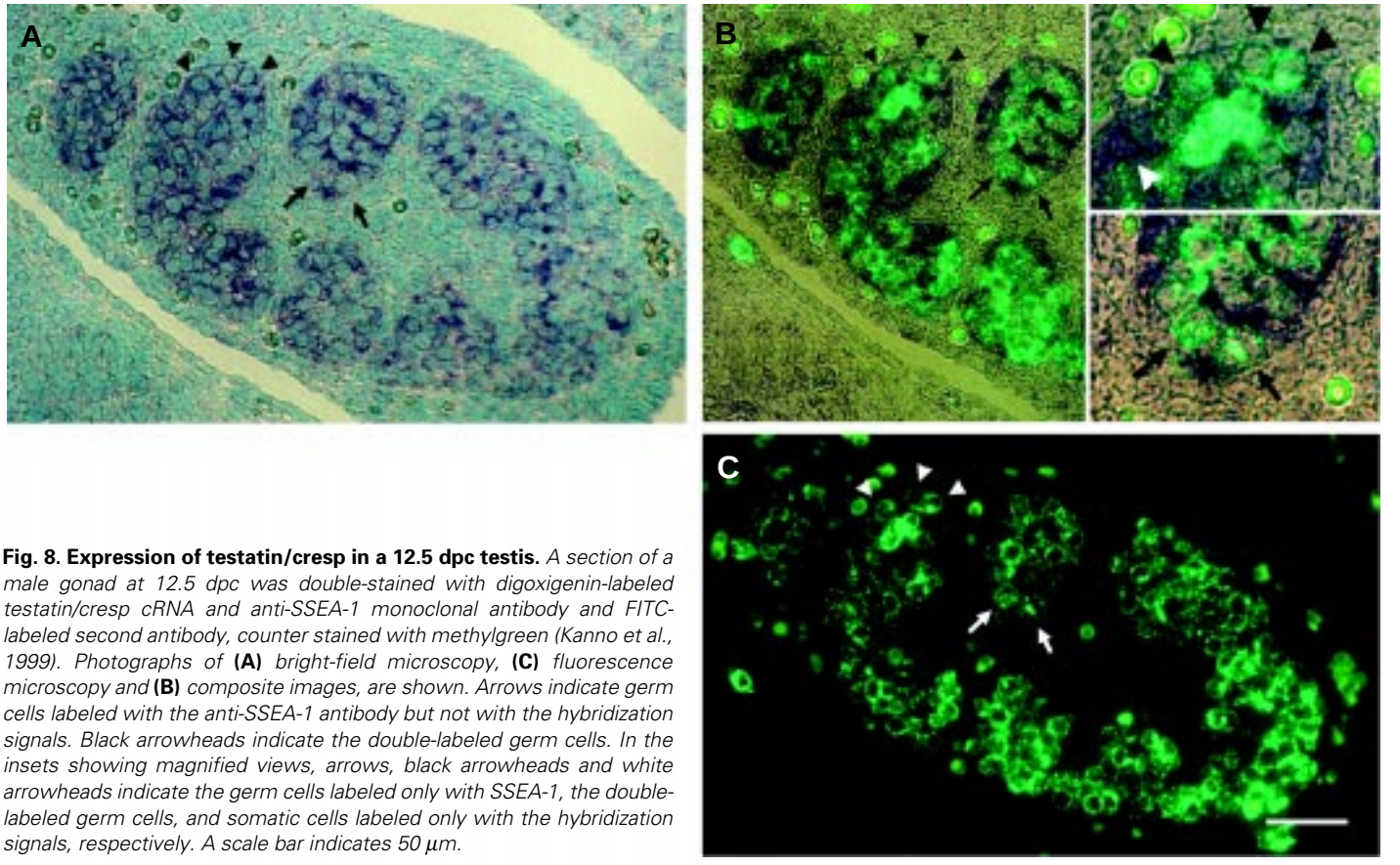

Fig. 8. Expression of testatin/cresp in a 12.5 dpc testis. *A section of a male gonad at 12.5 dpc was double-stained with digoxigenin-labeled testatin/cresp cRNA and anti-SSEA-1 monoclonal antibody and FITC-labeled second antibody, counter stained with methylgreen (Kanno et al., 1999). Photographs of* **(A)** *bright-field microscopy,* **(C)** *fluorescence microscopy and* **(B)** *composite images, are shown. Arrows indicate germ cells labeled with the anti-SSEA-1 antibody but not with the hybridization signals. Black arrowheads indicate the double-labeled germ cells. In the insets showing magnified views, arrows, black arrowheads and white arrowheads indicate the germ cells labeled only with SSEA-1, the double-labeled germ cells, and somatic cells labeled only with the hybridization signals, respectively. A scale bar indicates 50 μm.*

in situ hybridization and immunohistochemical analyses demonstrated that the *testatin/cresp* mRNA was localized both in the germ and Sertoli cells in the fetal and adult testes (Fig. 8). (Kanno *et al.,* 1999). Germ cells take different developmental pathways depending on the sex of the gonadal somatic cells. The upregulation of *testatin/cresp* expression in the male gonad and its downregulation in the female takes place immediately before such sex-differentiation of the germ cells, thus suggesting its important function.

