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[REVIEW]

Hormonal Control of Meiosis Initiation in the Testis from Japanese Newt, *Cynops pyrrhogaster*

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ABSTRACT—Meiosis is an event that occurs prerequisitely and specifically in gametogenesis. However, the mechanisms of conversion from mitosis to meiosis are poorly understood. I will review the results so far obtained by us using newt testis as a model system, and discuss about the extrinsic mechanism(s) controlling the conversion from mitosis to meiosis. In the newt spermatogonia enter meiosis in the 8th generation after 7 mitotic divisions. We developed organ and reaggregate culture systems with a chemically defined medium in which porcine follicle-stimulating hormone (pFSH) promotes spermatogonial proliferation and differentiation into primary spermatocytes. Human recombinant stem cell factor (RhSCF) *in vitro* stimulates the spermatogonial proliferation and progression to the 7th generation, but not the differentiation into primary spermatocytes; instead they die of apoptosis. The reason why rhSCF does not stimulate meiosis entrance seems to be due to the low level expression of c-kit protein at the 7th generation of spermatogonia. Ovine PRL induces apoptosis in the 7th generation of spermatogonia *in vivo* and *in vitro*. Incubation of newts at low temperature causes spermatogonial apoptosis by the elevation of plasma PRL titer. In the absence of FSH in organ culture spermatogonia can progress until the 7th generation, but the 8th generation never appear due to the apoptosis. Altogether there seems to be a regulatory checkpoint for entrance into meiosis in the 7th generation. Spermatogonia could circumvent the checkpoint by the influence of some factor(s) produced by Sertoli cells upon activation by FSH. Trial to isolate factor(s) responsible for the meiosis-initiation is now underway.

Key words: meiosis entrance, spermatogenesis, FSH, prolactin, apoptosis

INTRODUCTION

Meiosis is an event that occurrs prerequisitely and specifically in gametogenesis. During spermatogenesis spermatogonia proliferate and enter meiosis after species-specific number of somatic divisions (Fig. 1A) (Roosen-Runge, 1977). These processes involve interactions between germ cells and somatic cells, especially Sertoli cells, in the testis and actions of hormones secreted by the pituitary and testis (Parvinen *et al.,* 1986; Skinner, 1991; Jegou, 1993).

The studies in *Drosophila* suggest that cell-extrinsic signaling, together with cell-intrinsic factors, regulates progression through these processes. *bag-of-marbles* (*Bam)* and *benign-gonial-cell-neoplasm* (*bgcn)* are indicated to act cell-intrinsically to restrict the amplification of spermatogonial cells or to promote their entry into the meiotic cell cycle (Gonzy *et al.,* 1997). Schnurri, a transcription factor required for response to *dpp*like ligands (Arora *et al.,* 1995; Grieder *et al.,* 1995; Staehling-Hampton *et al.,* 1995), and Punt, a type II receptor (Letsou *et al.,* 1995; Ruberte *et al.,* 1995), are found to regulate progression through the male germline stem cell lineage and that the activity of these genes is not required in the germline cells, but rather within the somatic cyst cell lineage which corresponds to Sertoli cells in vertebrates (Matunis *et al.,* 1997). Soma-to-germ line signal produced by the cyst cell lineage limits the amplification of committed male germline precursor cells. The involvement of both schnurri and punt indicates the involvement of an initiating signal that targets the cyst cell lineage and belongs to the TGF-β/*dpp*/ BMP2 superfamily. TGF-β signal transduction within the cyst lineage specifically activates a second, soma-to germline signal required for germ cells to exit mitosis and enter the meiotic cell cycle. However, the soma to

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germline signal has yet to be identified.

Intrinsic and extrinsic mechanisms may also be involved in meiosis entrance in vertebrates. However, the mechanisms of the conversion from mitosis to meiosis are poorly understood. To address these questions, especially extrinsic mechanism, our laboratory has performed several approaches using organ culture of testicular fragments from Japanese newt, *Cynops pyrrhogaster*. Newt testis is an ideal vertebrate model for investigating the mechanism(s) controlling the conversion from mitosis to meiosis. I will review the results so far obtained by us and discuss about the mechanism(s) controlling the conversion from mitosis to meiosis.

Structure of newt testis and spermatogenesis

As in other anamniote species, spermatogenesis in urodeles occurs within spermatocysts comprising germ and Sertoli cells (Fig. 1B) (Callard *et al*. 1978). Germ cells within a cyst proliferate and differentiate synchronously; even the stage during cell cycle is synchronous. A larger unit of the testis is a lobule that consists of some cysts and is ensheathed by basement membranes. A lobule corresponds to a seminiferous tubule in amniote species. Within a lobule the stage of the germ cells is almost the same, but not as much as in cysts; cell cycle stages are asynchronous among cysts in a lobule and two successive generations of spermatogonia are within a lobule. Outside the lobules are Leydig-like cells that are also called pericystic cells or lobule boundary cells. Also blood vessels and red blood cells are present outside the lobules. A newt testis consists of a couple of lobes, though the number is various. Each lobe is connected by a slender cord and has an entire structure and function as an independent testis.

In adult mammals, Sertoli cells are permanent elements of the seminiferous tubule and a single Sertoli cell is associated with several germ cell generations, nurturing generation after generation of the germ cells. In contrast, urodelen Sertoli cells in each cyst make contact with germ cells in the same stage of differentiation. The Sertoli cells degenerate after spermiation and proliferate again along with the proliferation of the spermatogonia.

The testis displays well-marked zones of spermatogenic cell types because the lobules formed at the cephalic region gradually acquire more caudal positions as the cells mature (Fig. 1A) (Callard *et al*., 1978). When longitudinal sections of newt testis are made, all spermatogenic stages from spermatogonia to the most advanced stage for the season can be observed. These characteristics permit us to isolate germ cells in specific stages of spermatogenesis.

