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Mammalian Spermatogenesis *in Vivo* and *in Vitro*: A Partnership of Spermatogenic and Somatic Cell Lineages *

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I. Historical Overview

Is gametogenesis possible *in vitro*? This question was one of the relevant topics in a comprehensive review published in 1965 by Etienne Wolff and Katy Haffen (1), describing results of pioneering organ culture work on vertebrate and invertebrate gonads carried out between 1913 and 1965. Wolff and Haffen provided a partial answer to the above question by indicating that organ culture methods allow "far greater differentiation of the germ cells to occur since they are surrounded by their natural environment, namely the somatic cells of the gonads." The early recognition that spermatogenic cells can differentiate *in vitro* in close association with somatic cells can be regarded as the most forward-looking aspect of this statement. Another important remark in Wolff and Haffen's review was the anticipated potential use of *in vitro* organ and cell culture systems for determining the role of various factors, including hormones, in the regulation of the spermatogenic process.

In the early 1960s, Anna Steinberger and co-workers (2–5) reported organ culture conditions resulting in limited differentiation of rat spermatogenic cells in the presence of Sertoli cells. This work demonstrated that *partial* spermatogenesis was indeed possible *in vitro*. In the early 1970s, several investigators (6–12) reported the use of velocity sedimentation methods for the separation of nearly homogeneous testicular cell populations. Techniques for the separation of testicular cells according to their stage of differentiation remain particularly valuable for the analysis of somatic and testis-specific histone transitions during spermatogenesis, for the construction of cell-specific complementary DNA (cDNA) libraries, for cell reaggregation experiments, and for the study of cell-specific gene expression. Coincident with progress in spermatogenic cell fractionation techniques, methods were developed for the isolation and culture of Sertoli cells to study the hormonal regulation of androgen-binding protein (13–15). Significant gains in both the understanding of Sertoli cell physiological responses *in vitro* and in the characterization of biochemical and molecular markers in fractionated spermatogenic cells led research back to the starting question: Is spermatogenesis possible *in vitro*? In the early 1980s, we reported the long-term coculture of interconnected spermatogonial and spermatocyte cell progenies undergoing synchronous DNA synthesis and cell division in close association with Sertoli cell surfaces (16). Parvinen *et al.* (17) reported the completion of meiosis and initiation of early spermiogenesis in incubated, spermatogenic stage-specific seminiferous tubular segments. These studies demonstrated the usefulness of *in vitro* systems for the study of spermatogenesis. Additional gains were made by the development of polarized Sertoli cell cultures using extracellular matrix-coated and uncoated permeable substrates and perifusion techniques (18–22) in an attempt to duplicate *in vitro* conditions prevalent in the intact seminiferous tubules. This review briefly summarizes key features of testicular devel-

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This paper is dedicated to the memory of H. Stanley Bennett, a scientist, a teacher, and a friend.

opment and spermatogenesis *in vivo* and *in vitro* in mammals and then focuses on how culture systems can provide the framework for analyzing the complexity of spermatogenic-somatic cell interactions and their regulatory molecules.

A. Sequential gene activation triggers testicular organogenesis and sexual dimorphism

An sry gene-encoded protein triggers testicular organogenesis and ensures the development of the male phenotype. Coincident with continuous improvements in cell culture techniques and with remarkable advances in cellular and molecular biology, it was realized that, in addition to the classical androgen and gonadotropic hormones, diverse families of novel molecules could act as potent regulators of testicular organogenesis, development of the male phenotype, and initiation and maintenance of spermatogenesis. Of particular significance is the role of *sry* protein, a nuclear transcription factor encoded by the *SRY* gene (for *sex-determining region of the Y* chromosome). *sry* Protein contains a highly conserved amino acid DNA binding motif known as the HMG box (because of its similarity to members of an abundant nuclear protein class, the High Mobility Group proteins), that recognizes a discrete binding site on the upstream regulatory region of both the *Müllerian Inhibiting Substance* (MIS) gene, the *Cytochrome P_{450} aromatase* gene and other genes (23, Fig. 1). The *MIS* gene is expressed by fetal Sertoli cells as soon as the testicular cords develop and is responsible for the regression of the female (Müllerian) reproductive duct primordium in male embryos (Fig. 2). The *Cytochrome P_{450} aromatase* gene, in turn, is responsible for the conversion of testosterone to estradiol. Testosterone is required for the development of Wolffian duct derivatives at the time when Müllerian duct development is inhibited by the Sertoli cell-expressed MIS gene (Figs. 2 and 3). It has been proposed that *sry protein* is the master switch that simultaneously triggers the expression of MIS gene and the repression of the cytochrome P_{450} aromatase gene. By this mechanism, *sry protein* leads the autonomous female developmental program toward the male phenotype.

Although the *SRY* gene provides a coherent picture of how male-specific organs develop and how the female developmental program is inactivated, our understanding of how germinal cells and somatic cells interact with each other in the developing testis and during spermatogenesis is less refined.

II. In the Fetal Testis, Testicular Cords Result from the Aggregation of Sertoli Cell Precursors and Primordial Germinal Cells

Migrating primordial germinal cells use an extracellular matrix pathway to reach the gonadal ridge. In mammalian embryos, gonads develop in the bilateral *gonadal ridges* from the interaction of primordial germinal cells (*PGCs*) with local somatic cells represented by two different populations: mesenchymal cells of the mesonephric region and epithelial cells of the overlying coelomic epithelium. In the mouse, about eight PGCs depart from their site of origin, the yolk sac, and translocate, by a nonmigratory mechanism, to the epithelium of the hindgut adjacent to the yolk sac. PGCs leave the hindgut by *active migration* to enter the dorsal mesentery and finally settle in the gonadal ridge (see Refs. 24–26 for reviews). Soon after reaching the gonadal ridges, after a journey of approximately 4 days in the mouse, migrating PGCs increase in number to a maximum of 20,000–25,000 cells (27) and begin their cell-cell interaction with coelomic epithelial and mesenchymal cells to organize *testicular cords* (Fig. 2). Male PGCs become *gonocytes*, the cell precursor of spermatogonia, and enter a mitotic arrest stage. In contrast, female PGCs give rise to mitotically dividing oogonia that enter meiotic prophase I and mature up to the diplotene stage.

Important questions are: What causes PGCs to begin moving? What factors guide them to the gonadal ridge? What causes them to stop when they reach the gonadal ridges? Recent *in vitro* studies have explored the dynamic process of PGCs locomotion and conditions for survival and proliferation. PGCs can be dissociated from embryos to study their adhesion to and migration on various substrates (28). PGCs can be identified by their surface alkaline phosphatase activity and by their active locomotion patterns (29). After entering the dorsal mesentery, migration of PGCs toward the gonadal ridges depends on two factors: 1) the transient interaction between *fibronectin* molecules and corresponding PGC receptors, presumably integrins, and 2) the release of chemo-attractant factors by gonadal ridges (30).

One approach to define the mechanism of PGC migration is to examine *in vitro* the distribution of cell adhesion molecules in PGCs, their interaction with extracellular matrix components, and the mechanism by which adhesive signals are transduced to the cytoskeleton. Similar studies were conducted *in vivo* and *in vitro* to evaluate neural crest cell migration elicited by extracellular matrix substrates of differing binding affinity for integrin receptors (31). Fibronectin surrounds migrating PGCs in amphibians (32), birds (33), and mice (34, 35). During their nonmigratory phase, within the endoderm of the hindgut, PGCs attach tightly to fibronectin. When PGCs leave the hindgut by active migration, their adhesion to fibronectin becomes transient (28). Once within the gonadal ridge, PGCs become quiescent and initiate active proliferation. Although mesenchymal cells within the dorsal mesentery synthesize fibronectin (36, 28), migrating PGCs do not express fibronectin messenger RNA (mRNA) (28). Moreover, fibronectin is present at very low levels in the gonadal ridge (25, 36). These findings suggest that PGCs can break away from the hindgut by crawling on a fibronectin tract within the dorsal mesentery. Thus, a contact guidance mechanism mediated by specific cell surface receptors may regulate the directional migration and final localization of PGCs *in vivo*. This mechanism implies the developmental regulation of cell adhesion molecules to allow cycles of cytoplasmic streaming and cell cortical changes on a permissive extracellular matrix.

A second phenomenon is the ability of PGCs to migrate toward a distant source guided by a chemo-attractant that defines the end-point of the PGC migratory pathway. Sup-

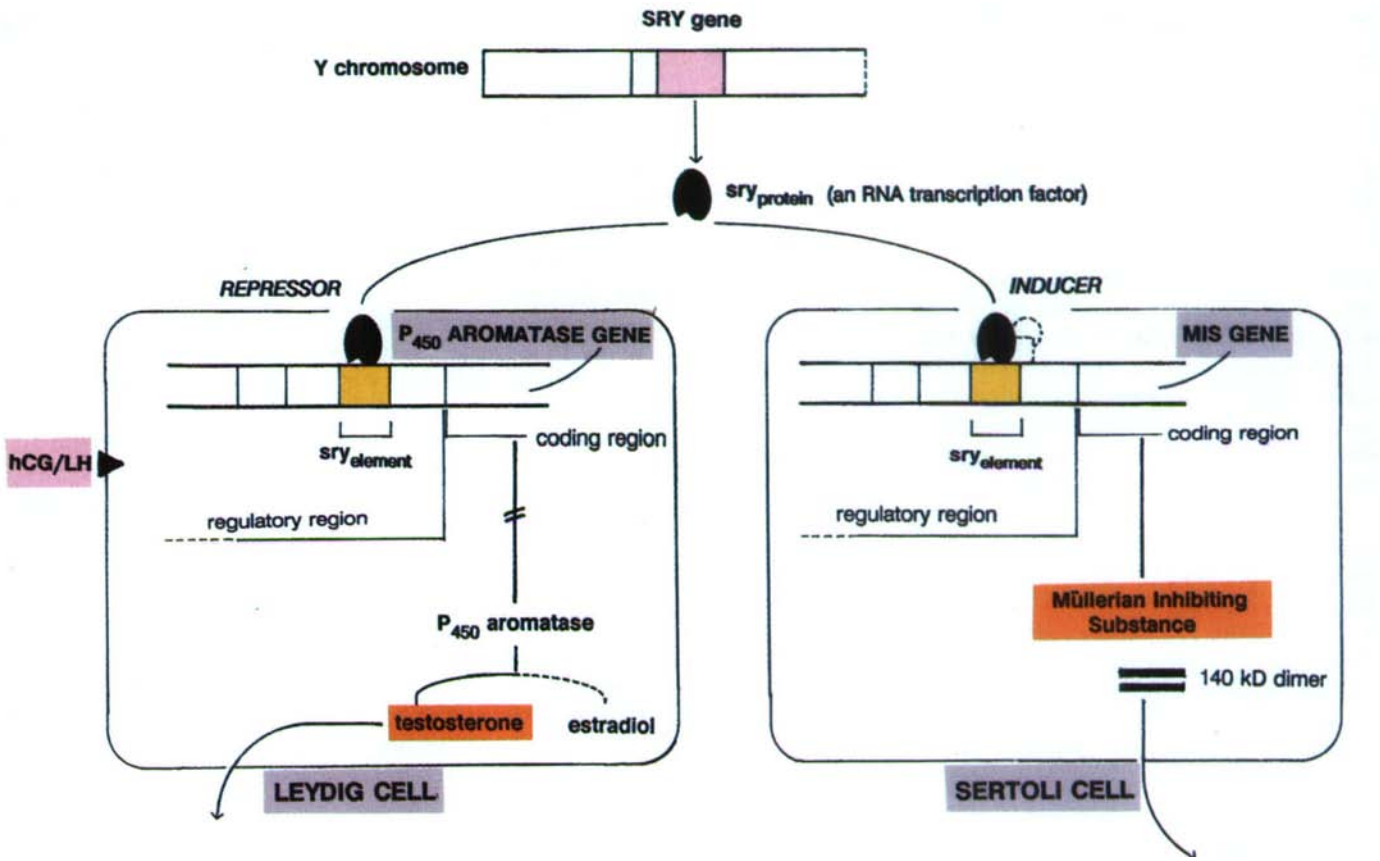

FIG. 1. Regulatory effect of the Y-chromosome encoded *sry* protein on the expression of Müllerian Inhibiting Substance (MIS) and cytochrome P_{450} aromatase genes. *sry* Protein, an RNA transcription factor, binds to the sry element in the regulatory region of the MIS gene and induces the expression of the 140 kilodalton (kD) MIS dimer in fetal Sertoli cells, causing regression of the paramesonephric (Müllerian) ducts. *sry* protein binds to the sry element of the P_{450} aromatase gene and down-regulates its expression in fetal Leydig cells stimulated by hCG/LH, preventing the conversion of testosterone to estradiol. Testosterone is required for the development of the mesonephric (Wolffian) ducts into the male phenotype. [Derived from data in Refs. 23 and 60.]

