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Role of c-*kit* in mouse spermatogenesis: identification of spermatogonia as a specific site of c-*kit* expression and function

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Summary

Recent studies have shown that the *dominant white spotting (W)* locus encodes the proto-oncogene c-*kit*, a member of the tyrosine kinase receptor family. One symptom of mice bearing mutation within this gene is sterility due to developmental failure of the primordial germ cells during early embryogenesis. To elucidate the role of the c-*kit* in gametogenesis, we used an anti-c-*kit* monoclonal antibody, ACK2, as an antagonistic blocker for c-*kit* function to interfere with the development of male and female germ cells during postnatal life. ACK2 enabled us to detect the expression of c-*kit* in the gonadal tissue and also to determine the functional status of c-*kit*, which is expressed on the surface of a particular cell lineage. Consistent with our immunohistochemical findings, the intravenous injection of ACK2 into adult mice caused a depletion in the differentiating type A spermatogonia from the testis during 24–36 h, while the undifferentiated type A spermatogonia were basically unaffected. Intraperitoneal injections of ACK2 into prepuberal mice could completely block the mitosis of mature (differentiating) type A spermatogonia, but not the mitosis of the gonocytes and primitive type A spermatogonia, or the meiosis of spermatocytes. Our results indicate that the survival and/or proliferation of the differentiating type A spermatogonia requires c-*kit*, but the primitive (undifferentiated) type A spermatogonia or spermatogenic stem cells are independent from c-*kit*. Moreover, the antibody administration had no significant effect on oocyte maturation despite its intense expression of c-*kit*.

Key words: c-*kit*, *W* locus, monoclonal anti-c-*kit* antibody, mouse, spermatogonia, oocyte, postnatal development.

Introduction

c-*kit* is a proto-oncogene encoding a receptor tyrosine kinase in the member of PDGF receptor/CSF-1 receptor family (Yarden *et al.* 1987; Qiu *et al.* 1988). Recent studies have demonstrated that c-*kit* is allelic with *dominant white spotting (W)* locus of mouse, and a number of mutant alleles have been analyzed at nucleotide sequence level (Chabot *et al.* 1988; Geissler *et al.* 1988; Tan *et al.* 1990). In addition to the effects of c-*kit*/*W* locus on coat color and hematopoiesis, the typical symptom of the mice bearing mutation within the gene is sterility due to developmental failure of primordial germ cells during early embryogenesis (Mintz and Russell, 1957; for review, Russell, 1979). Thus it is clear that c-*kit* and its ligand play an essential role in normal development of the germ cells in early mouse embryos. Very recently, the molecular cloning of the ligand for c-*kit*, SCF/KL/MGF has been carried out and it was mapped to *Steel (Sl)* locus, which has the identical phenotype as *W* mice (Anderson *et al.* 1990; Huang *et al.* 1990; Zsebo *et al.* 1990).

Although phenotype analysis of *W* or *Sl* mice proved that c-*kit* and its ligand are indispensable for the germ cells during early embryonic life, many questions still remain to be resolved. For instance, whether c-*kit* and its ligand are required for the maintenance of the germ cells in postnatal life is difficult to address by phenotype analysis, because virtually no germ cells are present in the gonads of *W* and *Sl* mice. Recent *in situ* hybridization studies demonstrated that c-*kit* mRNA is detected not only in the primordial germ cells of fetal gonads (Orr-Urtreger *et al.* 1990) but also in oocytes or spermatogonia of postnatal gonads (Manova *et al.* 1990), suggesting that c-*kit* may function in gametogenesis not only during early embryogenesis but also in postnatal development. However, the expression does not necessarily indicate its functional status. In fact, c-*kit* and its ligand appear to be expressed in a variety of other cells/tissues/organs that are normal in *W* and *Sl*

mice (Nocka *et al.* 1989; Manova *et al.* 1990; Matsui *et al.* 1990; Orr-Urtreger *et al.* 1990).

The purpose of the present study is to elucidate the role of c-*kit* in postnatal gametogenesis. To assess the actual role of c-*kit* in a given tissue at a given stage of differentiation, it is necessary to control the function of c-*kit* in the living organism. Since c-*kit* is a transmembrane receptor, one possibility is to use an anti-c-*kit* monoclonal antibody capable of antagonizing the function of c-*kit in vivo*. Here, we produced anti-c-*kit* monoclonal antibodies and attempted to use them to specify the function of c-*kit* in gonadal tissues during postnatal development of mice. Our results indicate that the survival and/or proliferation of the differentiating type A spermatogonia is dependent on c-*kit*. Moreover, although oocytes express high level of c-*kit*, antibody administration had no significant effect on the oocyte maturation and ovulation.

Materials and methods

Animals

Adult C57BL/6 mice (10- to 12-weeks-old), purchased from Japan SLC Inc. (Shizuoka, Japan), were used in the present study. To obtain prepuberal animals, female mice were paired with males and were checked daily for vaginal plugs early in the morning. The majority of litters were born on the 19th day after the plug day, and the day of birth was designated as day 0 of age.

Antibodies

Antibody preparation is described in the section on results. The monoclonal antibody (ACK2), which defines c-*kit* molecules is of the IgG2b class, and was derived from hybridomas prepared by fusing X63-Ag8 myeloma cells with spleen cells from Wistar rats immunized with IL-3-dependent mast cells (Nishikawa *et al.* 1991). As a class-matched control monoclonal antibody, we used purified anti-Mac-1 antibody (Springer *et al.* 1979), which does not bind to germ cells.

Immunohistochemistry

Freshly dissected ovaries and testes were covered with OCT (Lab-Tek Products), quickly frozen and sectioned at 6–8 μm in a cryostat. The sections were fixed for 10 min with cold acetone, washed in phosphate-buffered saline (PBS) at pH 7.5 and incubated with purified ACK2 antibody at the concentration of 10 $\mu g\,ml^{-1}$ in PBS at 4°C overnight. Antibody binding to tissue sections was detected by a streptavidin–biotin–peroxidase technique, using Histostain-SP kit (Zymed Labs), according to the manufacturer's recommendations, and the sections were counterstained with hematoxylin. For demonstrating the tissue localization of ACK2, acetone-fixed frozen sections of the gonads from ACK2-injected mice were stained directly with biotin-labeled anti-rat IgG antibody using a streptavidin–biotin staining kit (Zymed Labs). Specificity of antibody binding was checked by comparing the samples with controls that had been treated with rabbit non-immune serum or anti-Mac-1 antibody instead of ACK2. The control sections did not show any reactivity except for occasional cells in connective tissues probably anti-Mac-1 antibody-positive macrophages (not shown).

ACK2-injection experiment and histological analysis

Prepuberal (0- to 28-day-old) mice were injected intraperitoneally with 5 $mg\,kg^{-1}$ of purified ACK2 per mouse at 48 h intervals as indicated in Fig. 5. Adult (10- to 12-week-old) mice were injected intravenously with 100 μg to 1 mg of purified ACK2 every second day. Injection of anti-Mac1 antibody used as a class-matched non-binding control antibody did not show any effect on germ cells including other somatic cells in ovaries and testes (not shown).