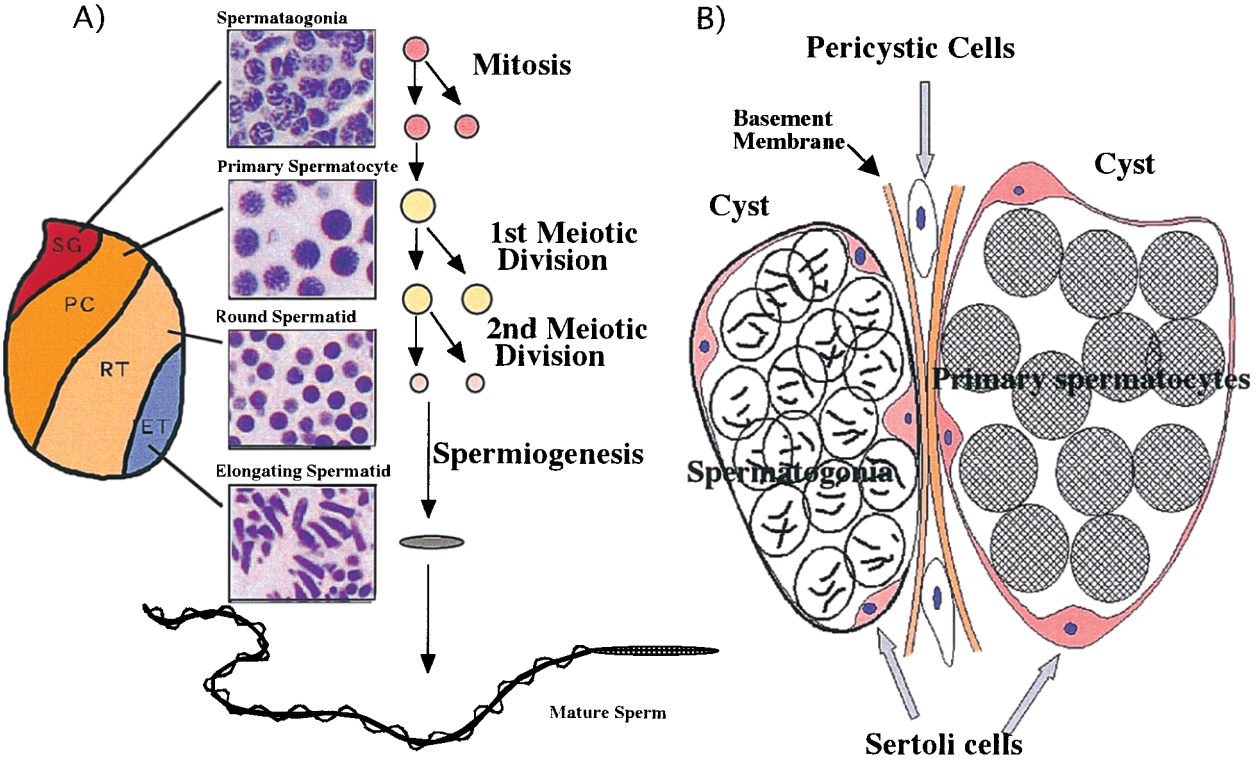

Fig. 1. Spermatogenesis and testicular structure in newt. A) Newt testis consists of several zones each consisting of the same stage of spermatogenesis; spermatogonial stage (SG), primary spermatocyte stage (PC), round spermatid stage (RT) and elongating spermatid stage (ET). The ratio of volume among several stages depends on seasons. Mature part is neglected. After several rounds of mitosis, spermatogonia differentiate into primary spermatocytes that undergo two meiotic divisions, giving rise to round spermatids that proceed through spermiogenesis and eventually differentiate into mature sperm. B) The smallest unit of the testis is a cyst consisting of a germ cell clone derived from single spermatogonia, and some somatic cells, Sertoli cells. Some cysts are packed in a lobule that is surrounded by basement membrane. Outside the lobules are some Leydig-like cells called pericystic cells.

Throughout winter and early spring, the testis consists largely of mature part with creamy color that contains lobules with almost mature sperm embedded in Sertoli cells, and a thin layer of translucent immature part that contains lobules only with spermatogonial stage (Tanaka and Iwasawa, 1979). When ambient temperature rises, spermatogonia proliferate and the immature part increases in size, while the mature part degenerates. Proliferation of spermatogonia is followed by entrance into meiosis in early summer and extensive meiotic divisions are observed in mid-summer. Completing two successive meiotic divisions, the resultant haploid round spermatids undergo morphogenetic processes called spermiogenesis, such as formation of flagella and acrosomes, nuclear elongation and condensation, mitochondrial assembly, etc. In late fall spermiogenesis almost completes. After spermiation, the caudal part of the mature part changes into yellowish glandular tissue in which Sertoli cells degenerate and pericystic cells proliferate.

These annual changes of spermatogenesis depend mainly on temperature, because when the newts obtained during winter or early spring are transferred to room temperature and fed regularly, spermatogonia begin to proliferate and differentiate into primary spermatocytes that undergo meiotic divisions and subsequently differentiate into elongate spermatids.

Seasonal changes in gonadotropic activity and steroids

Tanaka and Takikawa (1984) compared biological properties of newt gonadotropins (GTHs) using isoelectric focusing (IEF) profiles and *Xenopus* RRA assay (LH-like activity) and *Anolis* RRA assay (FSH-like activity). Only in July when the activities of GTH in the pituitary and spermatogenesis are very high, *Anolis* RRA gives two additional peaks, neutral to acidic region, while no such peaks were found by *Xenopus* RRA, indicating that FSH is excreted by the pituitary. On the other hand, in February the IEF patterns of the pituitary GTH assayed by *Xenopus* RRA is very similar to that found by *Anolis* RRA. Presence of both FSH and LH in newt pituitary was recently demonstrated by isolation of newt *FSHβ* and *LHβ* cDNAs and immunocytochemical detection of the antigens in the pituitary (Saito *et al.,* 2002).

Plasma testosterone (T) levels measured by radioimmunoassay (RIA) show two distinct peaks, one in early spring and the other in autumn (Tanaka and Takikawa, 1984). Maximum levels are in February and October, and the minimum levels are in July. On the other hand, 5α-dihydrotestosterone (DHT) levels show three peaks; the highest peak is in May and two lower peaks are in early spring and autumn. Remarkable increase in May is of special interest, but the significance is unknown. Seasonal changes of plasma 17β-estradiol levels also show two distinct peaks, one in May and another one in summer. In urodele amphibian *Necturus maculosus* testis, aromatizing enzyme is very active and ovine FSH primarily stimulates secretion of estrogen (Callard *et al.,* 1978; Bolaffi and Callard, 1981). Δ^5-3β-hydroxysteroid dehydrogenase (3β-HSD) reaction is detected strongly in pericystic cells in glandular tissue from October to June, but barely in germ cells or Sertoli cells (Imai and Tanaka, 1978). These results may indicate a functional relationship between estrogen production by testis and regulation of spermatogenesiss in urodele amphibia (Tanaka and Takikawa, 1984), but the role of estrogen in newt spermatogenesis has not been clarified yet.

Hormonal regulation in newt spermatogenesis

Since recombinant newt FSH and LH have not been available yet, effects of mammalian FSH and LH were examined by several investigators. Treatment of hypophysectomized adult *Triturus cristatus carnifex* with FSH plus LH stimulated maturation of spermatogonia to the pachytene primary spermatocytes (Vellano *et al.,* 1974). Injections of FSH to hypophysectomized *Pleurodeles* promoted spermatogenesis but caused no effect on glandular tissue, while addition of LH caused 3β-HSD reactions in the glandular tissue but no change in germ cells (Andrieux *et al.,* 1973). Injections of FSH to hypophysectomized *Ambystoma tigrinum* larvae stimulated proliferation of secondary spermatogonia and their differentiation into spermatocytes (Moore, 1975). In *Cynops pyrrhogaster*, ovine FSH promoted spermatogenesis, while LH has potency in testosterone production (Tanaka and Takikawa, 1984). Also the authors showed that FSH is more potent at a higher temperature (18°C), whereas LH has potency at a lower temperature (8°C).

Morphological changes during spermatogenesis

A primary spermatogonium (G1) of the newt is surrounded by a couple of Sertoli cells and undergoes 7 mitotic divisions in a cyst before entering meiosis (Fig. 2) (Yazawa *et al*., 2000a). The primary spermatogonial nuclei are lightly stained and the largest among the spermatogenic cells (Figs. 2A). On the other hand, cysts with secondary spermatogonia contain multiple germ cells surrounded by Sertoli cells (arrowheads in Figs. 2B, C and D). The nuclear diameters of secondary spermatogonia decrease from G2 to G5, but then remain almost constant from G5 to G8. Subsequently, the spermatogonia differentiate into preleptotene spermatocytes (Fig. 3). Active preleptotene spermatocytes are in the premeiotic S phase, and their nuclei are morphologically intermediate between secondary spermatogonia and leptotene spermatocytes; the early preleptotene spermatocytes contain granular heterochromatin similar to that of secondary spermatogonia, whereas the late ones contain fine reticular chromatin similar to leptotene spermatocytes (Fig. 3). The nuclear diameters of the leptotene spermatocytes increase until the late pachytene stage. Thereafter the spermatocytes undergo two successive divisions, and the nuclear diameters in round spermatids are about one half that of late pachytene spermatocytes.

That germ cells develop synchronously within each cyst permits us to estimate the number of spermatogonial generations by estimating the number of germ cells (N) in a given cyst, using Abercrombie's formula (Abercrombie, 1946).