portive evidence for chemotaxis derives from two experimental examples during gonadogenesis in avians, and in the nematode, *Caenorhabditis elegans*. First, avian PGCs detach from the epiblast and temporarily circulate through the blood vascular system before reaching the gonadal ridges (37). Donor chick PGCs injected into the blood stream of recipient quail embryos can reach and settle in the gonadal ridges, even while the migration of homologous PGCs is still active (38). An ectopic colonization of PGCs (*e.g.* in the head region around the neural tube) was observed in chick embryos when the future gonadal region was removed (39). Second, specialized gonadal muscle precursors of *C. elegans* migrate to the gonads. If the gonad is removed experimentally, myoblasts stop their migration at various sites along their normal pathway (40). One gene involved in the migration of gonadal myoblasts is *sem-5*, which encodes a protein with one SH2 (for *src* homology 2) and two SH3 domains, characteristic of many proteins involved in cell signaling. These observations have been strengthened by *in vitro* experiments showing that chemotropic factors released by incubated gonadal ridges can enable the migration of mouse PGCs (30). The implication of these observations is that PGC migration to gonadal ridges depends on the release of an attractive signal from the target to achieve oriented migration and colonization in the gonadal ridges.

PGCs require both the c-kit receptor and its Steel factor ligand for their survival. In addition to PGC migration to the gonadal ridges, the development of a normal testis depends on: 1) the proliferative activity of PGCs, 2) the aggregation of PGCs with Sertoli cell precursors, and 3) the contribution of the mesonephros to the interstitial cell population. The significance of the first two events has been recognized by the study of mice bearing the mutations designated *Sl/Sl*^{d} (*Steel-Dickie*) and *W/W*^{v} (*dominant white-spotting*) characterized by the depletion of three embryonic migratory lineages: hematopoietic stem cells, neural crest-derived melanocytes, and PGCs (see Ref. 41 for a review). Both mutant mice are black-eyed with white hair, sterile, and severely anemic. Transplantation experiments have shown that the depletion of red blood cells, melanocytes, and germinal cells (ovary and testis) in *W/W*^{v} mice is caused by an intrinsic defect in the migration of precursor cells to the affected organs. By contrast, defects in *Sl/Sl*^{d} mice are ascribed to the site where precursor cells settle, grow, and differentiate.

The defects of these mutants have been characterized at the molecular level. The *W* locus encodes the c-*kit* transmem-

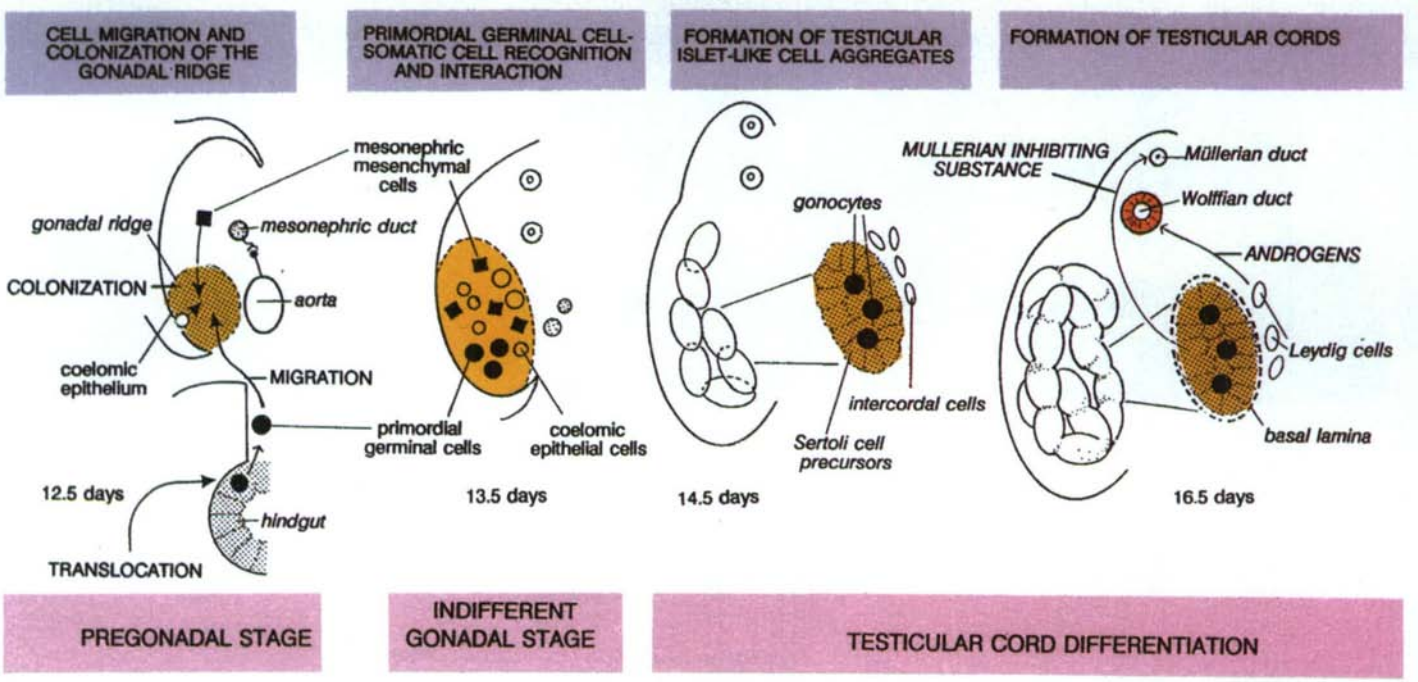

FIG. 2. The *labels at the bottom* indicate the major stages of testicular organogenesis in the rat. These are: the *pregonadal stage* (embryonic 12.5 days), the *indifferent gonadal stage* (embryonic 13.5 days), and the *testicular cord differentiation stage* (embryonic 14.5–16.5 days). The *pregonadal stage* is characterized by the migration and colonization of the gonadal ridge by primordial germinal cells (●) as a three-step event: the *TRANSLOCATION* of primordial germinal cells from the yolk sac to the hindgut, the *MIGRATION* of primordial germinal cells through the dorsal mesentery, and the *COLONIZATION* of the gonadal ridges, composed of epithelial cells derived from the coelomic epithelium (○) and mesonephric mesenchymal cells (■). The prevalent event of the second stage, the indifferent gonadal stage, is the recognition and interaction of primordial germinal cells, coelomic epithelial cells, and mesonephric mesenchymal cells in the gonadal ridge region. The third stage, when testicular cord differentiation occurs, consists of two consecutive events. The first event is the *formation of testicular islet-like cell aggregates* consisting of gonocytes (●), derived from mitotically dividing primordial germinal cells) and Sertoli cell precursors (derived from the coelomic epithelium). Intercordal mesenchymal cells will give rise to Leydig cells, peritubular cells, and vascular structures. The second event is the formation of a basal lamina around the islet-like aggregates, that fuse with each other to form the testicular cords. These two morphogenetic events correlate with an *sry protein* switching effect: the induction of MIS expression in Sertoli cells and the repression of P_{450} aromatase expression in Leydig cells to prevent the conversion of testosterone to estradiol (see Fig. 1). MIS induces the regression of the Müllerian duct; testosterone stimulates the differentiation of the Wolffian duct. [Derived from data in Refs. 23 and 55.]

brane tyrosine kinase receptor, while the *Sl* locus encodes a ligand for this receptor. A ligand for the c-*kit* receptor was cloned and designated by different names (mast cell growth factor, stem cell factor, c-*kit* ligand, or *Steel* factor). The designation *Steel factor* will be used in this paper. The mutation *Steel-Dickie* (Sl^d) is a small deletion of the *Sl* gene, which results in *Steel factor* lacking its transmembrane domain (42, 43). This mutant protein retains some biological activity. The membrane-bound *Steel factor* that is missing in Sl^d homozygous mice is required for the survival of melanoblasts but not for their migration and initial differentiation (44). In mice, c-*kit* and its ligand are expressed in PGCs of 12.5 day-old embryos (45), correlating with their colonization in the gonadal ridges (between 10 and 13.5 days of gestation). The *Steel factor*, in turn, is expressed from day 9 of gestation onward along the migratory pathway of PGCs and in the gonadal ridges (46). After birth, c-*kit* is expressed as early as type A_2 spermatogonia through type B spermatogonia and into preleptotene spermatocytes, as well as by Leydig cells at all ages (47, 48). Sertoli cells also express *Steel factor* (49).

Several lines of evidence provided by *W* and *Sl* mutants suggest that c-*kit* is linked to the generation of the PGC lineage, whereas *Steel factor* fosters the proliferation and differentiation of PGCs and spermatogonia. Similarities in c-*kit* and *Steel factor* expression between testis and ovary are significant. For example, Sertoli and granulosa cells express *Steel factor* while germinal cells (spermatogonia or oogonia-oocytes) and steroid-producing cells (Leydig cells and theca-interstitial cells) express c-*kit* (48, 50). A striking distinction is the expression of c-*kit* in oocytes arrested at the diplotene stage of meiotic prophase I and in ovulated oocytes, compared with the lack of c-*kit* expression beyond the preleptotene stage in spermatogenic cells (47, 48). Essentially, c-*kit* expression is down-regulated at the initiation of male meiotic prophase.

