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Introduction

Oogenesis is an area that has long been of interest in medicine, as well as biology, economics, sociology, and public policy. Almost four centuries ago, the English physician William Harvey (1578–1657) wrote *ex ovo omnia*—“all that is alive comes from the egg.”

During a women’s reproductive life span only 300–400 of the nearly 1–2 million oocytes present in her ovaries at birth are ovulated. The process of oogenesis begins with migratory primordial germ cells (PGCs). It results in the production of meiotically competent oocytes containing the correct genetic material, proteins, mRNA transcripts, and organelles that are necessary to create a viable embryo. This is a tightly controlled process involving not only ovarian paracrine factors but also signaling from gonadotropins secreted by the pituitary.

The contribution of the male to the biology of reproduction is to produce a genetically intact spermatozoa that will fertilize an oocyte. The end product of male gametogenesis, the mature spermatozoa, is designed for one purpose: to deliver the male contribution of the genetic makeup to the embryo. The biology of gamete production is different in males compared to females. Gamete production in females is

intimately part of the endocrine responsibility of the ovary. If there are no gametes, then hormone production is drastically curtailed. Depletion of oocytes implies depletion of the major hormones of the ovary. In the male this is not the case. Androgen production will proceed normally without a single spermatozoa in the testes.

This chapter presents basic aspects of human ovarian follicle growth, oogenesis, and some of the regulatory mechanisms involved [1], as well as some of the basic structural morphology of the testes and the process of development to obtain mature spermatozoa.

Structure of the Ovary

The ovary, which contains the germ cells, is the main reproductive organ in the female. It also functions as an endocrine organ, secreting estrogen and progesterone in response to gonadotropin and paracrine signaling. Ovaries exist as a pair of glands, approximately the size of almonds, on either side of the uterus. Within the abdominal cavity, ovaries are found closest to the lateral wall of the pelvis, attached to the back portion of the broad ligament of the uterus [2]. This area is known as the ovarian fossa and is surrounded by the external iliac vessels, the umbilical artery, and the ureter [2, 3].

The ovary comprises several different layers and types of tissues, shown in Fig. 3.1. The innermost layer is the medulla, which houses the blood vessels essential to supporting the ovary. To the outside of this is the ovarian cortex, which is made up of follicles and stromal tissue. The outermost layer of the ovary consists of a thin layer of epithelial cells. Known as the *germinal epithelium*, this layer produces thousands of primordial follicles during fetal growth [4]. Underlying the germinal epithelium is a strong connective tissue layer known as the tunica albuginea (TA). Ovum production and oocyte maturation occur within the cortex of the ovary [5]. As primordial follicles are recruited and develop, they move closer to the outer edge of the ovary, eventually bursting through the surface during ovulation [3].

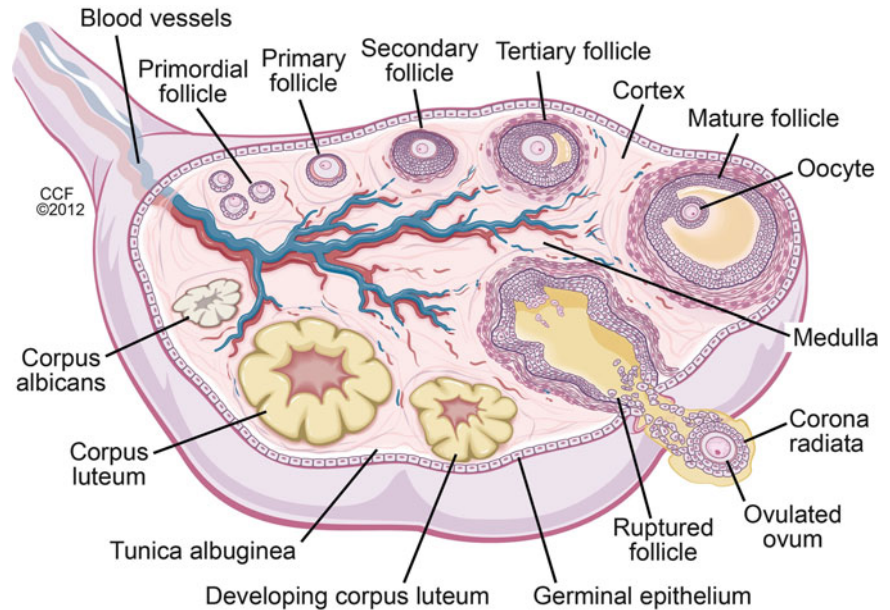
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Fig. 3.1 This schematic of the ovary depicts the developing follicle and oocyte in the ovarian cortex. The outermost layer comprises a thin layer of epithelial cells known as the germinal epithelium, which gives rise to primordial oocytes during fetal growth. Just below this is a strong connective tissue layer known as the tunica albuginea. The medulla, located at the center of the ovary, houses blood vessels and ligaments that are vital to the survival and function of the ovary