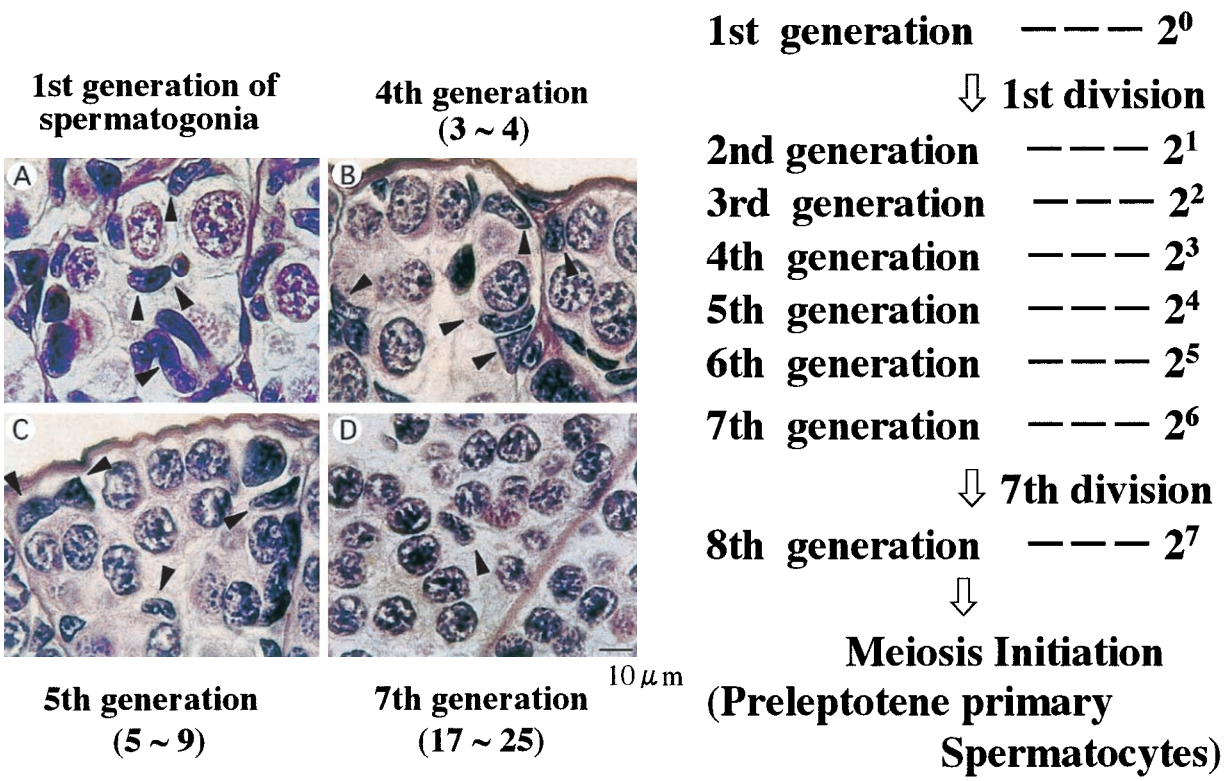

Fig. 2. Progression of spermatogonial generations and meiosis initiation. Testes were fixed by Bouin, embedded in paraffin and the sections were stained by hematoxylin and eosin. Arrowheads show nuclei of Sertoli cells. After the 7th division, spermatogonia initiate meiosis in the 8th generation and differentiate into preleptotene spermatocytes. Reproduced by permission of Blackwell Publishing Ltd from Fig. 1 in a paper by Yazawa *et al*. (2000a).

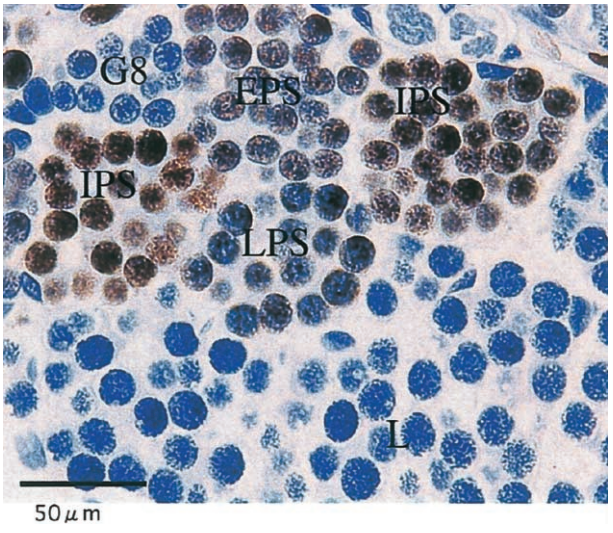

Fig. 3. Meiosis initiation in the 8th generation of spermatogonia (G8). BrdU incorporated into spermatogonia during organ culture of testicular fragments was detected by monoclonal antibody against BrdU and horseradish peroxidase-conjugated anti-mouse IgG, followed by incubation in 3, 3'-diaminobenzidine. The sections were counterstained by hematoxylin. Intermediate preleptotene spermatocytes (IPS) incorporated BrdU to high extent, while early preleptotene spermatocytes (EPS) and late preleptotene spermatocytes (LPS) incorporate BrdU to less extent. L, leptotene primary spermatocytes. Reproduced by permission of Blackwell Publishing Ltd from Fig. 3 in a paper by Yazawa *et al*. (2000a).

Conventionally we can estimate the generation of the spermatogonial cyst by counting the number of spermatogonial nuclei in a cyst in a section containing the maximum cyst size as follows; 3–4, 2^3/cyst (fourth generation); 5–9, 2^4/cyst (fifth generation); 10–16, 2^5/cyst (sixth generation); 17–25, 2^6/cyst (seventh generation); 26-33, 2^7/cyst (eighth generation). Spermatogonia enter meiosis in the eighth generation after seven mitotic divisions.

Expression of *Dmc1* mRNA during newt spermatogenesis

To get a molecular marker for meiosis initiation, we cloned a cDNA fragment for newt DMC1 from the newt testis, based on the conserved sequence of mammalian *Dmc1* homologue (Yazawa *et al.,* 2000a). *Dmc1* is an *Eschericia coli* RecA-like gene that is specifically active in the meiotic processs (Habu *et al.,* 1996; Pittman *et al.,* 1998; Yoshida *et al.,* 1998). With this cDNA probe, we isolated the newt *Dmc1* cDNA which is 2017 nucleotides in length. The predicted newt DMC1 protein consists of 342 amino acids and shows 88.3, 89.5 and 52.9% protein sequence similarity to mouse DMC1, human DMC1 homologue and newt RAD51, respectively. Two ATP binding motifs conserved in yeast and mammalian RAD51 and DMC1 were observed in the predicted newt DMC1 protein.

Northern blot analysis shows that 2.0 kb mRNA is strongly expressed in testes fragments enriched with pri-

mary spermatocytes, but not in fragments rich in spermatogonia (Yazawa *et al.,* 2000a). RT-PCR performed using oligo(dT)-primed cDNAs as a template prepared from total RNA derived from highly purified fractions of germ or somatic cells (mostly Sertoli cells) showed that PCR products were detected in germ cells but barely in somatic cells.

In situ hybridization shows expression of newt *Dmc1* mRNA at high level in leptotene-zygotene spermatocytes (Figs. 4A, C), weakly in pachytene spermatocytes (Fig. 4D) and spermatids (Fig. 4E), and barely in secondary spermatogonia (Fig. 4A) (Yazawa *et al.,* 2000a). The earliest expression occurs as dots, though quite weak, in cysts containing 2^7 germ cells that correspond to preleptotene spermatocytes (G8) (Figs. 4B, b). Thus, newt *Dmc1* mRNA is initially expressed at the beginning of meiosis. This finding indicates that the *Dmc1* mRNA could serve as an excellent marker for conversion from spermatogonia into primary spermatocytes.