Steel factor is required for the limited survival of PGCs in culture (51, 52). *Steel factor* ligand neither stimulates proliferation nor has a chemo-attractant effect on PGCs during their migration to the gonadal ridges. The membrane-bound form of *Steel factor* ligand is the survival factor for cultured PGCs (52). PGCs and melanoblasts appear to be similar in their requirement of membrane-bound *Steel factor* for survival. However, mast cells survive and proliferate in the presence of either the soluble or membrane-bound form of the *Steel factor* (52). Murine PGCs can be maintained in a proliferating and undifferentiated state *in vitro* when cultured on a feeder cell layer and maintained in culture medium containing various growth factors, including *basic fibroblast*

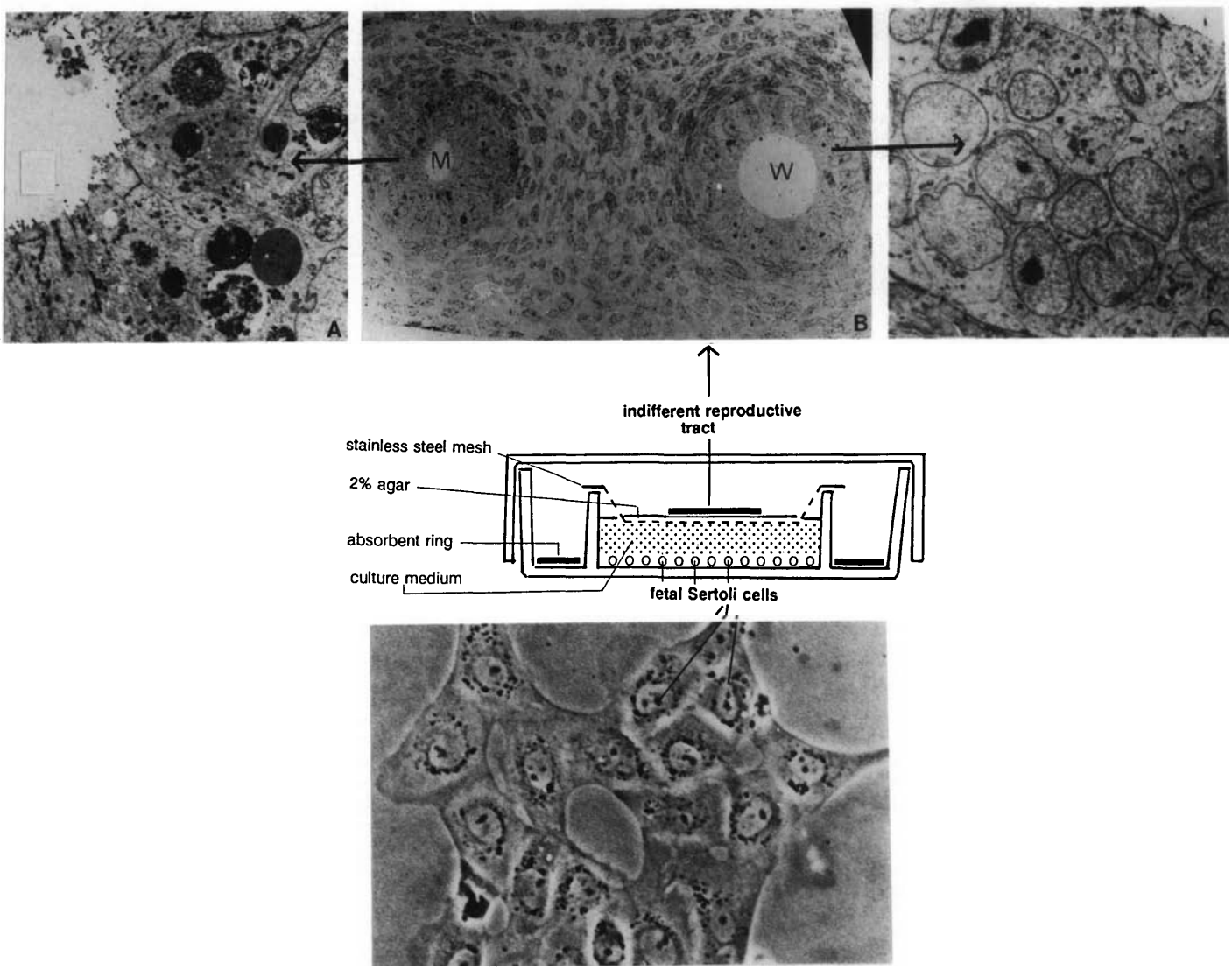

FIG. 3. MIS-mediated effect of fetal rat Sertoli cells on the Müllerian (M) and Wolffian (W) ducts. Primary Sertoli cell cultures were prepared from fetal 16.5-day-old rats. Indifferent reproductive tract tissue was collected from fetal 14.5-day-old rats, incubated with [^{3}H]thymidine for 12 h (to demonstrate viability of the explant) and then cocultured with fetal Sertoli cells for 2–3 days as shown in the diagram. The diagram illustrates a dual organ-cell coculture chamber consisting of dissected reproductive tracts in an indifferent developmental stage, placed on a layer of 2% agarose supported by a stainless steel mesh. Fetal Sertoli cells, cultured at a distance of 5 mm from the explanted reproductive tracts, release *Müllerian Inhibiting Substance* which induces the regression of the Müllerian duct (shown in panel A). Reproductive tract explants were processed for light microscopic autoradiography (B) and transmission electron microscopy (A and C). The epithelial lining of the regressing Müllerian duct (A) displays abundant secondary lysosomes (intracellular circular dense bodies). The epithelial lining of the Wolffian duct (C) appears devoid of lysosomes. [Derived from data in Ref. 59.]

growth factor (bFGF) (53, 54). Permanent pluripotent embryonic stem cell lines can be directly derived from mouse PGCs *in vitro* (53).

How do migrating PGCs and somatic cells of the gonadal ridge interact with each other during the initial steps of testicular cord organization? The critical period of testicular organization starts soon after 13.5 days of gestation in the developing rat embryo (55). At about 14.5 days of gestation somatic cells differentiate into Sertoli cell precursors, which interact with PGCs to organize islet-like cell aggregates devoid of a basal lamina (Fig. 2). By 16.5 days of gestation, cell aggregates fuse to form *testicular cords* that acquire a basal lamina and exclude Leydig cell precursors from the evolving cords. Leydig cells differentiate shortly thereafter and display the characteristics of typical steroid-producing cells, including the activity of 3β-hydroxysteroid dehydrogenase (56). In cultures of intact fetal testes containing well developed testicular cords, extracordal Leydig cells produce high concentrations of testosterone (57). Recent *in vitro* studies have shown that cells migrating from the mesonephric region into the developing testis are necessary for the establishment of testicular cords (58). Testes from 11.5-day-old mice cultured in the absence of the mesonephric region failed to develop testicular cords. However, testes grafted to mesonephric regions carrying a transgenic marker display the marker in some peri-

tubular and interstitial cells and organize testicular cords (58).

Fetal rat Sertoli cells can be isolated from developing testes and cultured under conditions that enable the production of *MIS* (59). The biological activity of *MIS* can be demonstrated *in vitro* by its regressive effect on the Müllerian ducts in an explant coculture system (Fig. 3). Müllerian duct regression is quickly followed by the development of the Wolffian ducts into the male urogenital tract under control of androgens produced by Leydig cells (60).

Cell reaggregation experiments using dissociated gonadal cells of both sexes have been carried out to define factors determining the organization of testicular cords (61). These studies were conducted to test the role of the *H-Y antigen*, a Y chromosome-encoded protein. Sertoli cells secrete the H-Y antigen, and gonadal somatic and germinal cells have specific H-Y receptors (62). Up to 20% of testicular cells, but not male nongonadal cells, were able to induce ovarian cells to form islet-like aggregates. A reduction in the number of testicular cells (below 15%) resulted in the formation of follicle-like structures (61). It was proposed that the specific binding of H-Y antigen to a cell surface receptor for this product accounts for testicular differentiation (see Ref. 63 for a review). However, other sex-linked antigens and/or sex-unrelated molecules may be required for inducing gonadal masculinization. Of special interest is the role of cell adhesion molecules during embryogenesis (see Ref. 64 for a review). Little is known about the patterns of expression and the functional roles of the various adhesive receptors during PGC migration and testicular cord organization. These studies can be carried out *in vitro* in order to define whether PGCs can integrate a number of different signals from the extracellular matrix. Both stimulatory and inhibitory signals can modulate not only PGCs' adhesiveness but also PGC migration, growth, and interaction with somatic cells upon their arrival in the gonadal ridges.

III. The Timing of c-*kit* and bFGF Expression Correlates with the First Surge of Spermatogenesis

At birth, rat gonocytes are separated from the surface of the testicular cords by Sertoli cells and remain in mitotic arrest from fetal day 17 onward (65). After a quiescent period of about 1 week, the first gonocytes reenter the mitotic cell cycle on postnatal day 3, establish contact with the basal lamina 1 day later (66), and then, as spermatogonial cell precursors, initiate the first wave of spermatogenesis. These structural and cell cycle events in rats and mice coincide with the temporal and cell-specific expression of the potent mitogen *bFGF* (67), c-*kit* (48), and *Steel factor* ligand mRNA by Sertoli cells (68, 69). Spermatogonial cell precursors in 5-day old rats display a *bFGF*-like immunoreactive product; in adult rats *bFGF* immunoreactivity is associated with pachytene spermatocytes (67).

As indicated above, *Steel factor* exists in both soluble and transmembrane forms encoded by alternatively spliced mRNAs (42). The transmembrane form of *Steel factor* supports the survival but not the proliferation of PGCs (52). In contrast, the soluble form of *Steel factor*, which slightly enhances PGC proliferation, stimulates mast cell proliferation (68) and DNA synthesis predominantly in type A spermatogonia (70). During postnatal testicular development, the expression of the *Steel factor* transmembrane form appears to change to the soluble form (69). Cultured mouse Sertoli cells express the soluble form of *Steel factor* (70). The ip injection of an anti-c-*kit* monoclonal antibody to prepuberal mice (to prevent c-*kit* binding to its specific *Steel factor* ligand) completely blocks mitosis of type A spermatogonia but not the mitosis of gonocytes, spermatogonial cell precursors, or Sertoli cells (48). Mouse gonocytes, spermatogonial cell precursors, and Sertoli cells do not express c-*kit*; Leydig cells express c-*kit* from day 3 onward (48). These observations indicate that although the re-initiation of mitosis by gonocytes and spermatogonial cell precursors can start in the absence of c-*kit* expression, mitotic activities in these cells correlate with their expression of *bFGF* (67). In addition to its function during early gonadal development, the c-*kit*/*Steel factor* complex continues to play a significant role during the early steps of the spermatogenic process, especially in the mitotic progression of type A spermatogonia. The differentiation of type B spermatogonia into preleptotene spermatocytes is independent of c-*kit* (48).

Methods for the preparation of Sertoli-spermatogenic cell cocultures from neonatal, pubertal, and adult testes. *In vitro* methods have been developed to study functional and structural properties of rat, mouse, and human spermatogenic and somatic cells using testicular fragments or suspension of mechanically or enzymatically dissociated cells (65, 66, 71, 72). Successful preparation of Sertoli-spermatogenic cell cocultures depends on several factors including: 1) minimal disruption during enzymatic dissociation of the structural interaction that Sertoli and spermatogenic cells maintain *in vivo*, 2) cell plating at maximum cell density ($\sim 1.5 \times 10^4$ cells/cm^2) to maintain Sertoli cells in a contact-inhibited state, 3) use of a serum-free medium supplemented with hormones and growth factors (Ref. 73 and Table 1), and 4) frequent replenishment of culture medium and simultaneous removal of metabolic waste products.