Overview of Oogenesis

In humans, oogenesis begins approximately 3 weeks after fertilization. PGCs arise from the yolk sac and migrate via amoeboid movements, through the hindgut, to the genital ridge [6–9]. PGCs undergo rapid mitotic division during this migration. The genital ridge, formed at around 3.5–4.5 weeks gestation, is composed of mesenchymal cells overlaid with coelomic epithelial cells. Upon arrival, the PGCs give rise to oogonia or germ stem cells (GSCs) that continue to proliferate to further expand the germ cell pool. The number of oogonia increases from 600,000 by the eighth week of gestation to over 10 times that number by 20 weeks. At around 7 weeks' gestation, these cells form the primitive medullary cords and the sex cords, respectively.

Follicle formation begins at around week 16–18 of fetal life. Oogonia are enveloped by somatic epithelial cells derived from genital ridge mesenchymal cells, forming primordial follicles. The oogonia then cease mitotic activity and enter meiosis [8, 10, 11].

Once meiosis has been initiated, the oogonial germ cell is termed a primary oocyte. The surrounding mesenchymal somatic cells secrete the follicle's basement membrane and give rise to the granulosa cell layer. By 4–5 months' gestation, the ovary has its maximum number of oocytes, between 5 and 8 million. This number decreases dramatically to 1–2 million at birth and less than 500,000 by puberty [12]. The primordial follicles containing these immature oocytes remain essentially dormant. Through the next 35–40 years of a women's reproductive life span, a small number of follicles are steadily released into the growing pool [13–15].

Oocyte Development and Meiosis

Oogenesis is the process by which the mature ovum is differentiated. It is not clear whether the signals controlling germline entry into meiosis are cell-autonomous or dependent on the mesonephric somatic cells. Meiosis is unique to germ cells. It results in the production of daughter cells with haploid DNA content as a result of a two-step division. During oogenesis, cell division is unequal. The result is a single ovum, with excess genetic material extruded as polar bodies [16]. This is quite unlike spermatogenesis, where meiosis results in four identical haploid daughter cells [17].

The four main stages of meiosis are prophase, metaphase, anaphase, and telophase (Fig. 3.2). DNA replication, chromosome pairing, and recombination, steps that are integral to sexual reproduction, all occur during prophase. Prophase can be subdivided into four stages, leptotene, zygotene, pachytene, and diplotene. DNA replication is finalized in preleptotene, while in leptotene, sister chromatids search for their homologous counterparts. Interaction between homologous chromosomes is facilitated by the formation of recombination nodules. In zygotene, homologous chromosomes pair and begin to synapse. The synapses are maintained by the synaptonemal complex. The crossing over and recombination of chromosomes occur in the pachytene stage, prior to the formation of ovarian follicles. Synapsis is completed in pachytene, and by the diplotene stage, homologous chromosomes are held together mainly at sites of chiasmata. Oocytes in primordial follicles are arrested at the diplotene stage of the first meiotic prophase [1, 18].