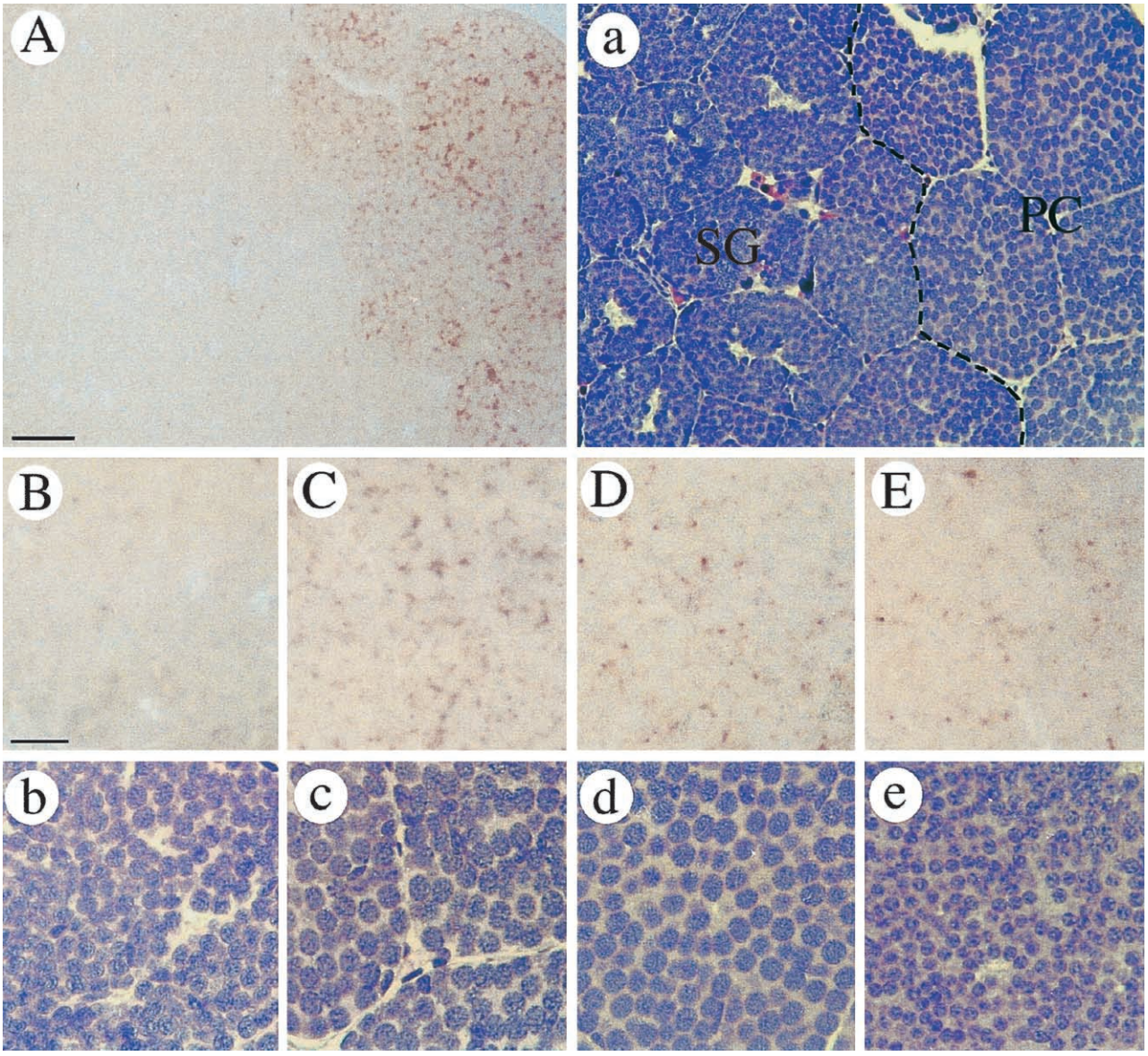

Fig. 4. *In situ* hybridization with anti-sense DIG-labeled *Dmc1* cRNA probes (A~E). The adjacent sections were stained with hematoxylin and eosin (a~e). (A) shows clear expression of *Dmc1* mRNA in primary spermatocyte stage, whereas no expression in spermatogonial stage. (a), Left side from the dotted line is spermatogonial stage (SG), while the right side is primary spermatocyte stage (PC). (B and b), preleptotene primary spermatocyte stage; (C and c), leptotene-zygotene stage; (D and d), pachytene stage; (E and e), round spermatid stage. Scale in (A and a), 100 μm. Scale in (B–E) and (b–e), 50 μm. Reproduced by permission of Blackwell Publishing Ltd from Fig. 8 in a paper by Yazawa *et al*. (2000a).

Induction of meiosis initiation by mammalian follicle-stimulating hormone (FSH) in organ culture of testicular fragments

In order to elucidate the molecular mechanisms whereby meiosis initiation is regulated, we developed an organ culture system with a chemically defined medium (Ji *et al.,* 1992; Abé and Ji, 1994). When testicular fragments containing only spermatogonial stage (Fig. 5A) were cultured in a basal medium (Leibovitz-15 alone) for 2 weeks, number of living germ cells decreased and most of the cysts remained as secondary spermatogonia (Fig. 5B). On the other hand, in the medium supplemented with porcine (or ovine) follicle-stimulating hormone (pFSH) alone, spermatogonia were stimulated to proliferate and differentiate into primary spermatocytes (Fig. 5C). Effective concentration range of pFSH (from Sigma) was 1~5 μg/ml. However, highly purified pFSH from NIH was recently tested and found to be effective in the range of 50~200 ng/ml. Neither the addition of highly purified luteinizing hormone nor androgens (testosterone and 5α-dihydrotestosterone) to the basal medium stimulated differentiation. Consistent with these findings was the fact that radioreceptor assays revealed high affinity specific binding sites for FSH but none for LH for the spermatogonial stage (Ji *et al.,* 1992). Binding experiments for fractionated germ and somatic cells indicated that FSH binds to somatic cells (mostly Sertoli cells) but not to germ cells (spermatogonia and primary spermaticytes) (Ji *et al.,* 1995). To check a possibility that steroids other than testosterone or 5α-dihydrotestosterone that might be produced by the fragments exposed to FSH promote the differentiation of spermatogonia to primay spermatocytes, the effect of cyanoketone (0.1~10 μg/ml), a specific inhibitor of 3β-hydroxy-Δ^5-steroid dehydrogenase (3β-HSD), was examined and found to be not inhibitory to the differentiation at all (Ji *et al.,* 1992).