TABLE 1. Composition of plating and culture medium[a] used for rat and human Sertoli-spermatogenic cell cocultures [Reproduced with permission from L. L. Tres *et al.*: *J Tiss Cult Meth* 14:265–270, 1992 (73)]

Eagle's minimal essential medium without phenol red supplemented with:	
Insulin (zinc-free)	5 μg/ml
Transferrin	5 μg/ml
Epidermal growth factor	10 ng/ml
Recombinant human GH[b]	133 μIU/ml
FSH[c]	500 ng/ml
Retinol	5 μM
Testosterone	0.1 μM
Dihydrotestosterone	0.1 μM
Glutamine	4 mM
Sodium pyruvate	1 mM
Nonessential amino acids	0.1 mM
Penicillin	100 U/ml
Streptomycin	100 μg/ml

[a] Cells attach overnight; culture medium changed every 24 h.
[b] Eli Lilly and Company, Indianapolis, IN.
[c] NIH ovine FSH-S16.

A. Progenies of spermatogenic cells connected by intercellular bridges have better growth and differentiation potential in vitro than single spermatogenic cells

Spermatogonial cell lineages, derived initially from spermatogonial cell precursors or stem cells, undergo successive mitotic cell divisions before entering meiosis. Members of each spermatogenic cell generation remain interconnected by intercellular bridges or channels. The persistence of bridges represents the remains of incomplete cell division, each bridge being the site of an earlier cell division. The development of *Drosophila* oogonia also involves the formation of intercellular bridges. Recent studies during *Drosophila* oogenesis (74) have shown that a protein expressed by the *kelch* gene becomes associated with the inner surface of the ring canal before the acquisition of an actin contractile ring required for the separation of two daughter cells (cytokinesis). A mutation of the *kelch* gene results in a defective ring canal, leading to a reduction in the transport of molecules between connected cells. It would be of particular interest to determine whether a specific gene product, like the *kelch* gene during *Drosophila* oogenesis, is operational during spermatogenesis.

In vitro studies have shown that the long-term viability and differentiation of spermatogenic cells in coculture with Sertoli cells depends in part on the preservation of intercellular bridges linking members of a spermatogenic cell lineage (16, 72). Support for this notion derives from 1) the autoradiographic analysis of spermatogenic cell proliferation, 2) the chain-like growth pattern of the spermatogonial cell lineage and the cluster-like growth pattern of the spermatocyte cell lineage, and 3) the use of molecular probes to evaluate differentiated spermatogenic cell function.

Rat and human spermatogonia and spermatocytes cocultured with Sertoli cells display distinctive growth patterns with characteristics similar to those observed in the intact seminiferous epithelium. For example, cells of the spermatogonial lineage undergo synchronous S-phase and mitosis (Fig. 4) and show active pulsatile movements while retaining their intercellular bridge connections (16). Meiotic prophase spermatocytes within a lineage exhibit clockwise and counterclockwise nuclear rotation patterns. Single spermatocytes detached from their putative progeny show typical ameboid movements seen in their ancestors, the PGCs and gonocytes. Round spermatids can grow a flagellum containing the characteristic 9 + 2 microtubular axonemal configuration. Spermatid axonemes developed *in vitro* lack outer dense fibers but display a typical wave-like motion (75).

Light microscopic autoradiographic techniques can demonstrate the progression of [^{3}H]thymidine-labeled cultured preleptotene spermatocyte progenies throughout meiotic prophase I stages. Because no significant DNA synthesis occurs after preleptotene, the demonstration of [^{3}H]thymidine-labeled pachytene spermatocytes with time in culture indicates that pulse-radiolabeled preleptotene spermatocytes have advanced in their differentiation from preleptotene to leptotene to zygotene to pachytene stages (16).

Phase-contrast microscopy provides additional details of spermatogenic cell growth patterns. During prolonged coculture, polygonal spermatogonia form long and branching chains of interconnected cells (Fig. 4). Spherical primary spermatocytes grow as compact cell clusters containing short chain-like cell formations (Ref. 76 and Fig. 5). Transmission electron microscopic studies indicate the polarized nature of Sertoli-spermatogenic cell cocultures (22). Additional information concerning the differentiation of spermatocytes *in vitro* derives from the study of the time-course replacement of somatic histones with testis-specific histone variants (Fig. 6 and 75–77). Histone variants TH2B and H1t are of particular interest because TH2B is expressed throughout meiotic prophase I, whereas H1t is prevalent at the end of meiotic prophase I.

These various examples support the emerging concept that the preservation of the putative interaction of Sertoli cells with the clonal-like spermatogenic cell lineages may be required for spermatogenesis *in vitro*.

B. During spermatogenesis in vitro multiple spermatogenic cell progenies coexist and interact with each other in an "open" system

Three distinct events occur in the seminiferous tubule during puberty: 1) the seminiferous tubules acquire a lumen that will allow the transport of testicular sperm and secretory products toward the rete testis, 2) a functional barrier is established at the basolateral region of adjacent Sertoli cells to restrict the bidirectional passage of substances (either blood-borne substances to developing meiotic and postmeiotic spermatogenic cells or spermatogenic cell antigens to the immune system), and 3) the Sertoli cell lineage reaches its maximum cell number and enters into a prolonged mitotic arrest phase. This "enclosed" structural organization is evolutionarily efficient because it assigns stretches of coexisting spermatogenic cell lineages to functionally cycling Sertoli cells (78, 79). However, the "enclosed" character of the seminiferous epithelium is compromised by the relatively "open" nature of the Sertoli-spermatogenic cell coculture system. This difficulty can be tempered by either using seminiferous tubular segments incubated in a culture medium or establishing cocultures from animals of different ages in which a spectrum of premeiotic, meiotic, and postmeiotic developmental stages of spermatogenesis can be accurately timed (17).

The organization of the spermatogenic process in the mammalian seminiferous tubule needs to be considered for determining potential uses and limitations of methods for spermatogenesis *in vitro*. Spermatogenesis is a cyclic process and any given generation of spermatogenic cells overlaps with an earlier or later generation to create a constant combination of spermatogenic cells known as *cell associations* (Fig. 7). Members of a cell association occupy both the basal and adluminal compartments of the seminiferous epithelium during their differentiation. Basal and adluminal compartments are limited by a boundary resulting from the alignment of occluding junctions between adjacent basolateral Sertoli cell surfaces.

Consecutive density patterns are observed when long stretches of isolated rat seminiferous tubules are examined under a microscope at very low magnification and illumi-

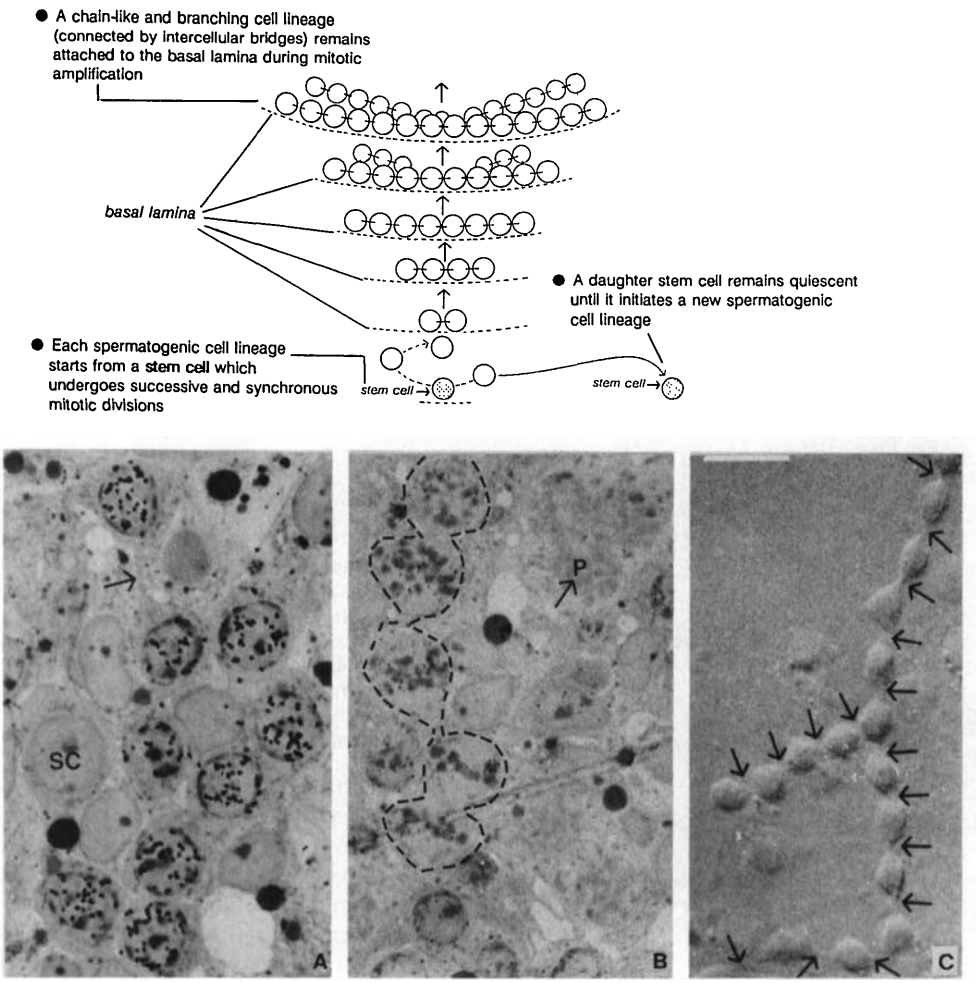

FIG. 4. The *line drawing* illustrates the clonal organization of a spermatogonial cell lineage initiated by the stem cell population undergoing a self-renewal cycle. *In vivo*, a chain-like growth pattern favors the spatial carpet-like arrangement of the spermatogonial cell lineage along the seminiferous tubular wall during mitotic amplification. *In vitro*, the spermatogonial cell lineage mimics *in vivo* functional activities by undergoing both synchronous DNA synthesis and mitosis in coculture with Sertoli cells (A and B, light microscopic autoradiography). Interconnected spermatogonia cocultured with Sertoli cells were exposed to a [^{3}H]thymidine pulse-labeling of 12 h. In panel A, all the nine components of a spermatogonial type B lineage in coculture with Sertoli cells (SC) traverse synchronously the *S-phase* while retaining their intercellular bridges (*arrow*). In panel B, a five-cell [^{3}H]thymidine-labeled spermatogonial lineage is engaged in synchronous *mitotic phase*, presumably generating a 10-cell progeny after completion of mitosis. A cluster of adjacent pachytene spermatocytes (*arrow*, P) are not involved in DNA synthetic activities. The lack of radiolabeling of pachytene spermatocytes serves as an internal control and indicates that these cells were beyond the preleptotene stage at the time of [^{3}H]thymidine labeling. [Adapted from data in Ref. 16.] In panel C (phase-contrast microscopy), a chain-like growth pattern of a spermatogonial lineage cocultured with Sertoli cells is seen after 12 days of plating. The *arrows* indicate the connecting intercellular bridges. Sertoli cells are out of focus in the background. [Adapted with permission from F. F. Smith *et al.*: *Develop Dynam* 193:49–57, 1992 (76).]

nated from below. Each transillumination pattern represents a specific cell association within a seminiferous tubular segment. The notion of "cell association" is linked to two additional designations: "wave" and "cycle" (Fig. 7). When an identical cell association reappears along the length of the same seminiferous tubule, the *distance* between these two identical cell associations is referred as *wave* of the seminiferous epithelium. The period of *time* (in days) that separates the initiation of two consecutive spermatogenic cell lineages from respective stem cell precursors in a given segment of a tubule comprises a *cycle* of the spermatogenic process. Figure 7 shows that wave and cycle share an identical component: the orderly sequence of cell associations. In the rat, it takes four consecutive cycles to release a crop of testicular sperm from a cell lineage (Fig. 7). Contrasting with the linear sequence of cellular associations along the seminiferous tubules of rodents, the seminiferous tubule of man displays a complex spiral-like progression of overlapping spermatogenic lineages. In both rodents and man, spermatogenesis proceeds in a coordinated and continuous manner in close association with the functionally cycling Sertoli cells which generate and propagate biological and mechanical signals within the seminiferous epithelium.