At the end of the experimental period, one side of whole testes or ovaries were fixed in Bouin's fluid. After embedding in paraffin, the tissue blocks were serially sectioned at 6 μm and stained with hematoxylin and eosin. To obtain a high degree of morphological information by light microscope, semi-thin sections were also prepared from the other side of the gonads. Tissue samples were fixed with 2.5 % glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 for at least 2 h at 4°C. After rinsing, they were post-fixed with cacodylate-buffered 1 % osmium tetroxide for 2 h and embedded in Epon through ethanols and propylene oxide. They were sectioned at 1 μm and stained with toluidine blue. In the testis, morphological staging of individual germ cell, in particular of spermatogonia and prophase spermatocytes, within the seminiferous tubule was identified using criteria described by Clermont and Perey (1957), Oakberg (1971), Hilscher *et al.* (1974), Hilscher and Hilscher (1976), Bellve *et al.* (1977), Huckins and Oakberg (1978), and Kluin and de Rooij (1981); each germ cell could be recognized by cell size, nuclear morphology, time of appearance during testis development and cycle of the tubule, and precise location within the tubule. The stage of each tubule cross section in the adult testis was determined according to criteria proposed by Oakberg (1956).

Testosterone measurement

To determine the effects of ACK2 on the function of Leydig cells, serum testosterone concentrations were measured. Male mice (11-week-old) were injected intravenously with 100 μg of ACK2. After 24 and 48 h, the mice were then anesthetized and bled by severing the jugular veins and carotid arteries. After centrifugation, serum samples were stored at −20°C until assayed. Concentrations of testosterone in the samples were determined by radioimmunoassay method using a TOTAL TESTOSTERONE kit (Japan DPC Co., Tokyo), according to the protocol. The sensitivity of the method was approximately 0.04 $ng\,ml^{-1}$. The mean intra- and interassay coefficients of variation for this assay were less than 10 %.

Results

*Production of anti-c-*kit *monoclonal antibody*

Previous studies on the expression of c-*kit* demonstrated that IL-3-dependent mast cell lines express high levels of c-*kit* on the cell surface (Tan *et al.* 1990; Reith *et al.* 1990). Since c-*kit* cDNA of *W/W* mouse has a point mutation in a splice donor site that results in a deletion including the transmembrane domain (Nocka *et al.* 1990; Hayashi *et al.* 1991), IL-3-dependent mast cells from *W/W* mouse are expected not to express c-*kit* on the cell surface. Based on these results, we immunized rats with IL-3-dependent mast cells and the spleen cells of the rats were fused with X63-Ag8. Hybridomas producing monoclonal antibody recognizing normal IL-3-dependent mast cells but not those from *W/W* mouse were screened, and we obtained four clones. All these four antibodies stained COS7 cells

that had been transfected with c-*kit* cDNA cloned into expression vector pSRα but not those transfected with insert-free pSRα, and detected 140 and 110×10^3 M_r protein of IL-3-dependent mast cells by western blotting (data not shown). These four monoclonal anti-c-*kit* antibodies were then screened for their ability to block *in vitro* hemopoiesis, and ACK2 was selected as an antagonistic antibody that has the ability to block completely the colony formation of bone marrow cells in response to recombinant MGF (data not shown).

Expression of c-kit *in postnatal ovaries and testes*

We made serial frozen sections of ovaries and testes from normal mice at varying days after birth, and immunostained with ACK2. Consistent with the previous study of Manova *et al.* (1990) and using *in situ* hybridization, strong c-*kit* expression could be detected in all oocytes at various follicle stages in ovaries from newborn to adult mice (Fig. 1A–C). The intensity of the expression varied, with primordial oocytes exhibiting a more intense level. Expression of c-*kit* was also found in the interstitial theca cells in ovaries from 14 days of age onward, mainly in cells of the theca interna outlining follicles (Fig. 1B, C). This pattern was most clearly demonstrated in hypertrophied theca cells in regressing ovarian follicles of a 5-week-old mouse (Fig. 1B). No c-*kit* expression could be found in the granulosa cells at any age examined, while weak expression was observed in cells of the corpus luteum in cycling adults (Fig. 1C).

In adult testes, consistent with the result of an *in situ* hybridization study (Manova *et al.* 1990), the interstitial Leydig cells expressed the most intense level of c-*kit* (Fig. 2F). c-*kit* was also expressed on some spermatogenic cells in the basal layer of the seminiferous tubules, and the number of the ACK2-positive cells varied with the stage of the cycle of the tubule (Fig. 2F). The significant expression of c-*kit* could be detected on the surface of the differentiating type A (A_{1-4}), intermediate, and type B spermatogonia and the earliest preleptotene spermatocytes, whereas no c-*kit* expression was detected in later spermatocytes (leptotene, zygotene and pachytene stages), spermatids and Sertoli cells. This result indicates that c-*kit* expression is down-regulated upon entering the meiotic cycle. To determine whether the undifferentiated type A spermatogonia express c-*kit*, we studied the immunostained sections of the testis from the mouse that was killed at 36 h after ACK2 injection as described in the next section. No c-*kit* expression could be detected on the serial cross-sections of the tubule where the ACK2-positive cells were depleted but the undifferentiated type A spermatogonia remained (Fig. 2G). This indi-