To corroborate that FSH works via Sertoli cells, co-cul-

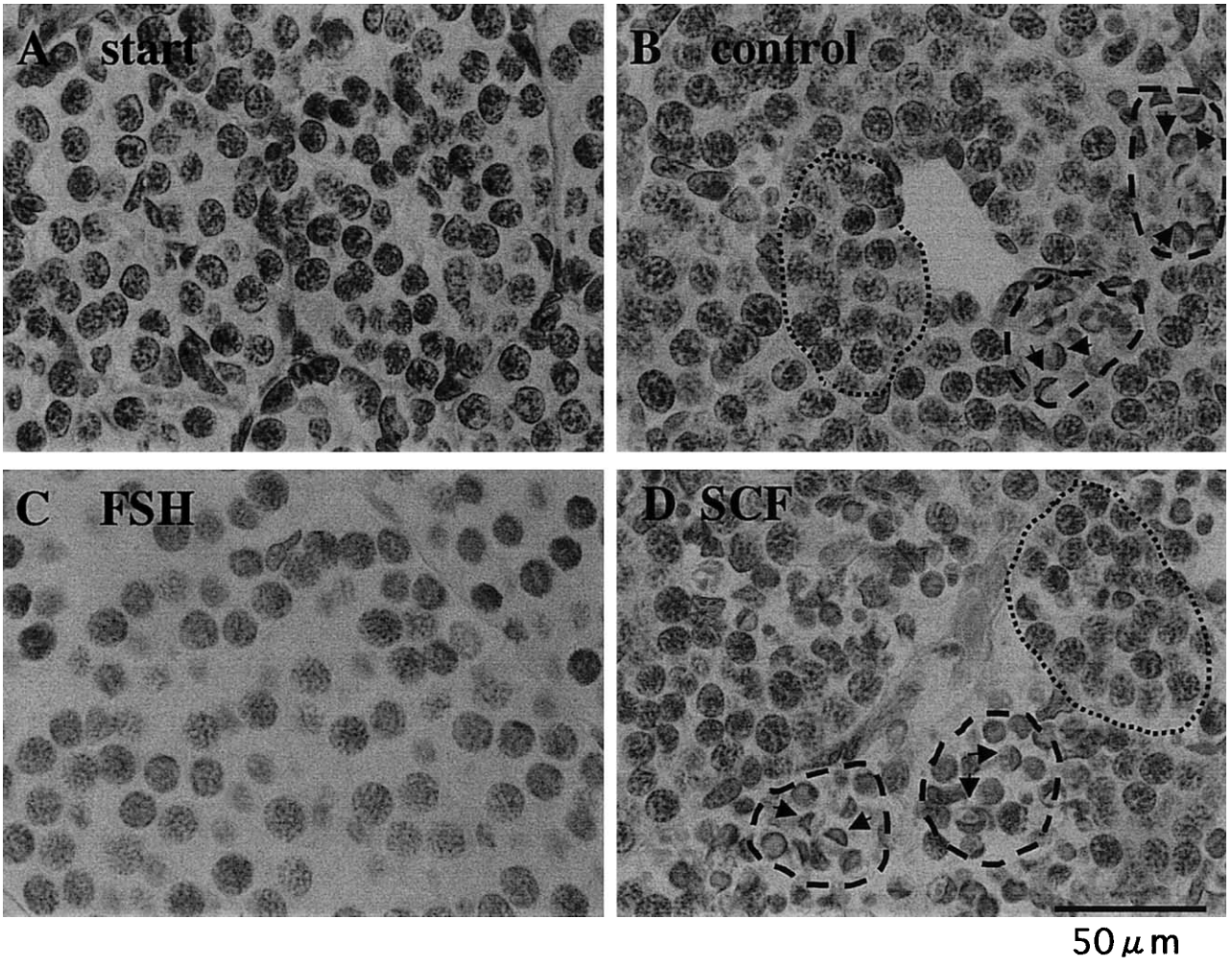

Fig. 5. Effect of FSH and SCF on the differentiation of spermatogonia in organ culture of testis fragments. A) 0 day of culture. B) ~D) two weeks of culture. B) Cultured in control medium. Some apoptotic nuclei of spermatogonia are observed (arrows). A cyst consisting of the 7th generation of spermatogonia is encircled by a thin dotted line. C) Cultured in the presence of FSH. Many primary spermatocytes are observed. D) Cultured in the presence of hSCF. Spermatogonia proceed until the 7th generation (encircled by a thin dotted line) but no primary spermatocytes appeared. Instead spermatogonia died due to apoptosis (arrows).

ture systems of spermatogonia and somatic cells (mainly Sertoli cells) were established. First, reaggregates of fractionated spermatogonia alone or with Sertoli cells were made by rotation cultures in suspension. In this system, [^{3}H]thymidine incorporation into spermatogonia was enhanced by FSH in the presence of Sertoli cells, but not in the absence of Sertoli cells (Maekawa *et al.,* 1995). However, viability of the spermatogonia rapidly declined and no primary spermatocytes differentiated.

Then we developed another culture system; larger reaggregates of cells were embedded within a collagen matrix and placed on a filter which floated on the medium like an organ culture (Ito and Abe, 1999). In reaggregates consisting of spermatogonia and somatic cells, spermatogonia were stimulated of their proliferation to more extent in the presence of FSH than in the absence, and differentiated into primary spermaticytes only in the presence of FSH. However, as the number of somatic cells contained in reaggregates of spermatogonia is reduced, the viability and proliferative activity of spermatogonia decreases even in the presence of FSH; spermatogonia barely differentiate into primary spermatocytes. These results indicate that proliferation of spermatogonia and their differentiation into primary spermatocytes are mediated by Sertoli cells that are activated by FSH.

We isolated a cDNA encoding a FSH receptor (FSH-R) from newt testis (Nakayama *et al.,* 2000). The total sequence homology in the deduced protein of the newt was approximately 70% with mammalian FSH-Rs. COS cells, transiently transfected with the cloned newt *FSH-R* cDNA, displayed specific binding to [^{125}I] human FSH and cAMP accumulation, indicating that the cloned cDNA encodes a functional newt FSH-R protein. Northern blot analysis revealed a single transcript of approximately 3.0 kb length that was synthesized in testicular somatic cells (mainly Sertoli cells) from spermatogonial to spermatid stages with the highest expression level during the primary spermatocyte stage. These results demonstrate that FSH stimulates newt spermatogenesis through the FSH-R.

Growth factors involved in spermatogonial proliferation (I): stem cell factor (SCF)

Stem cell factor (SCF)/c-kit system is known to play a pivotal role in survival (Packer *et al.,* 1995; Hakovirta *et al.,* 1999; Yan *et al.,* 2000a), proliferation, and differentiation of germ cells (Yoshinaga *et al.,* 1991; Manova *et al.,* 1993; Vincent *et al.,* 1998; Sette *et al.,* 2000) and Leydig cells (Yan *et al.,* 2000b) in mammalian testis. SCF is a ligand synthesized by Sertoli cells in the testis (Motro *et al.,* 1991; Rossi *et al.,* 1991; Tajima *et al.,* 1991) and the c-kit is a receptor for SCF that is expressed by spermatogonia and Leydig cells (Motro *et al.,* 1991; Manova *et al.,* 1990; Sorrentino *et al.,* 1991). Binding of SCF to the c-kit receptor activates the tyrosine receptor kinase, followed by activation of phosphoinositide 3-kinase (PI3-K) and p70 S6 kinase (Feng *et al.,* 2000). C-kit receptor is a conservative molecule and the cDNA is isolated in lower vertebrates such as chicken (Sasaki *et al.,* 1993), *Xenopus* (Baker *et al.,* 1995; Kao and Bernstein, 1995) and zebrafish (Parichy *et al.,* 1999a). *SCF* cDNA is also isolated in chicken (Zhou *et al.,* 1993), *Xenopus* (Yamamoto *et al.,* in preparation) and salamander (Parichy *et al.,* 1999b). So, SCF/c-kit system is also expected to be involved in newt spermatogenesis.

Addition of human recombinant SCF (RhSCF) to newt testicular organ culture stimulates the spermatogonial proliferation, and they progressed to the 7th generation that is the penultimate stage before primary spermatocyte stage. However, the spermatogonia do not differentiate into primary spermatocytes, but instead die due to apoptosis (Fig. 5D) (Abe *et al*., 2002). These results are consistent with the previous results in mice that addition of SCF to cultured male germ cells stimulates DNA synthesis in type A spermatogonia (Hakovirta *et al.,* 1999; Rossi *et al.,* 1993) and that intravenous injection of an anti-c-kit blocking antibody results in a loss of differentiating type A spermatogonia, but has no effect on the transition of intermediate spermatogonia to type B spermatogonia or to primary spermatocytes (Yoshinaga *et al.,* 1991).

We have recently isolated cDNAs for newt SCF (nSCF) and c-kit receptor (nc-kit) and raised specific antibodies against nSCF and nc-kit. Preliminary studies showed that the mRNA and protein of both SCF and c-kit are expressed in spermatogonial stage, and that addition of pFSH to organ culture of testis fragments elevated expression of *SCF* mRNA. Thus, our current results indicate that SCF-c-kit system plays a pivotal role in newt spermatogenesiss; SCF stimulates the proliferation of newt spermatogonia but not their entrance into meiosis, and FSH promotes spermatogonial proliferation mainly through activation of SCF expression in Sertoli cells.

Growth factors involved in spermatogonial proliferation (II): insulin-like growth factor(IGF)-I

Recombinant human insulin-like growth factors (rhIGF-I and rhIGF-II) and human insulin promote the differentiation of spermatogonia into primary spermatocytes in organ culture of newt testes fragments (Nakayama *et al.,* 1999). The biological potency for promoting differentiation is dose-dependent for all the ligands, with the highest potency displayed by IGF-I, followed by IGF-II, and the least by insulin. This order of biological potency accords well with the order of affinity in binding specificity of [^{125}I]IGF-I to the testicular membrane fractions: IGF-II and insulin competed the binding of [^{125}I]IGF-I only at concentrations 20-fold and 100-fold higher, respectively, than IGF-I. [^{125}I]IGF-I binds specifically to both somatic cells (mostly Sertoli cells) and germ cells (spermatogonia and primary spermatocytes), though the binding to somatic cells is about three times higher than that to germ cells. These results indicate that (1) specific binding sites for IGF-I are present in the newt testes, (2) IGF-II and insulin also bind to these receptors but to a lesser degree, and (3) IGF-II and insulin as well as IGF-I promote sper-

matogonial differentiation into primary spermatocytes by binding to the IGF-I receptor.