Regulation of apoptosis in fetal germ cells

Another aspect of germ cell development is regulation of apoptosis. It is well known that a large population of germ cells goes into apoptosis during development of female and male gonads, although its biological significance is unknown. PGCs obtained from the genital ridge undergo apoptosis in culture (Pesce *et al.,* 1993) and those in the extragonadal sites and within developing gonads show the hallmarks of apoptosis (Coucouvanis *et al.,* 1993).

Enforced expression of the adenovirus *E1B 19K* gene in cultured somatic cells can block apoptosis induced by various agents such as TNF-α and Fas antigen (Gooding *et al.,* 1991; Hashimoto *et al.,* 1991; White *et al.,* 1992). Also, the overexpression of *bcl-2* or *bcl-X*$_L$ gene can prevent apoptosis caused by various stimuli (Vaux *et al.,* 1988; Hockenbery *et al.,* 1990; Nunez *et al.,* 1990). Therefore, we tested if the forced expression of *E1B 19K, bcl-2,* or *bcl-X*$_L$ prevents the apoptosis of PGCs in culture (Watanabe *et al.,* 1997). The transient expression of these genes in PGCs obtained

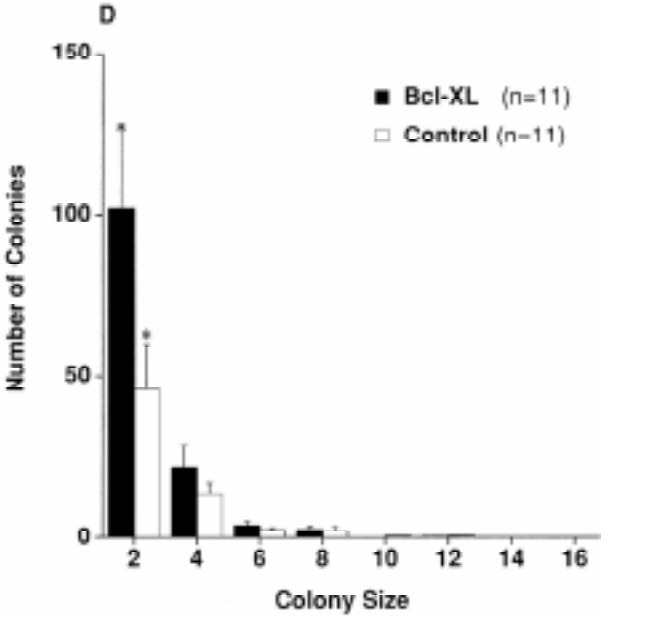

Fig. 9. Effects of the forced expression of bcl-xL in PGCs. *Histogram showing the distribution of the colony size of PGCs transfected with pCAGGS-cbcl-xL or pBSSK as control (Watanabe et al., 1997). PGCs obtained from 11.5 dpc embryos were cultured for 1 day, transfected and cultured for an additional 2 days.*

from the genital ridge at 11.5 dpc resulted in a remarkable increase of the surviving PGCs in culture (Table 1). Such effects of E1B 19K and bcl-xL were more pronounced in case of the round-shaped PGCs that were present as single or pairs of cells, than the polarized or spread type PGCs that made larger colonies (Fig. 9). Thus, the disappearance of the rounded PGCs that represent more advanced stages can be more effectively inhibited by the expression of these apoptosis inhibiting genes.

In good accordance with these results, a recent study using mice with hypomorphic mutation (Rucker *et al.,* 2000) of the *bcl-x* gene reported a large loss of germ cells in the fetal gonad and adult testis and ovary. Our study (Kasai *et al.,* unpublished results) also indicates a decrease of spermatogenic cells in the testis when gene dosage was halved in the heterozygotes for the *bcl-x* null mutation (Motoyama *et al.,* 1995).

As described in this review, many aspects of molecular mechanisms that regulate differentiation into female or male germ cells still remain unknown. Utilization of *in vitro* culture systems would facilitate such studies. Intracellular and intercellular signaling in the regulation of sex-differentiation of germ cells into oocytes or prospermatogonia remains to be one of interesting and unsolved problems of the mammalian germ-line development.

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