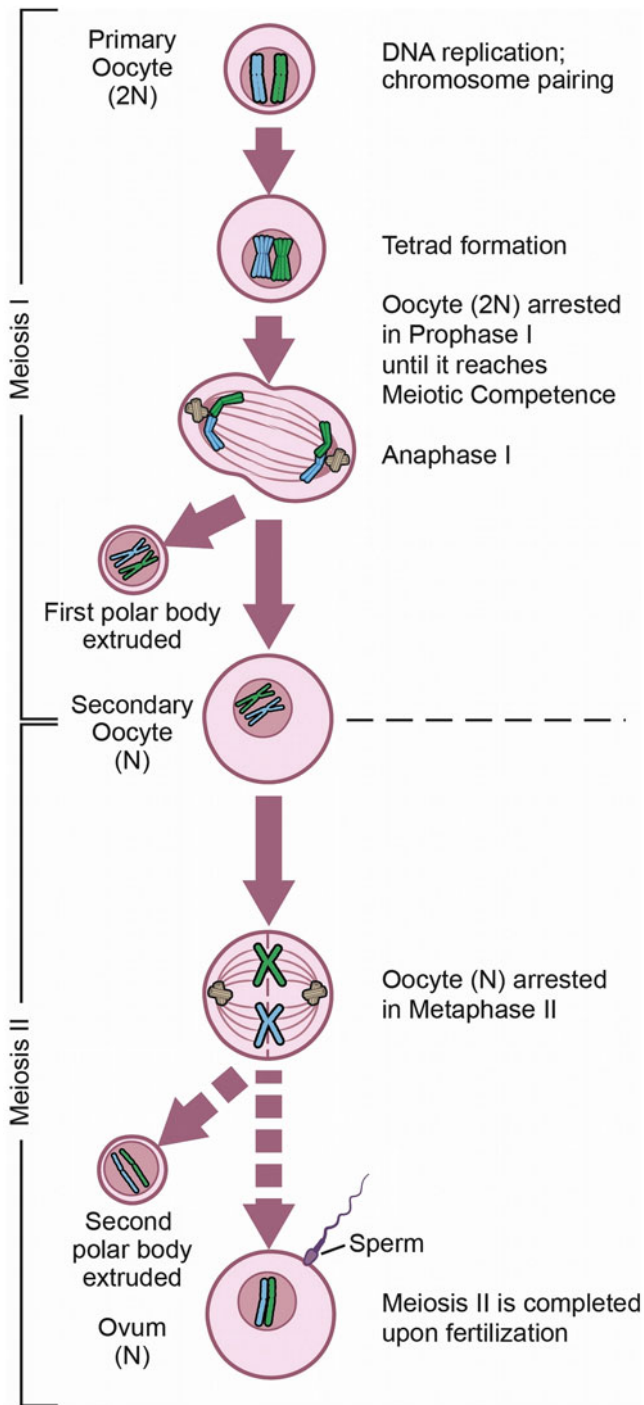


Fig. 3.2 The stages of meiosis I and II leading to the ovulation of a haploid ovum are depicted. Unlike with spermatogenesis, meiosis during oogenesis results in disproportionate cell division with the production of a single ovum with extraneous genetic material being extruded in the first and second polar bodies. During this division, the majority of the cytoplasm, containing important proteins, organelles, and growth factors, remains within the oocyte

The nucleus in the prophase oocyte is called the germinal vesicle. Ooplasmic factors prevent the resumption of meiosis in the prophase oocyte until it reaches a specific diameter and

stage [13, 19–22]. This stage, referred to as “meiotic competence,” occurs in the antral follicle. Once meiosis resumes, there is rapid progression through the metaphase, anaphase, and telophase stages of the first meiotic division. The oocyte then arrests at metaphase 2 of the second meiosis until sperm entry. Oocyte morphology at different stages of maturity is shown in Fig. 3.3.