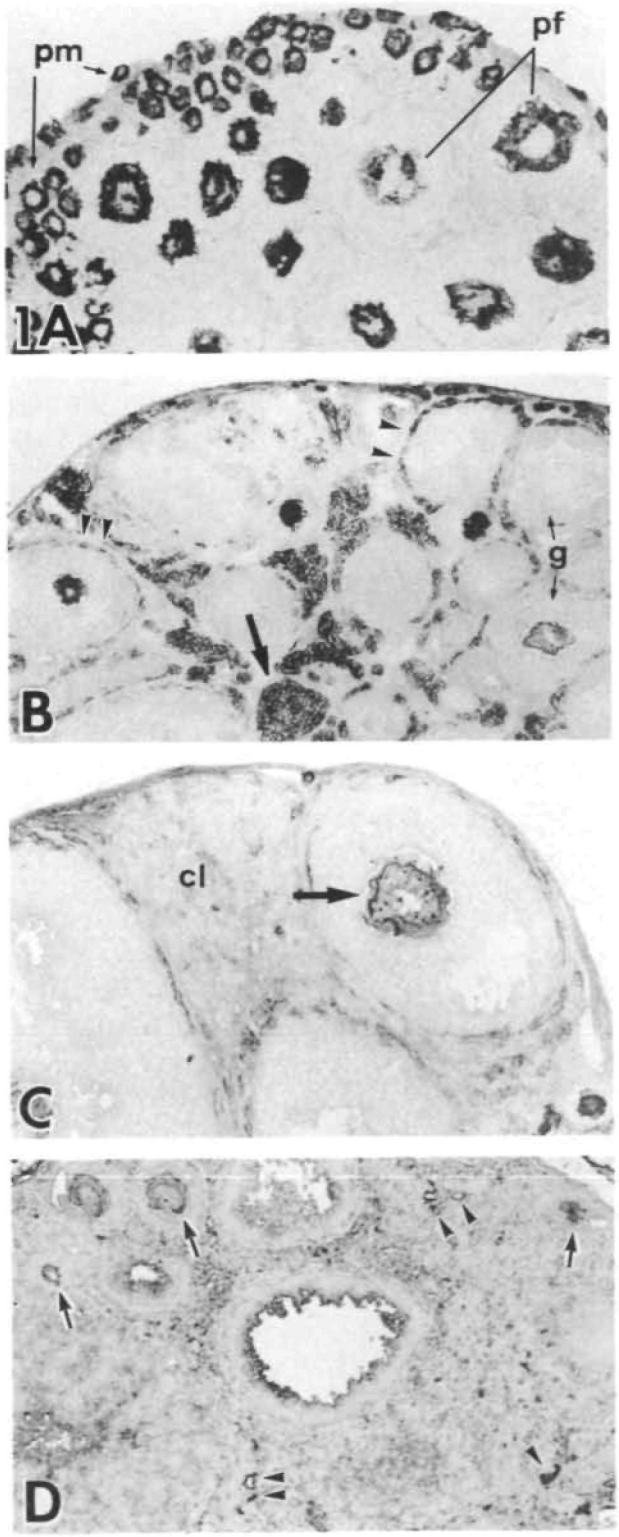

Fig. 1. Immunohistochemical staining of c-*kit* with ACK2 (A–C) and detection of injected ACK2 (D) in frozen sections of postnatal mouse ovaries. (A) Primordial (pm) and primary (pf) follicles with intensely stained numerous oocytes in 8-day ovary. ×170. (B) A young adult ovary (5 weeks old) with many developing follicles. Staining can be seen in oocytes and interstitial theca cells (arrowheads) surrounding the follicles but not in the granulosa (g) cells. Arrow indicates the intensely stained hypertrophied theca cells. ×170. (C) Maturing follicles with stained oocyte (arrow) in an adult ovary (12 weeks old). cl, corpus luteum. ×130. (D) Confirmation of ACK2 accession to oocytes in the ovary from 12-week-old mouse. Both serum components in the blood vessels (arrowheads) and oocytes (arrows) with positive reactions are incubated with second antibody only. ×80.

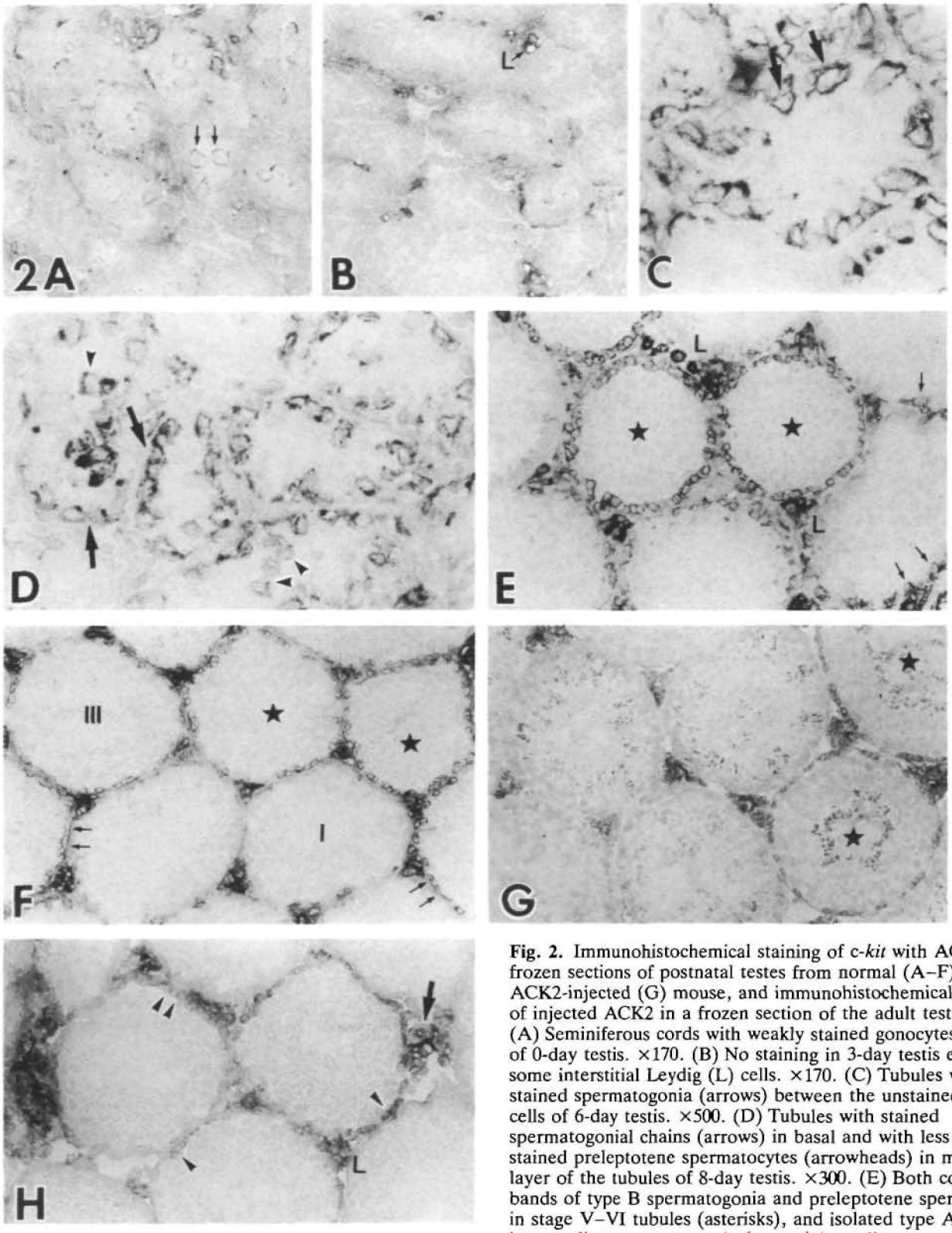

Fig. 2. Immunohistochemical staining of c-*kit* with ACK2 in frozen sections of postnatal testes from normal (A–F) and ACK2-injected (G) mouse, and immunohistochemical detection of injected ACK2 in a frozen section of the adult testis (H). (A) Seminiferous cords with weakly stained gonocytes (arrows) of 0-day testis. ×170. (B) No staining in 3-day testis except some interstitial Leydig (L) cells. ×170. (C) Tubules with stained spermatogonia (arrows) between the unstained Sertoli cells of 6-day testis. ×500. (D) Tubules with stained spermatogonial chains (arrows) in basal and with less intensely stained preleptotene spermatocytes (arrowheads) in more apical layer of the tubules of 8-day testis. ×300. (E) Both continuous bands of type B spermatogonia and preleptotene spermatocytes in stage V–VI tubules (asterisks), and isolated type A or intermediate spermatogonia (arrows) in earlier stage tubules are stained, as well as Leydig (L) cells in 28-day testis. ×140. (F) Early stage tubules (I–III) with stained type A or intermediate spermatogonia, and stage V–VII tubules (asterisks) with stained type B spermatogonia and preleptotene spermatocytes in adult (12 weeks old) testis. Arrows indicate the stained differentiating type A spermatogonial chains in later stage (IX–XI) tubules. ×140. (G) ACK2-treated adult testis showing no staining in tubules, except stage VI tubules (asterisks) with stained type B spermatogonia and preleptotene spermatocytes. ×140. (H) Confirmation of ACK2 accession to spermatogenic cells in the testis at 24 h after the injection. ACK2 retained within the blood vessels (arrow) and trapped on the surface (arrowheads) of spermatogonia and preleptotene spermatocytes are detectable. Interstitial Leydig cells (L) are also stained. ×140.