Northern blot and RT-PCR analyses show that *IGF-I* mRNA is highly expressed in somatic cells (mostly Sertoli cells) at the secondary spermatogonial stage but barely in germ cells, and that *IGF-IR* mRNA was expressed in both germ and somatic cells at all stages examined (Yamamoto *et al.,* 2001). Addition of pFSH to newt testis markedly increased *IGF-I* mRNA expression. Also, rhIGF-I increased *IGF-I* mRNA expression, whereas *IGF-IR* mRNA expression is decreased slightly. These results suggest that the ability of FSH to promote the differentiation of secondary spermatogonia into primary spermatocytes is at least partly mediated by somatic cell-derived IGF-I, and that *IGF-I* mRNA expression in somatic cells is auto-upregulated. These results are consistent with previous results in mammals: FSH increased *IGF-I* mRNA expression in the testis of immature hypophysectomized rats (Closset *et al.,* 1989), and stimulated IGF-I production in cultured porcine (Naville *et al.,* 1990) and rat (Cailleau *et al.,* 1990) Sertoli cells. Thus, FSH may regulate IGF-I production among vertebrate testes.

Previous studies of testes demonstrated the presence of IGF-IR on both somatic and germ cells, such as mammalian Leydig (Handelsman *et al*., 1985; Lin *et al*., 1986a, b; Kasson and Hsueh, 1987; Saez *et al*., 1988) and Sertoli cells (Borland *et al*., 1984; Oonk and Grootegoed, 1988), and trout Sertoli cells, spermatogonia and primary spermatocytes (Le Gac *et al*., 1996). In addition, IGF-I binding is observed on rat pachytene spermatocytes (Tres *et al*., 1986), and IGF-IR immunoreactivity is localized on human secondary spermatocytes and early spermatids (Vannelli *et al*., 1988). Similarly, our current studies detected [^{125}I]IGF-I binding to both spermatogonia-rich and Sertoli-rich fractions. Also *IGF-IR* mRNA is expressed both in germ cell fractions and Sertoli-rich fractions.

These results pose several interpretations regarding the mechanism of IGF-I action on spermatogonial differentiation. First possibility is that IGF-I may directly stimulate spermatogonial proliferation and/or differentiation into primary spermatocytes. Indeed, Loir (1994) showed firstly in vertebrates that IGF-I directly stimulates the proliferation of male trout germ cells, though the differentiation of spermatogonia into primary spermatocytes was not detected. Second is that IGF-I may activate Sertoli cells or pericystic cells which in turn produce factors that stimulate spermatogonial proliferation and/or differentiation into primary spermatocytes. Finally, the third possibility is that IGF-I may directly stimulate spermatogonial proliferation, whereas Sertoli cells or pericystic cells, activated by IGF-I, secrete factor(s) required for the differentiation of spermatogonia into primary spermatocytes.

Apoptosis induced by prolactin

Cell death occurs in the testes of various species (Roosen-Runge, 1977) including newts (Galgano, 1944) in which at the transition stage from spermatogonia to spermatocytes when the ambient temperature lowers naturally. This has been known to be the cause of cessation of spermatocytogenesis (differentiation of primary spermatocytes) in late fall when mature sperm is almost formed. Prolactin (PRL) is associated with various reproductive phenomena in urodeles such as adaptation to reproductive environment, sexual behavior, development of reproductive organs and sexual characters (Chadwick, 1941; Kikuyama and Toyoda, 1999; Kikuyama *et al.,* 1975; Kikuyama *et al.,* 1986). In the crested newt, administration of ovine PRL induces spermatogonial cell death, while co-injection of follicle-stimulating hormone (FSH) prevents it (Mazzi *et al.,* 1967; Mazzi and Vellano, 1968). In the Japanese red-bellied newt, intraperitoneal injection of ovine PRL induces pyknotic degeneration of spermatogonia in the cysts situated adjacently to the boundary line between zones of spermatogonia and spermatocytes (Fig. 6) (Yazawa *et al.,* 2000b). Addition of PRL to the medium of testicular organ culture also induces spermatogonial death, demonstrating that PRL acts on the testis

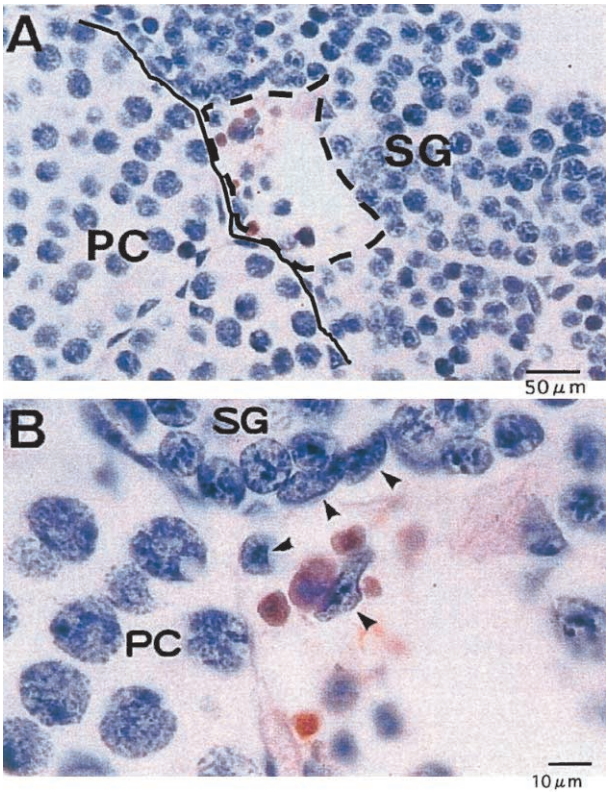

Fig. 6. Apoptosis of spermatogonia induced by prolactin. A) Intraperitorial injection of PRL caused apoptosis in all the spermatogonia in a cyst in the 7th generation (encircled by dotted lines). Stained by TUNEL method (brown color), and also by hematoxylin and eosin. The dead cysts are often located along or very close to the boundary line (straight line) between the region of primary spermatocytes (PC) and that of spermatogonia (SG). B) Enlarged figure of (A). Only spermatogonial nuclei are stained by TUNEL but no nuclei of Sertoli cells. Arrowheads show the nuclei of Sertoli cells that look alive. Reproduced by permission of The Endocrine Society from Fig. 1 in a paper by Yazawa *et al*. (2000b).

directly. All the spermatogonia in a given cyst die simultaneously, while the nuclei of Sertoli cells appear unaffected. This cell death was found to be apoptosis, based on the DNA ladder profile in the gel electrophoresis and TUNEL method. The stage of the dead cells is estimated to be in the 7^{th} generation of spermatogonia that is the penultimate stage before entrance into meiosis. Spermatogonial degeneration increases linearly with increased concentration of PRL, whereas FSH inhibits PRL action in a dose-dependent manner. Thus, FSH and PRL act on each other antagonistically.

The spermatogonial cell death is also induced when newts are incubated at low temperatures (12 and 8°C) but not at high temperatures (18 and 22°C) (Yazawa *et al.,* 1999). Also, plasma PRL concentrations are elevated by low temperatures. Anti-PRL antibody injection into newts that were incubated at low temperatures completely suppresses the cell death for as long as 3 days (Yazawa *et al.,* 1999). Injection of a dopamine antagonist (pimozide), which is known to increase the plasma PRL concentration, to the newt caused significant increase of spermatogonial degeneration, whereas treatment with an agonist (bromocryptin), which is known to decrease the PRL concentration, suppressed the cell death. These results may indicate that inhibitory control of PRL release by dopamine is predominantly operating in the summer newts. Thus, it is demonstrated that low temperature induces elevation of plasma PRL titer, which induces apoptosis of spermatogonia. These studies permit the following interpretation of the physiological events occurring *in vivo* and in nature. In the spring when the temperature rises, the FSH/PRL concentration ratio in the plasma increases because the amount of PRL secreted by the pituitary is reduced and that of FSH secreted is increased as mentioned earlier (Tanaka and Takikawa, 1984), preventing spermatogonial death and permitting them to proliferate and differentiate into primary spermatocytes (Fig. 7). On the other hand, in late fall when the ambient temperature lowers, the FSH/PRL ratio also decreases because the amount of PRL secreted from the pituitary increases and that of FSH decreases, causing spermatogonial death and cessation of spermatocytogenesis. The physiological significance of this phenomenon may be that as spermatogenesis completes in the fall, testes do not form primary spermatocytes that are unnecessary for the year.