C. The seclusion of spermatogenic progenies in germline cysts in amphibian and insect testis facilitates the progression in vitro of most of the spermatogenic process

In amphibians and insects, a single spermatogenic cell lineage of interconnected cells is confined to a germline cyst

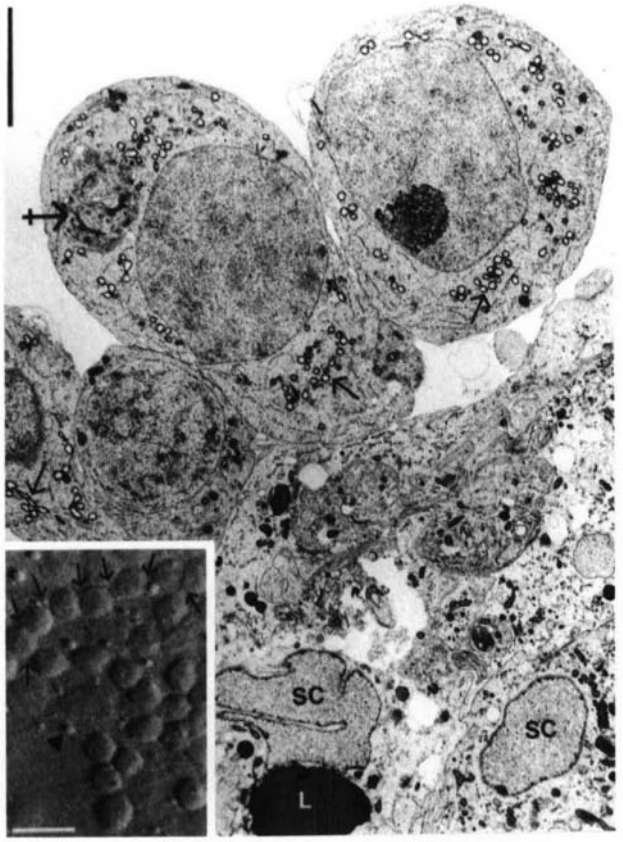

FIG. 5. *Inset* (phase-contrast microscopy), Cluster-like growth pattern of primary spermatocytes connected by intercellular bridges (*arrows*) cocultured with Sertoli cells with lipid droplets (*arrowhead*). Bar, 50 μm. Primary spermatocytes cocultured with Sertoli cells (SC) showing a characteristic lipid droplet (L) are depicted in a transmission electron micrograph. Spermatocytes display ring-shaped mitochondria (*arrows*) and a well developed Golgi apparatus (*crossed arrow*). After 7 days of coculture. Bar, 20 μm. [Reproduced with permission from F. F. Smith *et al.*: *Develop Dynam* 193:49–57, 1992 (76).]

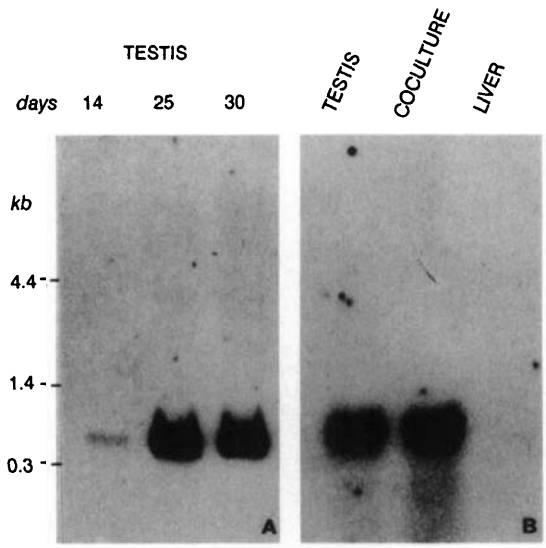

FIG. 6. Testis-specific TH2B histone mRNA shown in Northern blots. A, Temporal appearance of TH2B MRNA during testicular development reflecting a progressive increase in the primary spermatocyte population. B, Expression of TH2B mRNA in testis from 30-old rats and in Sertoli-spermatogenic cell cocultures prepared from testis of littermates and cocultured for 24 h. Liver RNA samples do not display TH2B mRNA. [Reproduced with permission from F. F. Smith *et al.*: *Develop Dynam* 193:49–57, 1992 (76).]

containing Sertoli cell-like nurse cells (80). Germline cysts can be maintained *in vitro* more easily than mammalian seminiferous tubules. For example, isolated spermatocytes from the frog *Xenopus laevis* can complete meiosis and progress to the elongated spermatid stage (81, 82). Primary spermatocytes from the newt *Cynops pyrrhogaster* differentiate to early-to-mid spermatid stages in cell suspension cultures (83). In cultured testes from *Drosophila* (84), primary spermatocytes develop into late spermatid stages. The continuous development of spermatogonia into spermatids and the final differentiation steps of the testicular sperm have not been observed during amphibian and insect spermatogenesis *in vitro*. However, the completion of the entire process of spermatogenesis *in vitro* (from spermatogonia to sperm) has been recently reported in testicular explants of male Japanese eel (*Anguilla japonica*) after incubation for 21 days with 11-ketotestosterone (85).

Contrasting with mammalian spermatogenesis *in vitro*, the progression of amphibian spermatogenic cell development *in vitro* does not require contact with Sertoli cells (82). Furthermore, a single spermatogenic cell lineage in amphibians and insects is limited to a germline cyst while, in mammals, several spermatogenic cell lineages coexist in a seminiferous tubule. However, germline cysts and seminiferous tubules retain an evolutionary conserved communal organization by displaying members of spermatogenic cell lineages linked by cytoplasmic bridges throughout spermatogenesis. The development of a mammalian spermatogenic cell progeny involves two kinds of cellular interactions: 1) an interaction between spermatogenic cells and Sertoli cells, and 2) an interaction between spermatogenic cells mediated by functional intercellular bridges. In the following sections we review additional mechanisms which may be critical for the partnership of the spermatogenic cell lineage with the somatic cell lineage (Sertoli cells, Leydig cells, and peritubular cells).

IV. Specific Proto-oncogene Transcripts Are Expressed During Premeiotic, Meiotic, and Postmeiotic Steps of Spermatogenic Cell Development

During the 1980s, it was recognized that proto-oncogenes are expressed in spermatogenic cells during their proliferation and differentiation. The specific significance of c-*kit* in testicular development and initiation of spermatogenesis is well defined (47–54); however, very little is known about the function of other proto-oncogenes expressed in the testis (Fig. 8).

Testicular proto-oncogenes are known to express at least three distinct classes of proteins. For example, proto-oncogenes in mouse spermatogenic cells express products belonging to the *protein kinase family* (c-*kit*, c-*abl*, c-*raf*, c-*mos*, *pim-1*), *GTP-binding proteins* with GTPase activity (c-*ras H, K and N*) and *nuclear transcription factors* (c-*fos*, c-*jun*). A fourth class of proto-oncogenes includes the *Wnt* proto-oncogene