Follicle Development

Folliculogenesis within the ovary is a very complex process with a high rate of follicle loss. Follicles periodically leave the resting primordial pool to join the growing pool but, in the absence of follicle-stimulating hormone (FSH), undergo atresia. After puberty, once the hypothalamus-pituitary-ovarian axis has been activated during the follicular phase, elevated FSH levels rescue the growing cohort of follicles. The ovarian paracrine signaling induces the continued growth of follicles from this cohort in a process called *initial recruitment* [23]. The recruited growing follicles, known as primary follicles, will subsequently grow into secondary and antral follicles. Figure 3.4 illustrates the different stages of follicle development. Ultimately, a single follicle will be selected from this cohort to become the dominant follicle. It will release a mature oocyte after exposure to increased levels of LH (luteinizing hormone). Almost 90 % of follicles undergo apoptosis or programmed cell death without ever becoming meiotically competent [24].

Critical to this process is the interaction between the somatic cell components and the oocyte itself. Follicle growth from the primordial to the preovulatory stage can be divided into two distinct stages based on responsiveness to the gonadotropins, FSH and LH. The first stage is relatively slow in humans, taking over 120 days [13, 25] and is not directly dependent on gonadotropin levels. Key growth mediators at this early stage may include TGF- β (beta), activin, bone morphogenetic proteins (BMPs), anti-Müllerian hormone (AMH), insulin, estrogen, and androgens. Follicle and oocyte diameters increase, follicles growing in size from 30 to 40 μm in primordial follicles to 100–200 μm in pre-antral follicles (see Fig. 3.4). The single layer of squamous granulosa cells present in the primordial follicles starts to proliferate and the oocyte becomes surrounded by several layers of cuboidal granulosa cells. Precursor thecal cells are recruited from surrounding stroma and a basement membrane forms around the follicle.

The second stage of follicular growth is far more rapid. The follicle is now responsive to the gonadotropins, FSH and LH. Granulosa cell secretions result in the formation of a fluid-filled cavity or antrum. During the early antral stage of follicle development, follicle size increases from 200 μm to