To determine whether caspase activity is required for the PRL-induced chromatin condensation, testis fragments were treated with various caspase inhibitors, followed by the addition of PRL (Yazawa *et al.,* 2001). Z-VAD-fmk, a broad-spectrum inhibitor of caspase-like proteases, completely

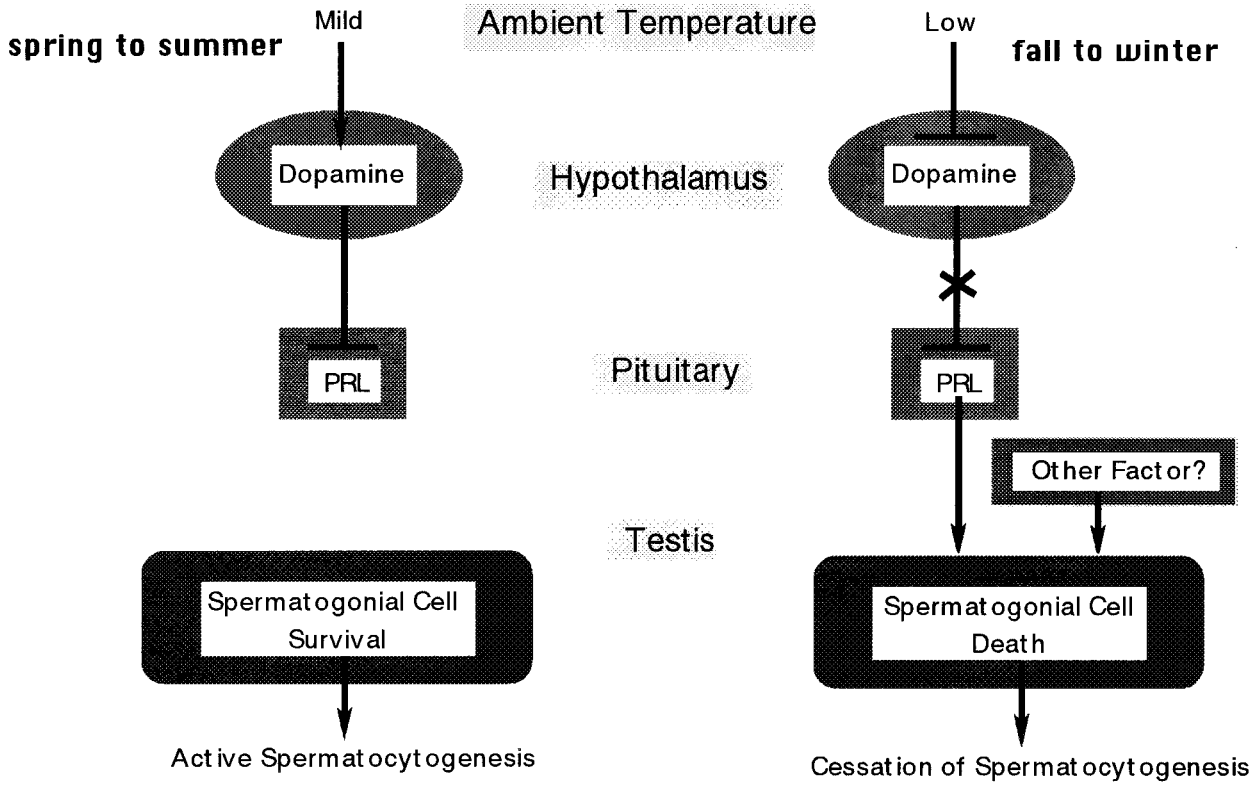

Fig. 7. Scheme of spermatocytogenesis regulation by PRL release from newt pituitary. In spring to summer when the ambient temperature is mild, dopamine is released from the hypothalamus to suppress PRL release from the pituitary. Then spermatogonia survive and undergo active spermatocytogenesis with the help of FSH. On the other hand, in fall to winter low temperature suppresses dopamine release from the hypothalamus, resulting in PRL release from the pituitary. Then PRL induces spermatogonial death and causes cessation of spermatocytogenesis.

prevented the spermatogonial chromatin condensation induced by PRL. Five other caspase inhibitors failed to prevent the chromatin condensation induced by PRL: Ac-YVAD-CHO, a caspase-1 inhibitor, and Ac-DEVD-CHO, a caspase-3/7 inhibitor, as well as Ac-VDVAD-CHO, a caspase-2 inhibitor, Ac-VEID-CHO, a caspase-6 inhibitor, or Ac-LEHD-CHO, a caspase-9 inhibitor. Furthermore, even a mixture of YVAD-CHO, DEVD-CHO, VDVAD-CHO, VEID-CHO and LEHD-CHO failed to inhibit apoptosis. As high caspase activity is present in extracts of testes treated with PRL, it is suggested that an unidentified caspase-like protease induces the morphological changes of apoptosis in newt spermatogonia. Trial to identify the unknown caspase-like protease is currently underway.

We also found cathepsin L activity in the newt testis that is elevated by PRL in organ culture, while E-64d, a lysosomal cysteine protease inhibitor, and Z-VAD-fmk suppressed it and chromosomal condensation; however the nuclei lost spots of heterochromatin that are otherwise observed in normal spermatogonia, and looked rather homogeneous (Fujimoto *et al.,* 2002). In the testis fragments treated with PRL+E-64d, the nuclei of these cells were TUNEL-negative. These results indicate that E-64d inhibits the PRL-induced chromatin condensation and DNA fragmentation in the spermatogonial apoptosis, suggesting that cathepsin is involved in chromatin condensation and DNA fragmentation during spermatogonial apoptosis. The inhibitory effect of Z-VAD-fmk on the spermatogonial apoptosis may be partly due to its cathepsin-inhibiting property, since it is recently reported that Z-VAD-fmk blocks activity of cysteine cathepsins as well.

It is said that there are at least two steps in apoptotic chromatin condensation (Susin *et al.,* 2000; Samejima *et al.,* 2001): the first is a peripheral partial condensation that does not seem to require caspase activation. The second is a tight compaction of chromatin, which involves caspase activation. Several chromatin condensation factors have been identified. Among them, Acinus (Sahara *et al.,* 1999) and CAD (Sakahira *et al.,* 1998) are known to be activated by caspases, and are likely to be involved in the second condensation step. However, it is not clear what factors are involved in the first step of chromatin condensation. In our study, it is shown that inhibition of cathepsin activities leads to blockade of the peripheral chromatin condensation. Therefore, this result may indicate that cysteine cathepsin is one of the factors required for the first step of chromatin condensation. However, further analysis is required to demonstrate whether caspase is involved in the second step of the chromatin condensation or whether cathepsin directly activates caspases in newt testis. On the other hand, as E-64d did not completely inhibit the morphological change of the spermatogonial nuclei, it is postulated that another factor exists for the step of chromatin condensation upstream of cathepsin.