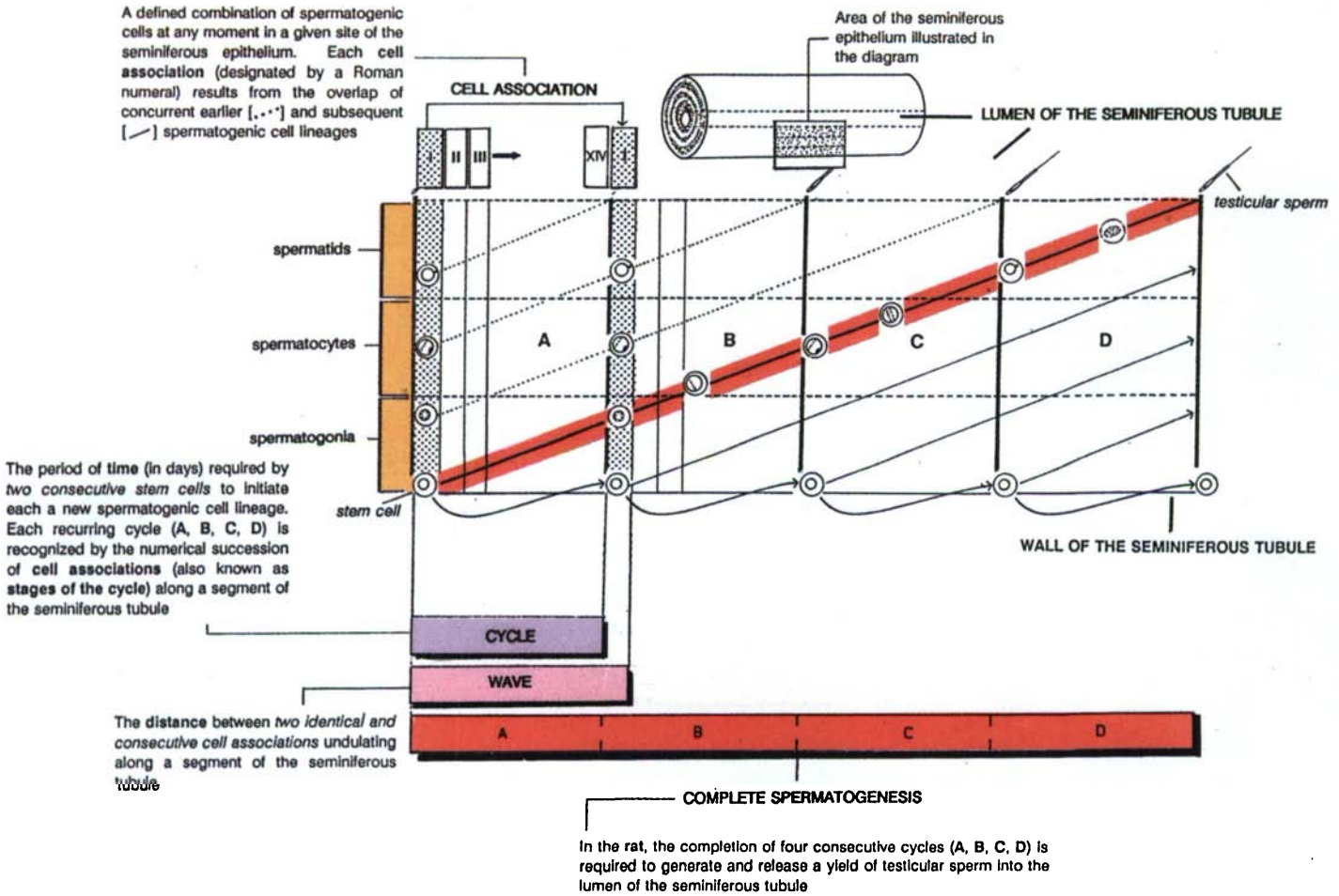

FIG. 7. Diagrammatic representation (not to scale) of rat spermatogenesis along a segment of a seminiferous tubule. A *complete spermatogenic cell lineage* is represented by an *orange diagonal line* interrupted by icons, each corresponding to relevant differentiating spermatogonia, spermatocytes, and spermatids. The *four boxes labeled A, B, C and D*, separated by *vertical thick lines*, represent consecutive *CYCLES* of a complete spermatogenic lineage. The starting point of each cycle is a *stem cell* (adjacent to the wall of the seminiferous tubule) which initiates the *mitotic, meiotic, and spermiogenic* development of a spermatogenic cell lineage (indicated by *diagonal lines*). Preceding and subsequent spermatogenic cell lineages are represented by *diagonal dotted and full lines*, respectively. The terms *CYCLE*, *WAVE*, and *CELL ASSOCIATIONS* are defined in the diagram. Note that: 1) cell associations are integral components of both a cycle and a wave, and 2) a cycle represents a *temporal* notion whereas a wave denotes a *spatial* notion. Icons shown in cell association I of cycles A and B emphasize the presence of identical cell combinations. Different cell combinations are present in subsequent cell associations II, III, . . . XIV (not shown). Rat spermatogenesis consists of 14 distinct cell combinations or stages per cycle. Because complete rat spermatogenesis consists of four cycles (A, B, C, and D), the 14 cell combinations are repeated four times during the period required for fulfillment of a spermatogenic cell lineage (*orange diagonal line*). See text for additional information. [Derived from data in Ref. 150.]

family represented in the testis by three prominent members: *Wnt-1*, *Wnt-5B* and *Wnt-6* (86). *Wnt-1* was formerly designated *Int-1* (for integration site-1) and is involved in mammary gland tumorigenesis and is normally expressed during mouse early neural development (86–88). In addition, *Wnt-1* is a homolog of *wingless*, a gene with a role in *Drosophila* embryonic segmentation. Related *Wnt-1* DNA sequences isolated from *Xenopus*, *Drosophila*, and *C. elegans* indicate multiple roles of this proto-oncogene family, in particular during mesoderm formation. In the mouse testis, *Wnt-1* expression is limited to round spermatids (87). Although the function of *Wnt*-encoded proteins is not well defined, it is known that the *Wnt-1* product is secreted (88) and binds firmly to the extracellular matrix and/or cell surfaces to elicit developmental changes, probably as a local growth factor or as a cell adhesion molecule. Different *Wnt* genes are expressed during early development of the central nervous system, but the testis is regarded as a unique site where *Wnt-1* expression continues in the adult. Both the selective expression of *Wnt-1* during mouse early spermiogenesis and the presence of high *Wnt-5B* and *Wnt-6* transcript levels in testis suggest that these proto-oncogenes may be involved in regulatory pathways during sperm formation.

The c-*mos* proto-oncogene is expressed during mouse and rat spermatogenesis and its product has serine-threonine protein kinase activity. The c-*mos* transcripts of different size were detected in mouse ovary and mouse and rat testis: transcripts in mouse oocytes are smaller in size (1.4 kilobases (kb) 89, 90) than in mouse spermatids (1.7). Rat spermatogenic cells express three c-*mos* transcripts: 5.0, 3.6, and the more abundant 1.7 kb (91) directed by seminiferous tubule-extracted transcription factors binding to the c-*mos* promoter

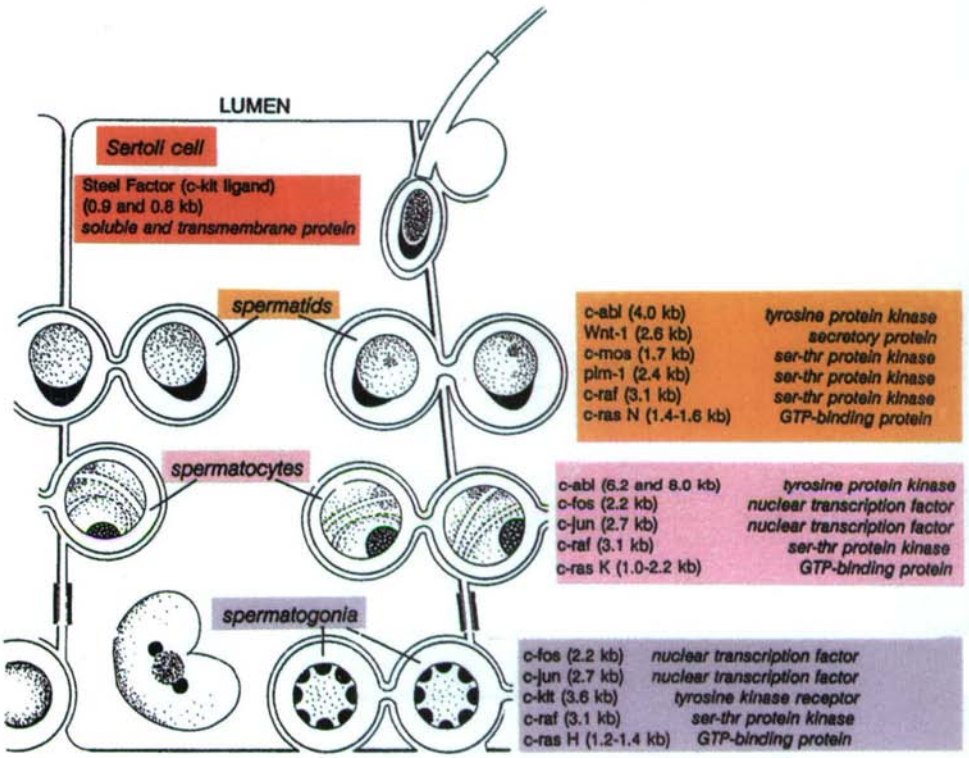

FIG. 8. Cell distribution of proto-oncogene mRNAs in the seminiferous epithelium. *Numbers in parentheses* indicate the mRNA size in kilobases (kb). Known encoded products are also listed for each proto-oncogene. See text for references.

region. In the ovary, oocytes contain about 100-fold higher concentration of c-*mos* transcripts than do round spermatids (92). The c-*mos* product is involved in the meiotic maturation of fully-grown oocytes as shown by the presence of $p39^{mos}$ in *Xenopus* and mouse oocytes (93, 94) and by the blocking of first meiotic division after microinjection of c-*mos* antisense oligonucleotides and antibody against c-*mos* peptide (94, 95). In mouse testis, c-*mos* encodes $p39^{mos}$ as well as a protein of slightly higher molecular mass [$p43^{mos}$ (96)]. The function of c-*mos*-encoded products during spermatogenesis in presently unknown.

The c-*abl* proto-oncogene encodes a protein with specific tyrosine kinase activity and is expressed throughout mouse prenatal development and during mouse spermatogenesis (97) but not during oogenesis (98). Primary spermatocytes contain larger size c-*abl* transcripts (8.0 and 6.2 kb) than early spermatids (4.7 kb) (98). Like the testis-specific transcripts of actin (99), tubulin (100), and protamine (101), c-*mos* and c-*abl* proto-oncogenes are expressed after completion of meiosis. The existence of a single copy of the c-*abl* proto-oncogene indicates that its expression is developmentally regulated during spermatogenesis. Mouse spermatids express the proto-oncogene *pim-1* which encodes a product with protein kinase activity (102). The size of *pim-1* transcripts (2.4 kb) differs from transcripts expressed in somatic cells (2.8 kb).

Three closely related and evolutionary conserved c-*ras* proto-oncogenes encoding a GTP-binding protein with GTPase activity are expressed during mouse spermatogenesis (102, 103). While c-*ras H* transcripts (1.2–1.4 kb) are expressed in spermatocytes and spermatids, c-*ras K* (2.0–2.2 kb) is detected in pachytene spermatocytes and c-*ras N* (1.4–1.6 kb) predominate in spermatids (102). Proto-oncogenes expressing nuclear transcription factors (c-*fos*, 2.2 kb, and c-*jun*, 2.7 kb) are expressed during premeiotic and meiotic steps of spermatogenesis (97).

By comparing both the temporal expression of testicular proto-oncogenes in distinct spermatogenic cell populations and the size variations of their transcripts, it is evident that proto-oncogene transcripts are valuable markers for monitoring spermatogenesis *in vitro*. However, the focus of interest in testicular proto-oncogenes is primarily concerned with their roles in spermatogenic cell development, control of cellular growth, and cell-cell interaction. Essential clues to the identification of proto-oncogene role in spermatogenesis can derive from *in vitro* approaches. Spermatogenesis *in vitro* methods are a useful alternative for the analysis of proto-oncogene cell specific expression and function because: 1) cocultures contain proliferating and differentiating progenies of premeiotic, meiotic, and postmeiotic round spermatids interacting with Sertoli cells, and 2) cell reaggregation and ligand binding experiments can be carried out to study the effects of proto-oncogene secretory products on spermatogenesis.

V. Families of Growth Factors Are Expressed During Testicular Development and Spermatogenesis

MIS and inhibin/activin are members of the transforming growth factor type β superfamily. One of the earliest indications of rat Sertoli cell differentiation is the peak level of *MIS* gene expression following testicular cord development (Fig. 2). The primary role of *MIS* in the male is the inhibition of Müllerian ducts but details of this inhibitory action are not

known. *MIS*, a disulfide-linked homodimer glycoprotein produced by Sertoli cells of the fetal and postnatal testis and by ovarian granulosa cells after birth, is a component of the *transforming growth factor type β* superfamily (*TGF-β*) whose members have effects on cell proliferation, differentiation and tissue organization (see Refs. 104 and 105 for a review).

Members of the TGF-β superfamily bind to a serine/threonine kinase receptor family characterized by a cytoplasmic kinase domain presumably used by ligands to signal their actions (105). In addition to the *MIS* family, the *TGF-β* superfamily includes the TGF-β family itself (with growth-inhibitory effects and control of cell differentiation and extracellular matrix formation), the *Vg*-related family [including bone morphogenetic, *Xenopus Vg-1*, and *Drosophila dpp* (decapentaplegic) proteins] and the *inhibin/activin* family (with hypophyseal regulatory effects and control of cell differentiation). A 2.5 kb transcript corresponding to the TGF-β_1 and TGF-β_3 genes [known to be expressed by rat and bovine Sertoli and peritubular cells (106)] and a TGF-β_1, 1.8 kb transcript [expressed by spermatogenic cells (107)] have been detected.