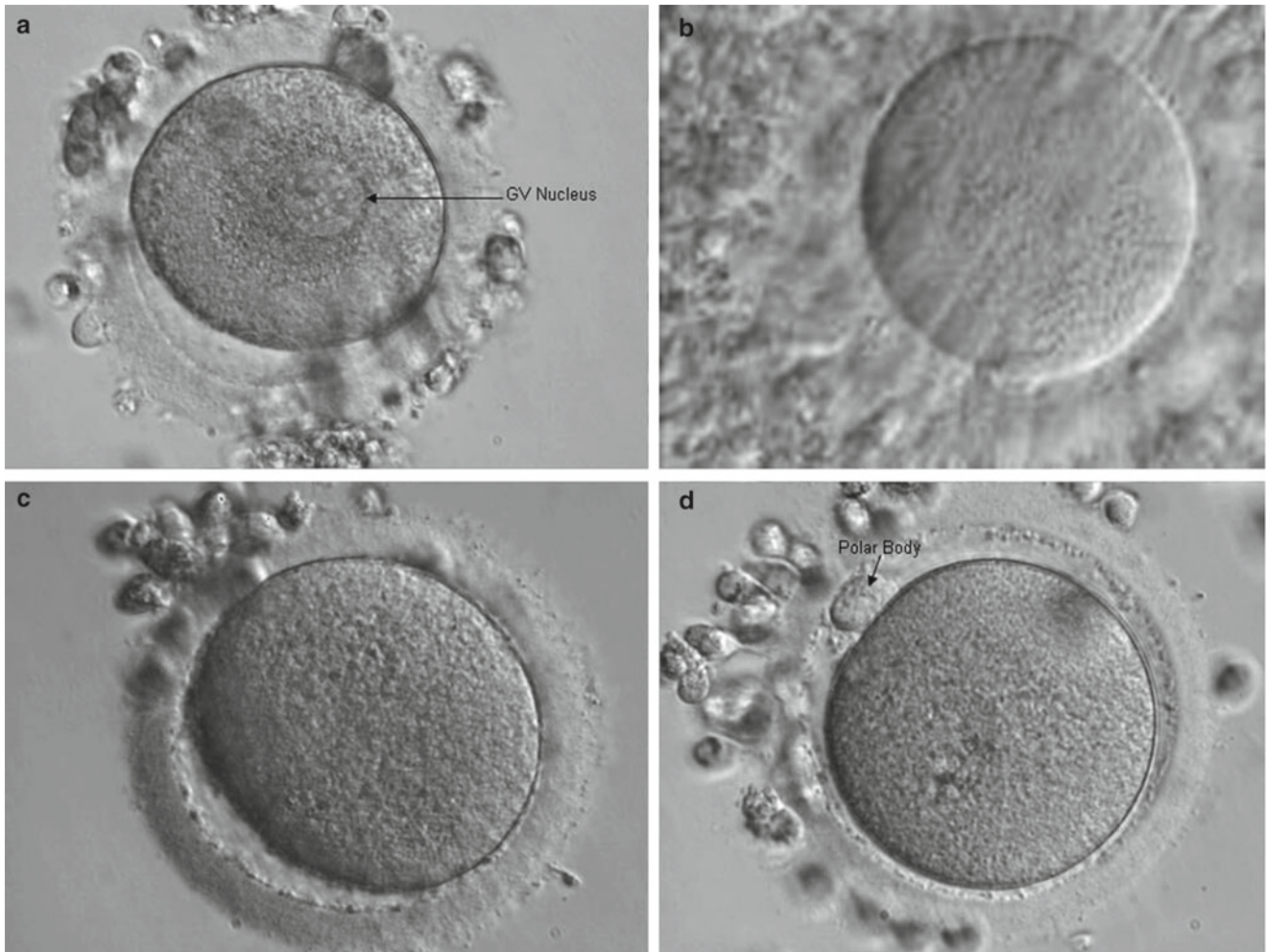


Fig. 3.3 Various stages of oocyte maturation: (a) immature oocyte in prophase I of meiosis I; (b) metaphase I oocyte; (c) cumulus: oocyte complex exhibiting morphology typically associated with mature oocytes at the time of ovulation; (d) mature metaphase II oocyte

2–5 mm. Follicle size increases to 20 mm by the time of oocyte ovulation.

The formation of a fluid-filled antrum and synthesis of steroid hormones mark the transition to the antral stage of follicle development. During this stage and with the influence of FSH, granulosa cells differentiate and become capable of aromatizing androgen, secreted by thecal cells, into estrogen. The local estrogenic environment, combined with high FSH levels, promotes further granulosa cell proliferation and an increase in FSH receptors. This, in turn, makes the follicles even more sensitive to FSH. The negative feedback of rising estrogen levels on the hypothalamus-pituitary axis limits FSH secretion. Thus, only follicles with increased FSH receptors will be able to continue to develop, while other follicles in the cohort will undergo atresia. It is through this mechanism that a single dominant follicle is selected. Continued growth of the selected follicle occurs despite the midcycle fall in FSH concentrations as a result of granulosa cells now acquiring LH receptors [26–28]. While granulosa cells of the early antral follicle are only responsive to FSH,

late antral stage follicles become responsive to both FSH and LH and continue to secrete high levels of estradiol [29, 30]. The layers of specialized granulosa cells bordering the oocyte are known as cumulus cells, which are also called *corona radiata*. These cells not only support cytoplasmic maturation, but are pivotal in maintenance of meiotic arrest and induction of ovulation. The preovulatory follicle, also known as a Graafian follicle, measures over 18 mm in size, and oocyte diameter is close to its final size of about 120 μm [31]. The multilayer follicle is enclosed in a basement membrane that separates it from the underlying vascularized thecal cell layer.