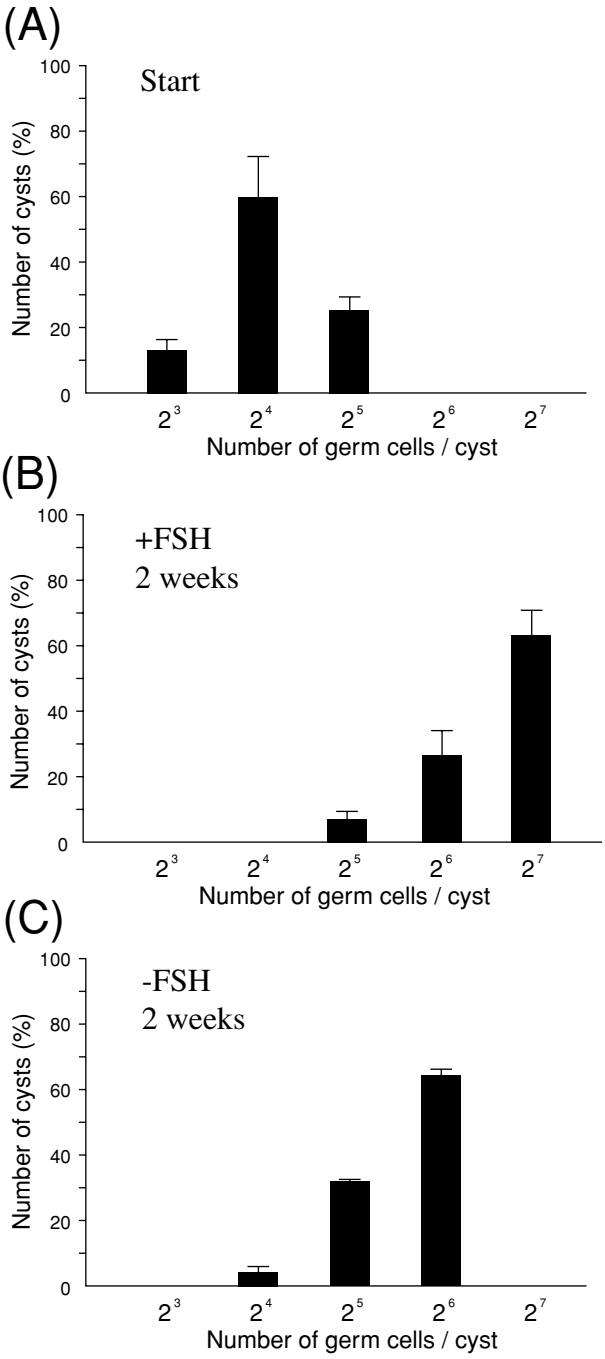

Fig. 8. Progression of spermatogonial generations during organ culture in the presence and absence of FSH. Histogram shows the number (percentage) of the cysts comprising 2^3 ~ 2^7 germ cells/cyst before culture (A), and after culture with (B) and without (C) FSH for 2 weeks at 18°C. In the presence of FSH spermatogonia progressed to the 8^{th} generation (2^7 cells/cyst) and differentiated into primary spermatocytes, while in the absence of FSH they reached the 7^{th} generation (2^6 cells/cyst) but no cells in the 8^{th} generation appeared. Reproduced by permission of Society for the Study of Reproduction, Inc. from Fig. 3 in a paper by Yazawa *et al.* (2002).

Checkpoint hypothesis

As mentioned in an earlier section, mammalian FSH induces differentiation of secondary spermatogonia into primary spermatocytes in organ culture of newt testicular fragments, whereas in medium lacking FSH primary sper-

matocytes never appear. Hence, we investigated why spermatogonia fail to form primary spermatocytes in the absence of FSH (Fig. 8) (Yazawa *et al.,* 2002). Spermatogonia maintain the proliferative activity and viability at about half the level of those cultured in the presence of FSH, progress into the 7th generation, but become moribund during the G2/M phase. Thus, the 8th generation of spermatogonia never appear, suggesting that cell death is the chief reason why primary spermatocytes fail to form in the absence of FSH. Thus, FSH is indispensable for the completion of the last spermatogonial mitosis, a prerequisite for the conversion of germ cells from mitosis to meiosis. Spermatogonial death in newts is induced by PRL both in vivo (Yazawa *et al.,* 2000b; Mazzi *et al.,* 1967; Kikuyama *et al.,* 2000; Yazawa *et al.,* 1999) and in vitro (Yazawa *et al.,* 2000b, 2001), and also at a specific time, the penultimate stage. These suggest that there is a regulatory checkpoint for spermatogonia to differentiate into spermatocytes during the penultimate stage of spermatogonia (Fig. 9). When FSH/PRL concentration ratio is high (in warm season), spermatogonia proceed through 7 mitotic divisions to enter meiosis, but when the ratio is low (in cool season) spermatogonia cannot complete the last mitosis and instead die by apoptosis. Enough amount of FSH can circumvent the checkpoint by activating Sertoli cells that may produce some factor(s) that prevent spermatogonia from apoptosis and drive them into meiosis.

Cessation of spermatocytogenesis by cell death also occurs in shark (Simpson and Wardle, 1967) and frog (Aron, 1926; Champy, 1913) testes in the spring month. So, we assume that this regulatory checkpoint is conserved in evolution, at least from chondrochytes to amphibians, and contributes to seasonal control of spermatogenesis in poikilothermic vertebrates. Whether this regulatory checkpoint is retained in mammals is unknown.

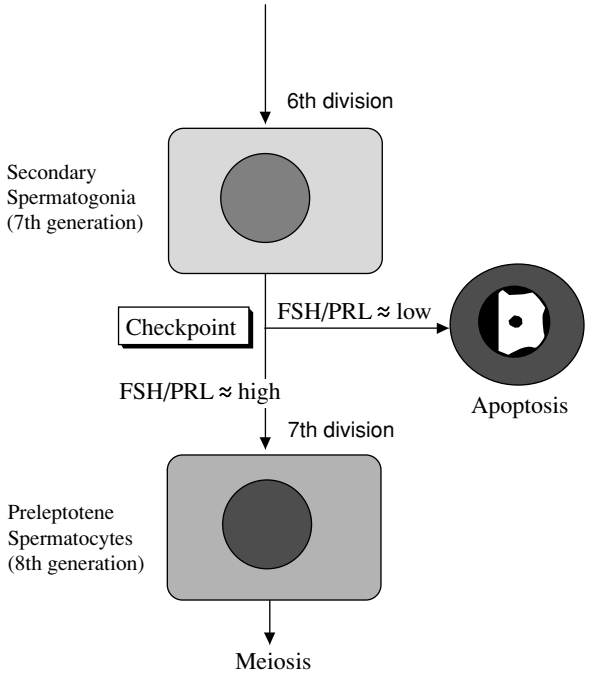

Fig. 9. Hypothesis on a regulation for entry into meiosis. When the FSH:PRL concentration ratio is high, spermatogonia proceed through the 7th division to enter meiosis, whereas they fail to do so and instead go to die when the ratio is low. Reproduced by permission of Society for the Study of Reproduction, Inc. from Fig. 6 in a paper by Yazawa *et al*. (2002).

Problems to be addressed and future perspectives

PRL induces apoptosis in spermatogonia by direct action on the testis. Indeed we have isolated a cDNA clone coding for newt PRL-R (nPRL-R) (Yamamoto *et al*., 1998). The predicted protein contained box 1 and box 2 sequences in the cytoplasmic domain that are considered to be required for the signal transduction of the cytokine/growth hormone/PRL-R family in mammals. The nPRL-R was demonstrated to be functional, because the expression in COS-7 cells showed specific binding of [^{125}I] rat PRL (Yamamoto *et al*., 1998). The mRNA is expressed at almost the same level from early spermatogonial stage, late spermatogonial stage and primary spermatocyte stage (our unpublished results). Then, why does apoptosis occur only in the 7th generation of spermatogonia? Is the signal transduction pathway active only in the critical stage, or is the apoptosis execution pathway stage-specific?

FSH is indispensable for the last mitosis of newt spermatogonia. Since FSH activates Sertoli cells which then act on spermatogonia by secreting some factors or by direct cell-cell contact. To screen out the factors, we have made EST library (about 5,000 non-overlapped clones) from newt testis and have performed microarray analysis using RNAs extracted from cultured testicular fragments in the presence and absence of FSH as probes. Several clones whose expressions were up-regulated or down-regulated were identified. From these studies, it is expected that factor(s) responsible for meiosis initiation is isolated in near future.

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