Inhibin A and B are heterodimers resulting from the combination of an α-subunit with a β_A- or β_B- subunit linked by disulfide bonds. Sertoli cells secrete inhibin to decrease FSH secretion from the hypophysis. Activin A and B are homodimers (β_A- or β_B-subunits) produced by Leydig cells and stimulate FSH production. Each of the inhibin/activin subunits is encoded by separate genes and the primary translation products undergo processing before they link to form mature inhibins or activins. Inhibin subunit gene expression can be detected in fetal testes, adrenals, and placenta (108). In primary cultures of human fetal testicular cells (second trimester), $(Bu)_2$cAMP (but not FSH) increases α- and β_A-subunit transcripts as well as *MIS* mRNA in Sertoli cells. In addition to testicular Leydig cells, activins have an extensive anatomical distribution and participate in the regulation of many biological processes such as early embryonic development and erythropoiesis (see Ref. 109 for a review). Activin A and B stimulate spermatogonial proliferation in Sertoli-spermatogenic cell cocultures prepared from pubertal rats (110). *In vivo* and *in vitro* studies have shown that the absence of spermatids is associated with a decrease of inhibin (111). The addition of a crude mixture of spermatogenic cells to cultured Sertoli cells enhanced inhibin secretion (112). It is clear that the evolutionary conserved *TGF-β* superfamily is represented in the developing testis by the family members *MIS* and *inhibin*. In the mature testis, *inhibin/activin* and *TGF-*β_1 and *TGF-*β_3 have been linked to the progression of the spermatogenic cell lineage. Taken together, these observations indicate that members of the *TGF-β* superfamily largely overlap in their expression, in particular their isoforms, to regulate sexual dimorphism and progression of spermatogenic cell progenies.

TGF-α, a member of the epidermal growth factor (EGF)-like peptide family. It is generally accepted that under certain experimental conditions, TGF-β can act as a growth-inhibitory factor that potentially offsets the actions of TGF-α. Therefore, TGF-β can be regarded as a bifunctional regulator of cell growth (113). *Type α TGFs* are single chain peptides sharing sequence homology with *EGF*. TGF-α and EGF both bind to the EGF receptor and have similar biological activities *in vitro*. Type β TGFs have different primary sequence and consists of two peptide chains. TGF-β binds to a unique cell surface receptor distinct from EGF receptor. Both TGF-α and EGF receptor genes are expressed in the pubertal testis (114). Functional EGF receptors have been found in peritubular cells but not in Sertoli cells, although low levels of EGF receptor gene expression can be detected in Sertoli and spermatogenic cells. The following findings add to the complexity of the TGF-α/EGF receptor relationship: 1) immunocytochemical studies have shown that Sertoli and Leydig cells, but not spermatogenic cells, possess EGF receptors (115, 116). 2) A transgenic mouse line overexpressing TGF-α lacks excessive testicular cell growth (114). 3) In neonatal mouse testis, EGF stimulates ornithine decarboxylase activity (117). 4) In rat Sertoli-spermatogenic cell cocultures, EGF also stimulates the activity of ornithine decarboxylase, an enzyme related to cell growth, differentiation, and proliferation. In the pubertal rat testis, ornithine decarboxylase activity peaks preceding synchronous DNA synthesis in spermatogonia and preleptotene spermatocytes (118).

Although the families of TGF-β and EGF (TGF-α) appear to have a still undefined role in spermatogenesis, the coexpression of several growth factors and proto-oncogene-encoded proteins adds to the intricacy of potential synergistic actions. In fact, other testicular growth factors have been reported. For example, insulin-like growth factor I (*IGF-I*) and IGF-I-binding protein are produced in the intact rat testis and by cultured Sertoli, Leydig, and peritubular cells (119–121). IGF-I binding sites have been observed in pachytene spermatocytes cocultured with Sertoli cells (119). *bFGF* has been detected in spermatogonial cell precursors, type A spermatogonia, pachytene spermatocytes, and in elongating spermatids (66). β-Nerve growth factor, a neurotrophic factor, is produced by spermatocytes and its receptor is found in Sertoli cells (122). The physiological functions of IGF-I, bFGF, and nerve growth factor in the spermatogenic process are presently unknown. Thus, the elucidation of the mechanism of action of growth factors has emerged as one of the most intriguing problems of spermatogenic regulation. Much of the information regarding growth factor-dependent cellular and molecular responses can benefit from the use of *in vitro* systems and specific biological markers which characterize the obligatory events of premeiosis, meiosis, and postmeiosis.

Hematopoietic and spermatogenic stem cells share common features: continuous self-renewal and differentiation of cell lineages after leaving a dormant phase in their cell cycle. Several striking structural and functional homologies exist during hematopoiesis and spermatogenesis. For example, hematopoietic and spermatogenic *stem cells* are responsible for the long-term repopulation of the bone marrow and the seminiferous epithelium, respectively. In these two systems stem cells are capable of: 1) *self-renewal*, to maintain the supply of lineages, and 2) *differentiation*, to provide a continuous yield of functional cells. Stem cells enter the cell cycle after

a "resting" period to initiate a new cell lineage. A significant difference is the *multilineage* potential of hematopoietic stem cells resulting in distinct colonies of fully separated erythrocyte and leukocyte lineages, and the *unilineage* potential of spermatogenic stem cells resulting in interconnected proliferating and differentiating spermatogenic cells (Fig. 4).

In vitro methods have lead to the identification of growth factors (cytokines) and their genes in human hematopoietic stem cells, enabling the understanding of stem cell kinetics and differentiation. Similar *in vitro* studies of postnatal spermatogenic stem cell populations are lagging, presumably because of the lack of specific markers for their detection and eventual isolation. At present, studies of the dynamic behavior of spermatogenic stem cell remain limited to the reconstitution of spermatogenic cell lineages *in vivo* under experimental conditions upholding the genetic integrity of stem cell and Sertoli cell populations (for example, the induced depletion of spermatogenic cell lineages by irradiation, cytotoxicants, and vitamin A deprivation). The development of techniques for the isolation and characterization of spermatogenic stem cells can provide the framework for the identification and timing of inhibitory or stimulatory factors regulating the cell cycle dormancy or commitment of a stem cell to the initiation of a spermatogenic cell lineage. Of particular interest is to determine whether the same growth factors controlling spermatogenic stem cell cycling have physiological roles later during spermatogonial mitotic expansion, meiosis, and spermiogenesis.

During hematopoiesis, *Steel factor*, the ligand for the c-*kit* proto-oncogene, is produced in the bone marrow by stromal cells (123). *Steel factor* appears to act synergistically with hematopoietic growth factors (cytokines) to induce proliferation of hematopoietic colony-forming cells growing in direct contact with stromal cells. Murine stromal cells, expressing both the membrane-bound and secreted form of the human homolog of *Steel* gene product, support human hematopoiesis in long-term bone marrow cultures (123). As indicated previously, Sertoli cells, the functional equivalent to bone marrow stromal cells, express *Steel factor*. During hematopoiesis, *Steel factor* induces the expression of members of the *cytokine* family, a group of regulatory proteins released by one cell to convey information to another cell. The cytokine family includes interleukins (*IL-1α, IL-1β, IL-2* and *IL-6*), tumor necrosis factor, several colony-stimulating factors, and erythropoietin (see Ref. 124 for a review), with binding affinity to cytokine receptors that do not contain a kinase domain. The *Steel factor/cytokine* relationship suggests that these regulatory molecules may modulate hematopoiesis by acting in synergy on target cells. *ILs* are produced in the testis by immunoactivated interstitial macrophages and may play a role in the local control of testosterone biosynthesis by Leydig cells (see Ref. 125 for a review). Further *in vitro* and *in vivo* studies should determine whether interacting *Steel factor*, cytokines, and other growth factors can trigger spermatogenic stem cells to self-renew, select quiescent stem cells to initiate a new spermatogenic cell lineage and control its proliferative (mitosis and meiosis) and differentiation (spermiogenesis) kinetics. The remarkable progress in the field of hematopoiesis during the last decade is a challenging precedent.

VI. Do Cell Adhesion Molecules Have an Exclusive Adhesive Role During Spermatogenesis? Do Growth Factors Have an Exclusive Growth Promoting Role?

In vitro and *in vivo* methods have shown that during their migration toward the gonadal ridges, PGCs establish transient interactions with components of the extracellular matrix, in particular with fibronectin. Upon their arrival at the gonadal ridge, PGCs interact with mesenchymal cells and cells derived from the coelomic epithelial cells to form cellular aggregates as a preparatory step for the development of testicular cords, resulting in the exclusion of Leydig cell precursors by a newly formed limiting basement membrane. During spermatogenesis, proliferating spermatogonial cell progenies, in close contact with extracellular matrix components of the basal lamina, migrate as interconnected cellular networks along Sertoli cell surfaces toward the adluminal compartment of the seminiferous tubule after disrupting extracellular matrix adhesive interactions and establishing new Sertoli-spermatogenic cell adhesive contacts. These developmental steps involve significant changes in the binding affinity of cell surface receptors of somatic and germinal cell lineages for various ligands. *In vitro* methods have shown that specific spermatogenic cell populations have preferential binding for Sertoli cell surfaces (126, 16, 127, 128) and that the actual attachment of spermatogenic cells to Sertoli cells contributes to the extended viability and differentiation of spermatogenic cells (16).

Clearly, cell adhesion molecules play critical roles in testicular development and spermatogenesis as shown by the ability of cells to sort out and aggregate in a histotypically specific manner. However, cell adhesion molecules may transcend their assigned role in tissue specification and remodeling during embryonic and adult life. In this context, it should be stressed that an increasing number of cell adhesion molecules (such as lectins, also called selectins) and extracellular matrix components [such as laminin, tenascin, and nidogen (entactin)] contain EGF-like domains although their exact function is not known. In addition, some growth factors behave as cell adhesion molecules. Several growth factors are synthesized as transmembrane polypeptides which are subsequently processed at the cell surface to produce soluble forms (cited in Ref. 113). An example relevant to gametogenesis is the stable transmembrane form of *Steel factor* which not only induces cell growth but also mediates cell adhesion to cells expressing the c-*kit* receptor (42). The mutant allele Sl^d, which affects the development of germ cells, encodes a soluble form of *Steel factor* that has retained its mitogenic function. Finally, some proteoglycans (composed of a core protein and chains of glycosaminoglycans: heparan, chondroitin and dermatan sulfate and hyaluronic acid) are expressed at the cell surface and behave as cell-cell adhesion molecules (cited in Ref. 113). Growth factors (bFGF, TGF-β) and the *Wnt-1* proto-oncogene product [expressed during mouse early spermiogenesis (87)] are known to bind to proteoglycans.

Different classes of cell adhesion molecules have been recognized in various tissues, including testis, and classified into structurally distinct families: 1) *integrins* (129), 2) *Ca^{2+}-dependent cadherins* (130), 3) *Ca^{2+}-independent cell adhesion molecules (CAM) of the immunoglobulin-like superfamily* (131), and 4) *selectins* (132) (Fig. 9). Both integrins and cadherins interact through mediator molecules with cytoskeletal actin filaments at the sites of cell adhesion: talin and α-actinin link an integrin subunit to actin filaments; α-, β-, and λ-catenins bridge cadherins to actin. Integrins are transmembrane glycoprotein complexes of one α-subunit and one β-subunit that can associate with each other in different combinations to confer ligand specificity of the heterodimer. Some 10 α-subunits and at least eight β-subunits have been reported although new subunits are currently being found. Adhesion molecules of the immunoglobulin-like superfamily mediate Ca^{2+}-independent cell-cell adhesion. Cadherins (E-cadherin or uvomorulin, P-cadherin and N-cadherin) display an extracellular domain that contains Ca^{2+}-binding sites. Selectins contain an NH_2-terminal carbohydrate-recognition domain stabilized by adjacent Ca^{2+}-binding domains. Therefore, selectins are Ca^{2+}-dependent cell surface molecules (also known as "C-type" lectins) and mediate cell-cell interaction by binding to carbohydrates. Three types of selectins have been recognized: E-selectin (endothelial), P-selectin (platelets), and L-selectin (leukocytes and lymphocytes).