cates that the undifferentiated type A spermatogonia do not express c-*kit*.

c-*kit* expression was also investigated in testes from prepuberal mice (Fig. 2A–E), where the first synchronous wave of spermatogenesis occurred. The morphological characteristics and the timetable of progressive development of spermatogenic cells in the prepuberal testes observed in the present study (e.g. Fig. 6A,C,E) were in general concordance with the findings of previous studies (Clermont and Perey, 1957; Nebel *et al.* 1961; McKinney and Desjardins, 1973; Bellve *et al.* 1977). In testes of newborn mice, the seminiferous cords contained only non-proliferating gonocytes (T_1 prospermatogonia) and Sertoli cells. Weak or faint expression of c-*kit* was found on the surface of the gonocytes but none in the Sertoli cells (Fig. 2A). During the following few days, c-*kit* expression became undetectable in the germ cells that started proliferating and differentiating into transitional primitive type A spermatogonia, whereas weak expression of c-*kit* was detected in some interstitial Leydig cells (Fig. 2B). The first appearance of type A spermatogonia was found in the tubule on day 6 after birth, and here we could detect significant expression of c-*kit* (Fig. 2C). The mitotic divisions of the differentiating type A spermatogonia continued to yield type B spermatogonia, and the earliest preleptotene spermatocytes appeared in the tubules on day 8. Fig. 2D demonstrates that c-*kit* expression appeared in the proliferating spermatogenic cells from the mature type A through type B spermatogonia to preleptotene primary spermatocytes in the testis on day 8. Expression of c-*kit* could not be detected in later spermatocytes with meiotic divisions and in spermatids in the testis of older mice (Fig. 2E). No c-*kit* expression was found in Sertoli cells during prepuberal life, while the interstitial Leydig cells expressed c-*kit* from day 3 onward.

ACK2 injection suppressed spermatogenesis but not oocytes maturation

The results in the preceding section demonstrated the site of c-*kit* expression in postnatal gonadal tissues, but are these c-*kit* molecules on the cell surface functioning? To answer this question, we used a monoclonal antibody, ACK2 which is able to block the function of c-*kit* upon injection. Varying doses of ACK2 were injected intravenously into adult mice and the histology of gonadal tissues of ACK2-injected mice was examined. As an initial attempt, we intravenously injected 1 mg of purified ACK2 every second day, and 12 days after the initiation of injection, the mice were killed for histological examination. The average concentration of free ACK2 in the serum was 293 $\mu g\,ml^{-1}$. Almost all myeloid and erythroid cells in various differentiation stages are eliminated from bone marrow by this treatment (Ogawa *et al.* 1991), so that more prolonged ACK2 injection was lethal to the mice. Consistent with our histological observation, almost all spermatogonia were selectively depleted from the seminiferous tubules of ACK2-treated mice (not shown). However, although a higher level of c-*kit* expression was detected in oocytes than spermatogonia, we could not detect any significant difference between the ovaries of ACK2-treated and -nontreated mice. This suggests that the process of oocyte maturation, which occurs at about 4- to 5-day cycle, is resistant to the ACK2 injection, although the role, if any, of c-*kit* in the long-term process would be difficult to detect in this experiment. In order to exclude the possibility that ACK2 is not accessible to oocytes, 1 mg of ACK2 was injected into mice and then the ovaries were immunostained with anti-rat IgG on next day. As shown in Fig. 1D, the antibody was detected not only in the blood vessels but also in the oocytes in various stages of ovarian follicles, suggesting that ACK2 is accessible to oocytes in the ovary.

After preliminary experiments determining the minimum dose of ACK2 for blocking spermatogenesis, it was found that 100 μg of ACK2 per injection was as effective as 1 mg in blocking the spermatogenesis, whereas the same dose could suppress hemopoiesis only partially (Ogawa *et al.* 1991). Thus, we used 100 μg for subsequent experiments. To confirm the antibody reaching its supposed sites of action in the testis, 100 μg of ACK2 were injected into mice and then the localization of ACK2 was demonstrated immunohistochemically by using anti-rat IgG antibody. The testes examined were at 24 h after the injection, because ACK2 treatment at this time affected the spermatogogenesis as describe later. As shown in Fig. 2H, the antibody was specifically detected on the surface of differentiating spermatogonia and early preleptotene spermatocytes situated in outer layer of the tubules, suggesting that ACK2 is accessible to ACK2-positive spermatogenic cells.

The time course of histological changes induced in adult testis by ACK2 injection was examined (Figs 2G, 3). As early as 24 h after the initiation of ACK2 injection, differentiating type A spermatogonia were already depleted in the testis, but intermediate and type B spermatogonia remained in stage IV tubule, where intermediate spermatogonia divide to form type B spermatogonia. During the next 12 h (Fig. 2G) and 24 h (Fig. 3A,B) almost all differentiating type A and intermediate spermatogonia disappeared, but the undifferentiated type A spermatogonia, which have slightly oval homogeneously grainy nuclei and are the stem cells of spermatogenesis in rodents (for review, De Rooij, 1983), remained in the testis (Fig. 3B). Despite the rapid disappearance of differentiating spermatogonia during 48 h after the initiation of ACK2 injection, type B spermatogonia with preleptotene spermatocytes of early meiotic prophase remained in the stage VI tubule (Fig. 3A,B), where type B spermatogonia divide to form preleptotene spermatocytes. This means that the differentiation of type B spermatogonia into preleptotene spermatocytes was not affected by the injection of ACK2, since the total cell-cycle time for all types of differentiating spermatogonia in the mouse was estimated to be 27–30 h (Monesi, 1962). During the next 2 days almost all spermatogonia and preleptotene spermatocytes disappeared from the testis, but the undifferentiated type A spermatogonia, leptotene or