Oocyte Growth

Coordinated growth of this diplotene-arrested oocyte and follicle is dependent on bi-directional communication between the oocyte and the surrounding granulosa cells [32, 33]. This communication occurs via gap junctions connecting the

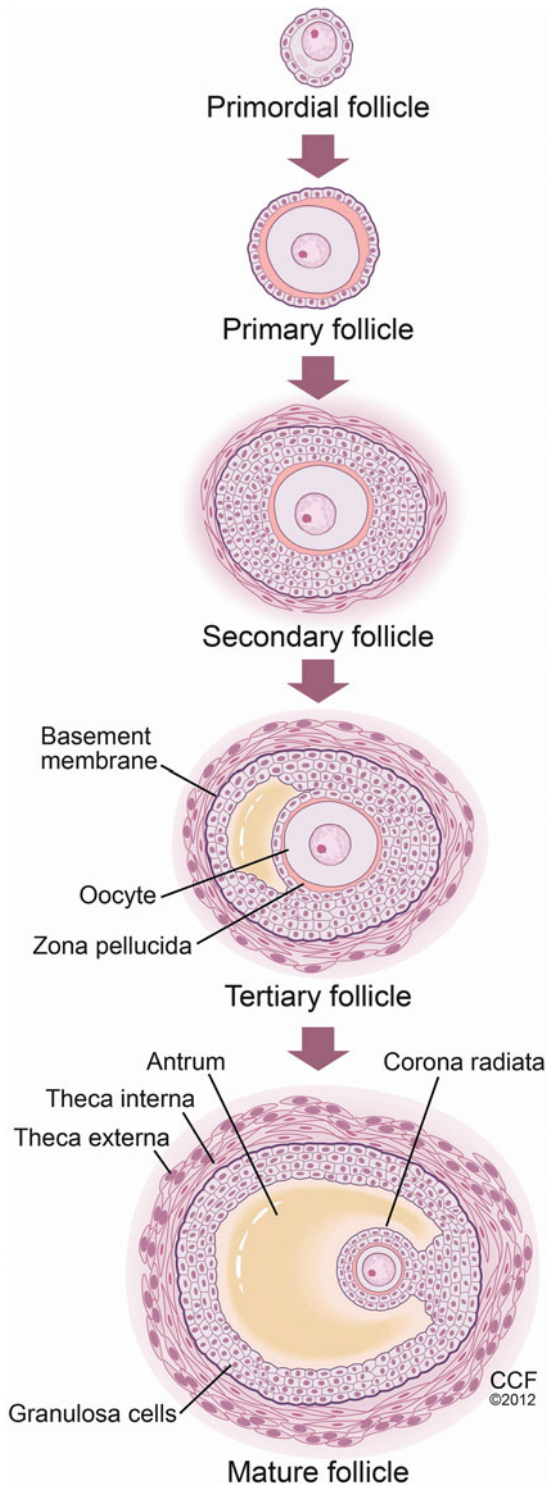


Fig. 3.4 Depicted is a sequential illustration of folliculogenesis within the human ovary. The process begins with the oocyte enclosed within single layer of granulosa cells known as the primordial follicle and ends with a fully developed multilayered follicle with antrum containing a secondary oocyte

granulosa cells and the oocyte [33–39]. These membrane channels enable the sharing of small essential molecules, including inorganic ions, second messengers, small metabolites,

and secreted paracrine factors that allow growth of both cell types [39–42].

The oocyte is unable to transport certain amino acids, carry out biosynthesis of cholesterol, or undergo glycolysis without a supply of the necessary factors by the granulosa cells. Similarly, evidence suggests that granulosa cell proliferation as well as select other metabolic processes require oocyte-derived secretions [43–46]. In vitro studies on cultured follicles demonstrate that severing of gap junctions and intercellular communications triggers premature ovulation and eventual degeneration of the released oocyte [47].

Gap junctions are comprised of a variety of connexin proteins [37]. The basic structure of connexins consists of four membrane-spanning domains, two extracellular loops, a cytoplasmic loop, and cytoplasm N- and C-termini. Different connexins contain different properties, providing increased complexity in the regulation of designated molecules. Gene knock-out experiments in the mouse model have identified specific gap junction proteins and their critical role in folliculogenesis. Absence of connexin-37 interferes with antral follicle formation [48, 49], while follicles in mice lacking the gene for connexin-43 arrest in the early pre-antral stage and are unable to produce meiotically competent oocytes [50]. Phosphorylation and several different protein kinases appear also to be associated with the activation and regulation of gap junctions [51, 52].