Several CAMs have been reported in the testis. Leydig cells *in vivo* and *in vitro* express N-CAM (neural CAM, 133). Antiserum against N-cadherin has been shown to inhibit *in vitro* the attachment of spermatogenic cells to Sertoli cells (134). CD4-like, a member of the CAM immunoglobulin-like superfamily and a receptor for the human immunodeficiency virus (HIV) and D2, homologous to N-CAM, have been detected by immunocytochemistry and immunoblotting in human testes (135, 136). Galactosyl receptor, a selectin-like homologous to the minor hepatic lectin 2/3 variant (137), and galactosyltransferase [GalTase (138)] have been observed on the surface of Sertoli cells, spermatocytes, and spermatids (135–139). Galactosyl receptor and GalTase are redistributed during late spermiogenesis to the dorsal region of the spermatid head overlying the acrosome (137, 138). This particular localization site suggests a role of these sperm surface molecules in sperm-egg recognition at fertilization. The cell adhesion molecule *D2*, originally detected in rat brain as an interneuronal adhesion molecule during synaptogenesis, has been observed in the head region of rat late spermatids, but absent from spermatogonia, mature sperm, and Sertoli cells (140).

The role of integrins in spermatogenesis is of particular interest. Recent data from both *in vivo* and cell culture systems have shown that integrins can transduce signals from the extracellular matrix and from other cells to regulate gene expression and cell growth. The extracellular domain of integrins can bind to more than one extracellular matrix molecule (Fig. 9). Fibronectin and laminin, both present in the seminiferous tubular wall [see Ref. 141 for a review (142)], are each recognized by at least six different integrin heterodimers as well as by nonintegrin receptors. The intracellular domain of integrins binds to cytoskeletal elements to transduce, in cooperation with other classes of adhesion receptors, structural information from the extracellular matrix to the cell interior via the cytoskeletal network. The cytoplasmic domain of the β1-subunit of an $\alpha_n\beta$1 integrin complex binds directly to talin and α-actinin and this interaction is presumably modulated by the α-subunit. Recent immunofluorescent studies have shown the presence of β1 integrin subunit in the rat seminiferous epithelium and in cultured Sertoli cells (143). β1-subunit was transiently present on apical surfaces of spermatogonial cells and along the heads of maturing and elongating spermatids. This last observation suggests an integrin-mediated adhesion mechanism between developing spermatogenic cells and Sertoli cells to influence adhesiveness, cell shape, and motion (Fig. 9).

Related to the integrin family is the *disintegrin* family of platelet aggregation inhibitors observed in snake venoms (144). The term disintegrin denotes a disruption of integrin-mediated interactions with the extracellular matrix. These interactions are mediated by the integrin consensus binding domain RGD (Arg-Gly-Asp) present in a variety of extracellular matrix components (129). A new development is the identification of *PH-30*, a guinea pig sperm cell surface glycoprotein involved in sperm-egg fusion and present at earlier stages of spermiogenesis (145). PH-30 consists of two subunits, α and β. PH-30α contains a potential fusion peptide whereas PH-30β contains a disintegrin domain. The binding/fusion potential of testicular PH-30 poses the intriguing question of whether soluble or membrane-bound disintegrins released by proteolysis can 1) mediate spermatogonial cell attachment to components of the seminiferous basal lamina, and 2) disrupt the adhesion of the spermatogonial cell lineage to facilitate displacement to the adluminal compartment (Fig. 9).

VII. Conclusion

Interest in developing methods for the study of spermatogenesis *in vitro* in our laboratory was initially triggered by a need to understand structural and functional aspects of mammalian spermatogenesis. Extensive cellular, molecular, and endocrinological data have reinforced the idea that success in achieving complete mammalian spermatogenesis *in vitro* is linked to further advances in signaling pathways that are now beginning to emerge. This review has emphasized conditions required by spermatogenic cell lineages interacting in long-term coculture with Sertoli cells that can lead to their proliferation and differentiation. Even though the complexity of interacting spermatogenic cell lineages with somatic Sertoli cells can hamper the understanding of their interrelationships, the possibilities for future research are varied. We must understand more completely what a spermatogenic cell lineage requires to complete the premeiotic-meiotic-postmeiotic sequence. Do lineages have reciprocal interactions or are they independent from each other? The compartmentalization of cellular associations within the mammalian seminiferous tubule indicates that this topographic arrangement is required by interacting Sertoli cells and spermatogenic cell lineages to sort out biological signals

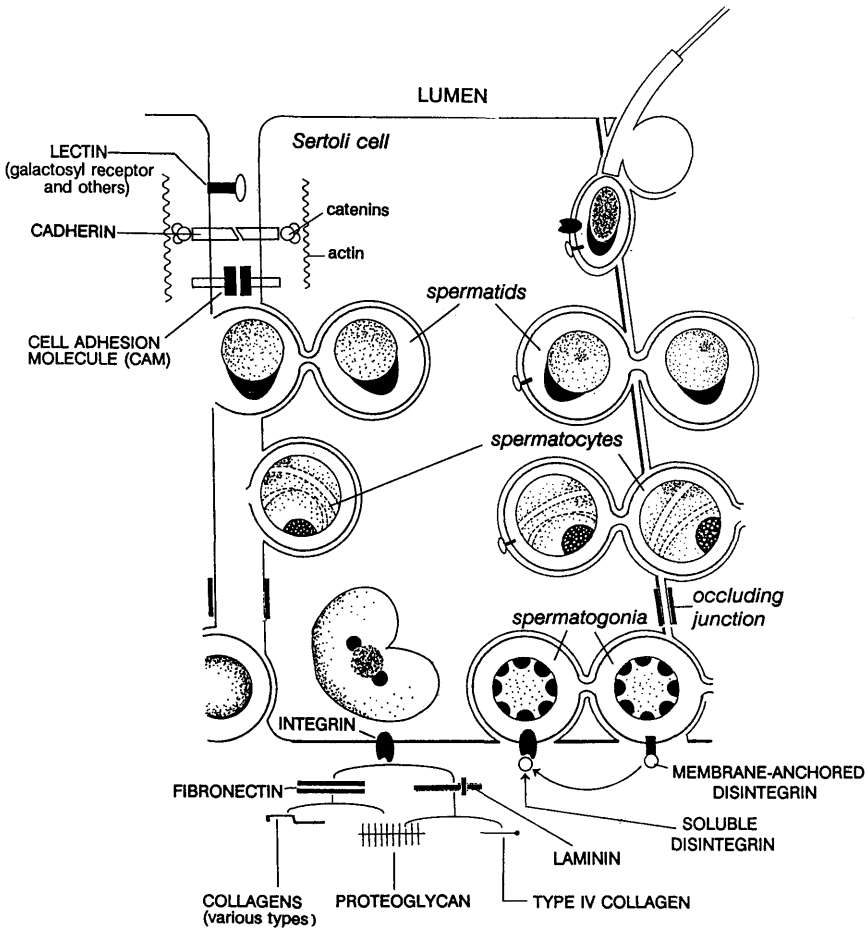

FIG. 9. Distribution of CAMs in the seminiferous epithelium. Note that integrin at the basal Sertoli cell surface is shown to interact with fibronectin and laminin which, in turn, interact with various types of collagens, proteoglycans (heparan sulfate), and type IV collagen. Although it is still debatable whether fibronectin is directly associated with Sertoli cells (142) or it is solely restricted to peritubular cells (141), fibronectin is tentatively included in this diagram pending further studies monitoring the expression of the fibronectin gene in the intact seminiferous tubule. Integrin $\beta 1$ subunit has been observed at the spermatid-Sertoli cell interface (138). The diagram illustrates the hypothesis that a soluble disintegrin (released from the extracellular matrix, or detached from a membrane-anchored molecule by proteolysis) can trigger the separation of spermatogonia from the basal lamina before translocation to the adluminal compartment across inter-Sertoli cell occluding junctions. The presence of CAMs, cadherins, and lectins/selectins is shown at a few sites for schematic purposes only. Other cell-cell localization sites are not precluded. See text for references.

and modulate specific gene expression. Success in germline cyst *in vitro* development in insects and amphibians stresses the territorial requirement of a spermatogenic cell lineage for survival. Alternatively, can spermatogenic and somatic cell lineages be genetically transformed to sustain a built-in differentiation program that can be lost upon coculturing? The immortalization of mouse spermatogenic cells (in particular, the type B spermatogonial-preleptotene transition) and somatic cells (Leydig, Sertoli, and peritubular cells) using the SV40 large T antigen (146) and the establishment of testicular epithelial cell lines [*TM4* of Sertoli cell origin and *TM3* of Leydig cell origin (147)] are efforts toward this goal. However, is it possible to immortalize a meiotic prophase spermatocyte undergoing complex and dynamic chromosomal pairing and genetic exchanging events? Another challenge is to understand how the polarity of the seminiferous epithelium can be maintained *in vitro* and tailored to the fluctuating demands of differentiating spermatogenic cell lineages. The "open" nature of a Sertoli-spermatogenic cell coculture system, in which functionally cycling Sertoli cell populations interact with spermatogenic cell lineages in a noncompartmentalized environment, remains as one of the most critical issues of spermatogenesis *in vitro*. The seminiferous epithelium is sealed by tight junctions at the basolateral surfaces between adjacent Sertoli cells (see Ref. 148 for a review). Sertoli cells act as functional and structural bridges linking the blood-lymphatic intertubular space to the well protected seminiferous luminal compartment where testicular sperm are transported to the epididymis. Studies involving a two-compartment culture system (19–20) have been restricted to the study of polarized secretory functions of Sertoli cells in the absence of spermatogenic cells; we have used a similar approach to study other functional properties of Sertoli cells in the presence of spermatogenic cells (21) and peritubular cells (22). The two-compartment culture system offers interesting experimental possibilities for the differentiation of spermatogenic cells *in vitro*. The development of new permeable substrates (149), the separation of apical and basal compartments by continuous Sertoli cell sheets, the reconstruction of a seminiferous tubular wall (22), and the use of perifusion methods have attempted to mimic *in vitro* the organization and functional polarity of the seminiferous epithelium *in vivo* (19–22). Extracellular matrices have been used to provide a structural and informational context to Sertoli and spermatogenic cells in culture. However, Matrigel, a basement membrane preparation extracted from EHS tumor, has a detrimental effect on spermatogenic cell survival (22) but favors the columnar cytoarchitecture of Sertoli cells (18). Answers to some of these questions will be complex, but the quest to establish complete spermatogenesis *in vitro* seems more feasible now, full of exciting possibilities.

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