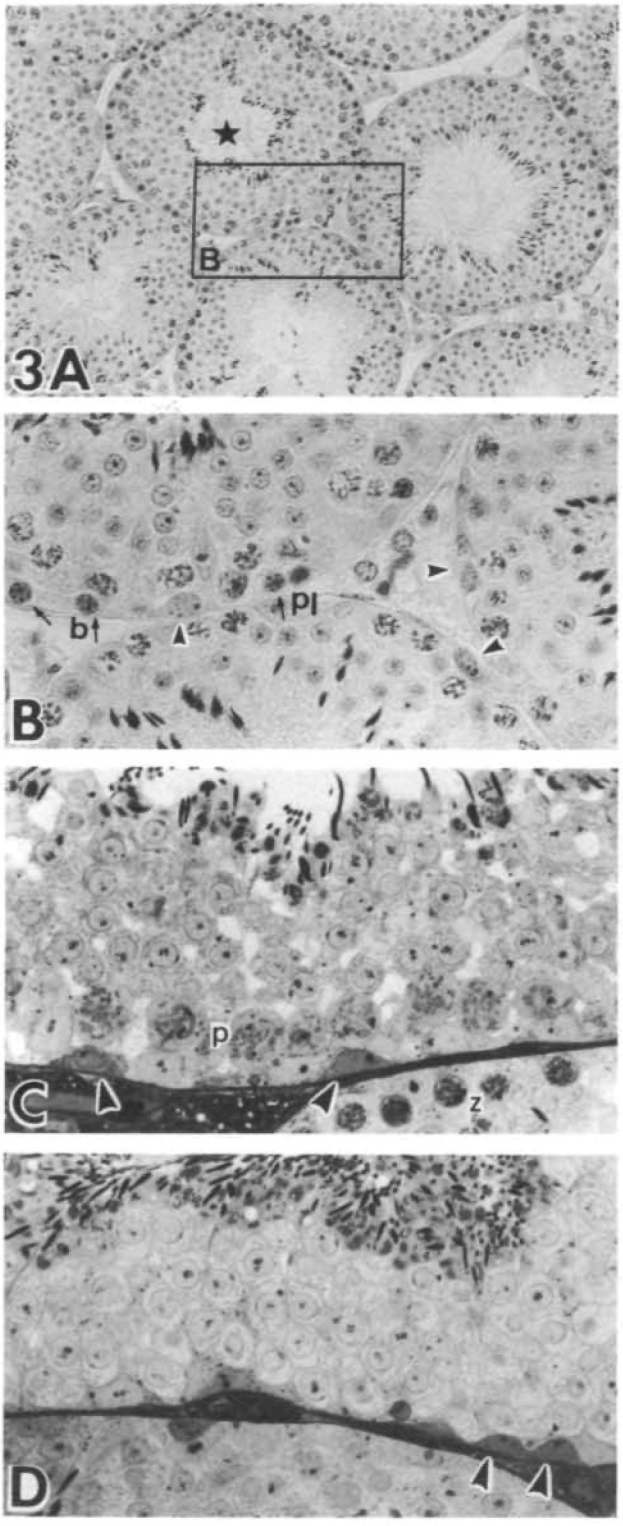

Fig. 3. Effects of ACK2 on adult testes. Paraffin sections (A, B) stained with hematoxylin and eosin and plastic sections (C, D) stained with toluidine blue. (A) 48 h after ACK2 injection. A normal tubule (asterisk) at stage VI with type B spermatogonia and preleptotene spermatocytes, and abnormal tubules without differentiating spermatogonia. ×160. (B) Arrowheads indicate three undifferentiated type A spermatogonia. b, type B spermatogonia; pl, preleptotene spermatocytes. ×470. (C) 4 days after two ACK2 injections. Only undifferentiated type A spermatogonia (arrowheads) remained among Sertoli cells in basal layer of stage VI tubule. p, pachytene spermatocytes; z, leptotene/zygotene spermatocytes. ×500. (D) 12 days after six ACK2 injections. Dividing the undifferentiated type spermatogonia (arrowheads) and normal development of spermatids. ×500.

zygotene spermatocytes remained intact (Fig. 3C). Thus, later meiotic or postmeiotic spermatogenic cells such as pachytene spermatocytes and spermatids were basically unaffected, although prolonged ACK2 injection eventually resulted in the orderly loss of spermatocytes according to the differentiation stage. Interestingly, the undifferentiated type A spermatogonia persisted and continued to divide in the testis of the mouse which had been ACK2 injected every other day 6 times, although other type spermatogonia and spermatocytes were absent (Fig. 3D). No effect of ACK2 was found on other somatic cell elements such as Sertoli cells and interstitial Leydig cells. In summary, it is clear from these findings that the spermatogenic block by ACK2-injection occurred during the early differentiating spermatogonial stages.

Effect of ACK2 on Leydig cell function

Our histological studies demonstrated that the ACK2 does not affect the Leydig cells despite its intense expression of c-*kit*. Since testosterone is indispensable for spermatogenesis, it is still possible that ACK2 affects the function of Leydig cells, namely the production of testosterone, thereby indirectly suppressed the proliferation of spermatogonia. To investigate whether ACK2 suppress the function of Leydig cells, the concentrations of serum testosterone were assayed after ACK2 injection. The levels of serum testosterone examined are shown in Fig. 4. Contrary to our expectation, the concentrations of testosterone in ACK2-injected mice were increased at 24 h after the injection (224±37.3 *vs* 25.4±5.5 ng dl^{-1}; means±S.E.) and during the next 24 h, the concentration of ACK2-injected mice decreased but remained higher than that of control mice (64.3±15.2 *vs* 25.4±3.7). This result indicates that the suppression of testosterone production was not the cause of ACK2-induced arrest of spermatogenesis. Although the serum testosterone level of ACK2-treated mice appear to be within the range of circadian variations (Coquelin and Desjardins, 1982), it could be that the elevation of serum testosterone may affect the spermatogonial proliferation in the seminiferous tubules. However, this is unlikely because we found the persistence of normal spermatogonial proliferation in the presence of higher concentration (600 to 1000 ng dl^{-1}) of serum testosterone exogenously administrated (unpublished data).

Effect of ACK2 in spermatogenesis of prepuberal mouse

Spermatogenesis is initiated shortly after birth; in prepuberal mouse, the mitosis and differentiation of

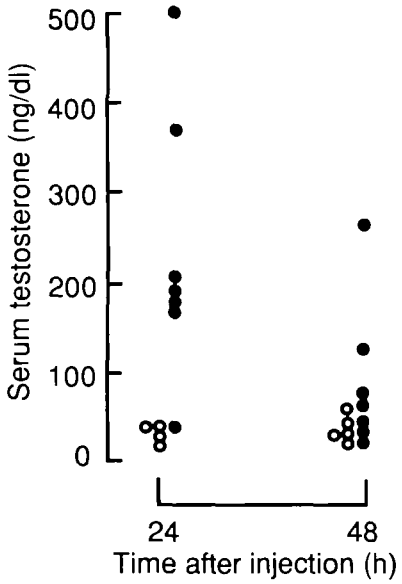

Fig. 4. Changes of serum testosterone concentration in ACK2-injected (●) and control (○) mice at different times after the injection.