The oocyte is metabolically active from its early growth stage, synthesizing the maternal RNA pool necessary to support early embryonic events after fertilization. Oocytes synthesize over 400 different proteins. Shortly after follicle activation and entry into the growing pool, the oocyte secretes a thick glycoprotein coat [53, 54]. This matrix coat, known as the zona pellucida, encircles the oocyte and is composed of three zona pellucida proteins, ZP1, ZP2, and ZP3. Thickness of the zona pellucida increases as the oocyte grows, reaching about 15 μm . The zona pellucida plays an important role in protecting the oocyte/embryo as it traverses the reproductive tract. The zona mediates sperm binding, confers species specificity, and serves as a block to polyspermic fertilization. Release of cortical granules by the oocyte cytoplasm at the time of fertilization results in a hardening of the zona and deters additional sperm from penetrating into the oocyte.

Resumption of Meiosis and Ovulation

Meiotic progression of the oocyte is highly dependent on a delicate balance between factors keeping the oocyte in meiotic rest and factors promoting oocyte maturation [55–61]. Cyclic AMP is one of the intracellular signaling molecules that keep the oocyte in meiotic arrest. Cyclic AMP, produced by granulosa cells, is transported via gap junctions to the oocytes. As long as the cAMP threshold is maintained, meiosis is inhibited.

Meiotic competence is also linked to oocyte size, presumably because increasing volume corresponds to increasing cytoplasmic accumulation of synthesized proteins. Oocytes that measure less than 70–80 μm have lower rates of meiotic competence compared to 100 μm fully grown follicles, which are usually able to resume meiosis. Activation of maturation or M-phase promoting factor (MPF) is required to resume meiosis [62, 63]. MPF levels increase with oocyte growth and eventually reach a threshold, at which point the oocyte becomes *meiotically competent*. MPF is composed of a regulatory unit, cyclin B, and its protein kinase CDK1 (also called p34^{cdc2}).

The onset of the LH surge triggers a cascade of events culminating in the ovulation of the oocyte from the Graafian follicle and initiation of oocyte maturation. LH induces a shift in steroidogenesis by granulosa cells to progesterone [64]. Cumulus cell expansion just prior to ovulation results in the severing of cumulus: oocyte connections, thus reducing intracellular cAMP levels in the oocyte levels and therein reducing its inhibitory influence on meiosis. Subsequent MPF activation drives the oocyte towards meiosis, starting first with breakdown of the germinal vesicle (GVBD). Neosynthesis of cyclin B may be a rate-limiting step, accounting for the observed lag time between resumption of meiosis and GVBD, as well as transition from metaphase I to metaphase II.

After the breakdown of the germinal vesicle, bivalents start to become organized and then align at the metaphase plate, forming a meiotic spindle. The oocyte remains arrested at the metaphase II stage until sperm penetration. An intracellular Ca^{2+} signal triggered by either the binding of sperm to oocyte receptors or else the release of a soluble sperm-derived factor during oocyte: sperm fusion initiates the destruction of endogenous cyclin [63]. The oocyte is now able to complete meiosis, with chromosome segregation beginning at the metaphase-anaphase transition. Defective chromosome segregation at this stage can lead to aneuploidy in the resulting egg and embryo.

Additional Regulatory Mechanisms

Clear maintenance of the primordial follicle pool, follicle recruitment, follicle atresia and selection of a dominant follicle, combined with controlling oocyte maturation, and synchronous growth of the follicular unit, is a complex process. In this section, we discuss a few of the most important ovarian paracrine factors involved in the coordination of events during folliculogenesis (Table 3.1).

cAMP

As mentioned earlier, FSH and LH, working in concert, are two of the most important hormones involved in folliculogenesis.