gonocytes start at day 3 and initiate a wave of spermatogenic cell differentiation leading to the formation of haploid spermatids at day 19–22 (for review, Bellve, 1979). Since all these processes occur synchronously and before the formation of blood–testis barrier which may prevent antibodies permeating to the target cells (Bellve, 1979), the prepuberal spermatogenesis provides a good system for identifying the target cells of ACK2 injection, and three distinct processes were examined for their sensitivity to ACK2 treatment. The first process is the initial phase occurring between days 3 and 5 after birth when mitosis of both gonocytes and primitive type A spermatogonia occurs but c-*kit* expression is undetectable. The second process was the mitosis of mature type A spermatogonia differentiating through intermediate into type B spermatogonia, which starts from day 6 to day 7 when c-*kit*-positive cells increase in number but meiosis has not started yet. The final process is the meiotic differentiation from preleptotene spermatocytes to condensing spermatids, which takes place from day 8 to day 28. Three schedules of ACK2 injection indicated in Fig. 5 were used to investigate the role of c-*kit* in each process. As shown in Fig. 6B, the first schedule (schedule 1) had no effect on the mitosis of either gonocytes or primitive type A spermatogonia. This indicates that the mitosis of the germ cells at this phase is not dependent on c-*kit*. However, the mitosis of more mature type A spermatogonia taking place between day 6 and day 7 was blocked completely by schedule 2 ACK2 injection. Fig. 6D shows that the seminiferous tubule of ACK2-treated mouse is aplastic and only the primitive type A spermatogonia or the undifferentiated type A spermatogonia with mitotic phase and Sertoli cells were detectable. In the testis of the mouse treated with injection schedule 3 in which ACK2 was administered after the appearance of the earliest preleptotene spermatocytes of meiotic prophase, maturing spermatids were present, although most of other germ cells except the undifferentiated type A spermatogonia were depleted (Fig. 6F). Thus, the earliest spermatocytes, which first appeared in the testis on day 8 after birth, could undergo formation of haploid spermatids even in the continuous presence of ACK2. These results indicate clearly that c-*kit* is required specifically for the mitosis of the mature type A or the differentiating type A spermatogonia, but the mitosis of earlier gonocytes and primitive spermatogonia, and the meiosis of spermatocytes are independent from c-*kit* (Fig. 7).

Discussion

Since c-*kit* was mapped to *W* locus (Chabot *et al.* 1988; Geissler *et al.* 1988), phenotype analysis of *W* mutant mice can reveal in which cell lineages c-*kit* is functionally required. Previous studies on *W* mice and also *Sl* mice, which have mutation within the gene encoding the ligand for c-*kit*, indicated clearly that c-*kit*/*W* and its ligand are involved in the process of germ cell development in both ovary and testis (for review, Russell, 1979). From detailed histological analysis of *W* and *Sl* embryos, it was demonstrated that c-*kit*/*W* and its ligand *(Sl)* play a role in the migration and/or proliferation of the primordial germ cells (Bennett, 1956; Mintz and Russell, 1957; McCoshen and McCallion, 1975). Consistent with this notion, recent *in situ* hybridization studies showed that c-*kit* is expressed during embryogenesis in the primordial germ cells before and after migration into gonadal ridges (Orr-Urtreger *et al.* 1990) and also that the ligand for c-*kit* is expressed along the migratory pathways of the primordial germ cells and in the gonadal ridges (gonads) (Matsui *et al.* 1990).

In contrast to the process of germ cell development during embryogenesis, the role of c-*kit*/*W* in postnatal gametogenesis is not so well understood. Previous histological examination of W/W^v and W^v/W^v mice indicated that some germ cells survive, but cease to grow and differentiate in postnatal gonads (Coulombre and Russell, 1954). In addition, recent studies showing that both ovary and testis express c-*kit* mRNA suggest a role of c-*kit* in postnatal development of the germ cells (Nocka *et al.* 1989; Manova *et al.* 1990; Orr-Urtreger *et al.* 1990). However, since virtually no germ cells were present in the gonads of *W* or *Sl* mice, phenotype analysis of mutant mice could not correlate the expression of c-*kit* and its ligand with their functional role in postnatal gonads. Thus, various approaches using *Sl* mice, including experimental cryptorchidism (Nishimune *et al.* 1980), *in vitro* organ culture (Nishimune *et al.* 1984), aggregation chimera (Kuroda *et al.* 1988; Nakayama *et al.* 1988), or transplantation of seminiferous tubules (Kuroda *et al.* 1989) have been taken to resolve this question but do not provide a direct answer. In this study, we used a monoclonal anti-c-*kit* antibody that not only recognizes the extracellular

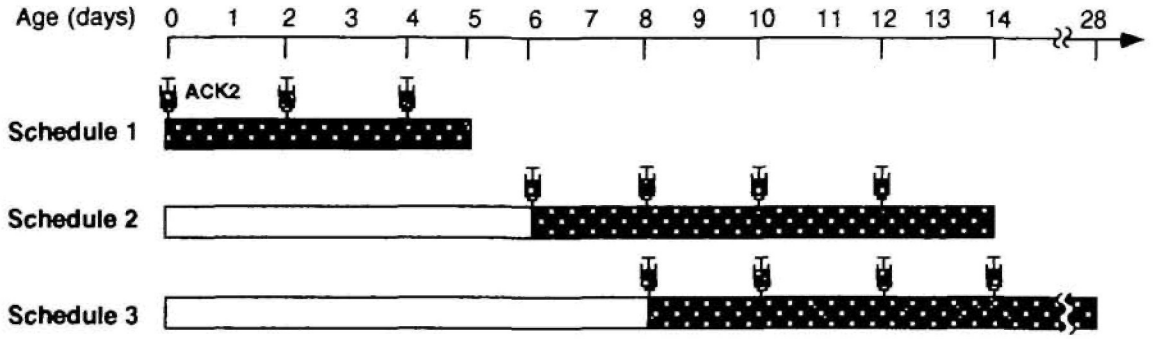

Fig. 5. Diagram illustrating the experimental schedule for the injection of ACK2 into the prepuberal mice.

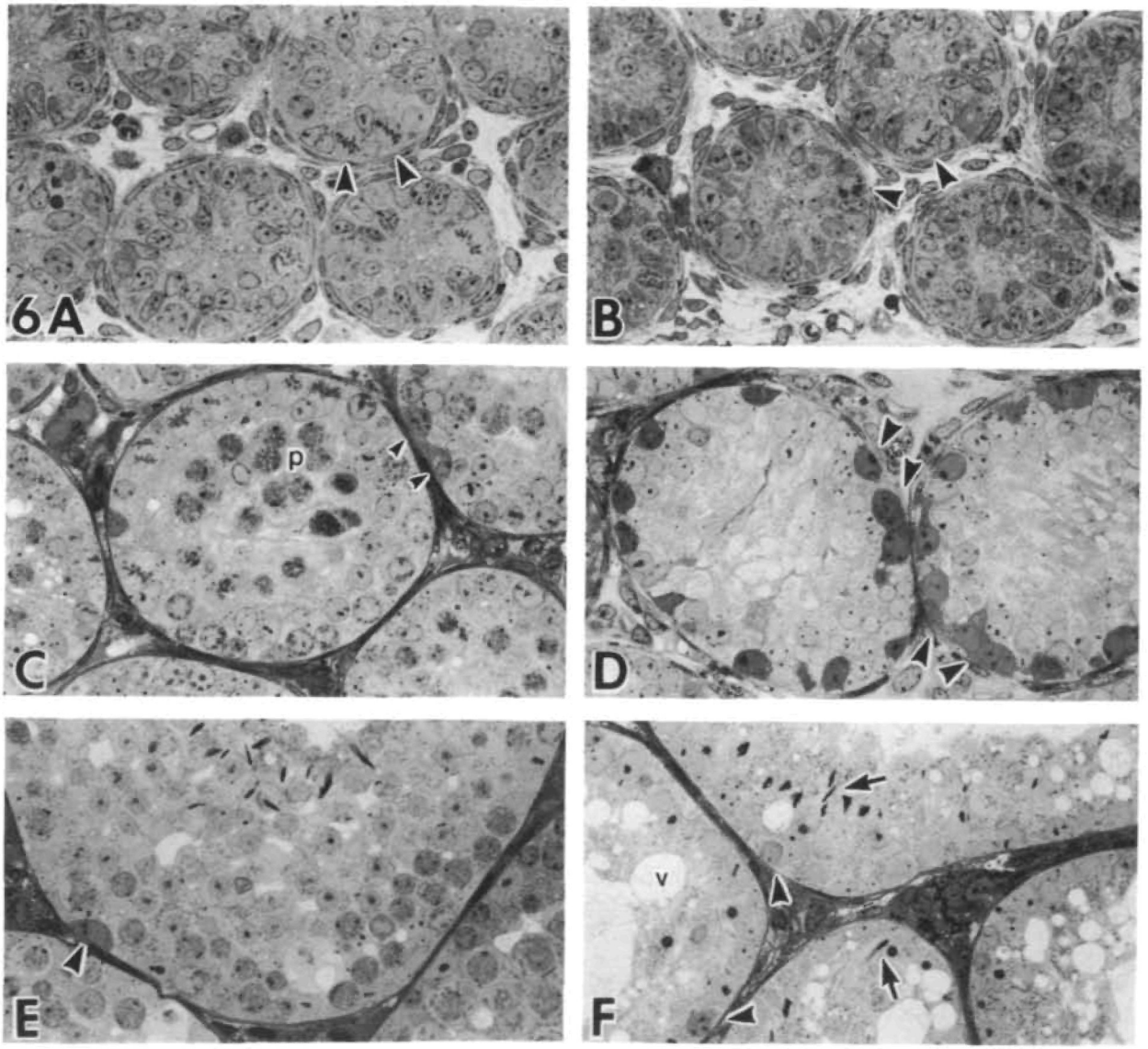

Fig. 6. Effects of ACK2 on prepuberal testes. Toluidine-blue-stained plastic sections of control (A, C, E) and ACK2-treated (B, D, F) mice. All animals in A–F are littermates. (A and B) 5-day-old testes. No significant difference in mice between control (A) and schedule 1 (B). Arrowheads indicate mitotic divisions of the primitive type A spermatogonia. (C and D) 14-day testes. Control tubules (C) with normal spermatogenesis differentiated into pachytene (p) stage, and schedule 2 tubules (D) with no spermatogenesis. Arrowheads indicate dividing primitive type A spermatogonia among numerous Sertoli cells. (E and F) 28-day testes. Control testis (E) with all stages of spermatogenesis, and schedule 3 tubule (F) with the undifferentiated type A spermatogonia (arrowheads) and the maturing spermatozoa (arrows) with numerous vacuoles (v) in the Sertoli cells. (A–F): ×440.

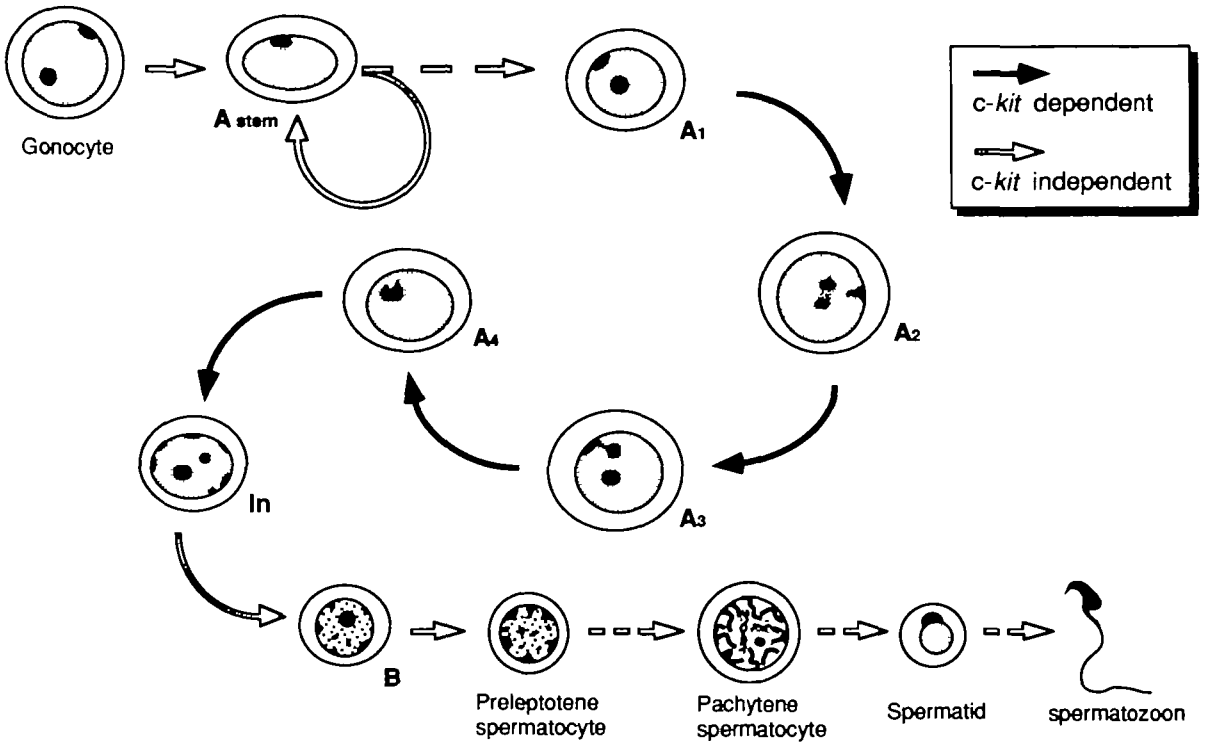

Fig. 7. Summary illustrating the function of c-*kit* in spermatogenesis of the postnatal mouse. Abbreviations: A_{stem}, primitive type A spermatogonium, undifferentiated type A spermatogonium or testicular stem cell; A_1–A_4, differentiating type A spermatogonia; In, intermediate spermatogonium; B, type B spermatogonium.

domain of c-*kit* specifically, but also is able to antagonize the function of c-*kit* both *in vitro* and *in vivo*. Consequently, ACK2 enables us to detect the expression of c-*kit* and also to determine the functional status of c-*kit* which is expressed on the surface of a particular cell lineage.

First, we examined the expression of c-*kit* in ovary and testis. In agreement with our immunohistochemical findings in the ovary, c-*kit* has been detected in both oocytes and interstitial theca cells by *in situ* hybridization (Manova *et al.* 1990). Since testicular interstitial Leydig cells are also c-*kit* positive, this result strongly supports a notion that the theca cell in the ovary is equivalent to the Leydig cell in the testis. We also identified the testicular germ cells as c-*kit* positive. In previous studies using *in situ* hybridization, while c-*kit* expression was found in both spermatogenic cells and Leydig cells (Manova *et al.* 1990), Orr-Utreger *et al.* (1990) reported that Leydig cells are the only sites of c-*kit* expression in the testis. However, this inconsistency could simply be a difference in the sensitivity of detection method. Thus, our results of c-*kit* expression in the testis support that of Manova *et al.* (1990). More interestingly, we demonstrated that the gonocytes and primitive type A spermatogonia in the testis of neonatal mice remained c-*kit* negative until the appearance of the differentiating type A spermatogonia at day 6 after birth. Considering that mitosis of spermatogonia has already started in the absence of c-*kit* expression, there must be a c-*kit*-independent phase of germ cell proliferation in the postnatal life. The significance of this phenomenon will be discussed later.

To investigate whether c-*kit* detected in the germ cells is actually functioning, we used the ability of ACK2 to antagonize c-*kit* function. As demonstrated in our previous studies (Ogawa *et al.* 1991; Nishikawa *et al.* 1991), this antibody exerts its effect by preventing the binding of c-*kit* to its ligand rather than inducing antibody-mediated cytotoxic reaction. Thus, ACK2 administration enables us to determine the functional status of c-*kit* expressed in a given cell. Although much higher expression of c-*kit* is detected in oocytes than spermatogonia, we could not detect any significant change in the histology of ovaries from mice that had been injected with high dose of ACK2 over 12 days. Since normal oocyte maturation repeats with a 4- to 5-day cycle, our results suggest that c-*kit* is not involved in this process. The following possibilities may account for the failure of ACK2 administration to alter the process in ovary: (1) oocyte is not accessible to antibody; (2) the ligand for c-*kit* is absent in the ovary or (3) c-*kit* expressed in the oocytes is not functioning. The first possibility is excluded by the experiment shown in Fig. 1D, demonstrating that intravenously administered ACK2 was present in the oocytes. Since a recent study demonstrated that the ligand for c-*kit* is in fact expressed in the ovary (Matsui *et al.* 1990), the second possibility may also be excluded. Thus, it is likely that, despite the presence of both c-*kit* and its ligand in ovary, c-*kit* expressed in oocyte is not

functioning. Furthermore, this observation strongly indicates that the expression of c-*kit* does not always correlate with its function. In fact, the expression of both c-*kit* and its ligand are detected in the tissues like brain, placenta and lungs which have no significant defect in *W* or *Sl* mutant mice (Nocka *et al.* 1989; Matsui *et al.* 1990). Consistent with the phenotype analysis, ACK2 injection could not induce a significant histological change in these tissues (our unpublished observation).

In contrast to the processes in ovary, spermatogenesis is a process that is highly sensitive to ACK2 administration. In fact, 100 μg of ACK2 per mouse was sufficient for the complete blockade of adult spermatogenesis. This dose is 10 times less than that required for blocking adult hemopoiesis (Ogawa *et al.* 1991). Although a much higher level of c-*kit* expression was observed in Leydig cells, they remained intact histologically in the ACK2-treated mice. It is somewhat surprising that ACK2 induced the elevation of serum testosterone. Since the elevation is within the range of circadian variations and much higher level of testosterone exogenously administrated had no significant effect on the proliferation of spermatogonia, the arrest of spematogonial proliferation was considered to be a direct effect of ACK2 rather than indirect effect *via* Leydig cells. The result in Fig. 4, however, suggests that c-*kit* and its ligand may play a role in a regulation of testosterone production by Leydig cells. The molecular mechanisms behind this phenomenon remains to be investigated.

Consistent with the immunostaining result with ACK2, the site of ACK2 action was very specific. Fig. 7 depicts a summary of the expression and function of c-*kit* in postnatal spermatogenesis derived from the present study. First, with regard to the male germ cells, the site of c-*kit* expression basically represents where c-*kit* is functionally required. Second, the gonocytes or primitive type A spermatogonia, including the self-renewal testicular stem cells, are independent from c-*kit*. However, these c-*kit* negative spermatogonia must be prepared by the c-*kit*-dependent proliferation of their precursors in the embryonic life, because virtually no germ cell was present in the testis of *W/W* mutant mouse. Thus, establishment of the undifferentiated type A spermatogonia which function as a stem cell pool for postnatal spermatogenesis involves a process downregulating the expression of c-*kit*, thereby rendering the stem cells under the control of other molecule(s) than the ligand for c-*kit*. In fact, the proliferation of c-*kit*-negative germ cells continues during the earliest phase of spermatogenesis from day 3 to 5 after birth even in the presence of ACK2. Furthermore, after long-term suppression of spermatogenesis by ACK2 injection, the aplastic seminiferous tubules were repopulated completely by germ cells generated from the remaining stem cells when ACK2 administration was interrupted (our unpublished observation).

Third, c-*kit* is reexpressed on the surface of the differentiating type A spermatogonia and functions as a receptor for mitotic signal. Since the differentiating spermatogonia in the mitotic phase disappeared abruptly 24–36 h after ACK2 injection, the mitosis of the differentiating type A spermatogonia is absolutely dependent on c-*kit* and its ligand. Furthermore, it is clear from our results that the differentiation of type B into preleptotene spermatocytes is independent of c-*kit*. This confirmed the previous observations on testicular tubules of *Sl/+* and *Sl/Sl*^{d} mice, where the differentiation of type A spermatogonia to type B spermatogonia is defective (Nishimune *et al.* 1980; Nakayama *et al.* 1988). Finally, when the meiotic division starts, c-*kit* is downregulated again and the later differentiation process into spermatozoa seems to be controlled by other unknown molecule(s).

In summary, our present study directly demonstrates that the maintenance and/or mitosis of the differentiating type A spermatogonia requires c-*kit* and its ligand, whereas oocyte maturation in adult ovary is independent of c-*kit* despite the high c-*kit* expression in oocytes. These conclusions, although previously suspected, could not be directly proven by phenotype analysis of *W* and *Sl* mutant mouse. Therefore, monoclonal antibodies that antagonize the function of a receptor molecule provide a useful tool for revealing its functional role in a given tissue. Recently, we showed that ACK2 injected into pregnant mice could enter the embryo through placenta and block melanocyte development (Nishikawa *et al.* 1991). Thus, the same strategy may enable us to dissect the role of c-*kit* in the embryonic development of germ cells.

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