Table 3.1 Human oogenesis and folliculogenesis beginning with the primordial germ cells (key modulators and their points of action are shown)

Developmental event	Key regulators
Primordial germ cells	
• 3 wk pc	BMP4, BMP8b
• Formation	c-KIT, SCF
• Migration and proliferation	
• Colonization of the forming gonad	
• Post-migratory survival	
Oogonia	
• Proliferation by mitosis	
Oocyte meiotic prophase I	
• Preleptotene: replication of DNA	
• Leptotene: start of homologous pairing	
• Zygotene	
◦ Pairing of homologs	
◦ Synapsis	
• Pachytene	
◦ Crossing over	
◦ Recombination	
◦ DNA repair	
• Diplotene: meiotic arrest	cAMP
Primordial follicle	Activins, AMH, TGF β , BMPs, insulin, estrogen, androgens, c-KIT
• 16–18 wk pc	
• Diplotene-arrested oocyte	
• Single flat granulosa cell layer	
Primary follicle	Activins, AMH
• Cuboidal granulosa cell layer	
Pre-antral secondary follicle	Activins, Inhibins, AMH
• Late folliculogenesis	
• Granulosa proliferation	GDF-9, BMP-15
• Theca precursor formation	
Antral/Graafian follicle	Inhibins, AMH, cAMP, FSH/FSH receptor, LH/LH receptor
• Formation of antrum	
• Formation of preovulatory follicles and corpus luteum	
Cumulus expansion	LH signaling, PGE2
Meiotic resumption	MPF
Ovulation	COX-2, LH
Oocyte maturation and fertilization	Ca^{2+}

wk pc weeks post coitum; *AMH* anti-Müllerian hormone; *BMP* bone morphogenetic protein; *GDF* growth differentiation factor; *FSH* follicle-stimulating hormone; *LH* luteinizing hormone; *SCF* stem cell factor; *COX-2* cyclooxygenase isoform; *PGE2* prostaglandin E(2)

An important component of this system is the second messenger cAMP, which amplifies the signal induced by FSH and LH. This amplification allows for a much larger response to the FSH and LH hormones than they are able to create alone [65]. Two important sets of enzymes control intracellular cAMP homeostasis: adenylyl cyclases, which generate cAMP, and the phosphodiesterases (PDEs), which break down cAMP. Binding of FSH and LH to their respective receptors, along with adenylyl cyclase, activates the generation of cAMP from ATP in follicular granulosa cells. The cAMP molecules activate a cascade of protein kinases,

dissociating their catalytic component, which then works to phosphorylate transcription factors. These factors bind to DNA upstream of genes at positions known as cAMP response elements, regulating various follicular events such as the growth of the dominant follicle [66]. In the oocyte, a high concentration of this second messenger prevents meiosis.

TGF- β Growth Factor Family

Several key regulators involved in folliculogenesis belong to the transforming growth factor- β (TGF- β) family, many of which utilize gap junctions for communications. Important members of this group include AMH, growth differentiation factor-9 (GDF-9), BMPs, activin, and inhibin.

Anti-Müllerian Hormone

AMH is secreted by granulosa cells when a woman is reproductively active. In studies utilizing the mouse model, AMH has been shown to have two important roles during folliculogenesis. First, AMH inhibits the recruitment of additional primordial follicles when there is already a growing cohort of follicles. By this means, AMH prevents women from speeding too quickly through their oocyte reserve. Second, AMH decreases the response of these growing follicles to FSH. Studies in humans have suggested that each follicle has a unique FSH threshold that must be met in order to proceed to the preovulatory stage. AMH may influence how responsive a follicle is to the FSH surge during cyclic recruitment [67].

AMH has also been used as a measure of a woman's ovarian reserve [68]. AMH levels decrease along with the size of a woman's follicle pool. Concomitantly, lower AMH levels fail to adequately inhibit the primordial pool, and as a result there is an increase in the rate of depletion of the follicular reserve [69].

Growth Differentiation Factor-9 (GDF-9) and Bone Morphogenetic Proteins

BMPs play a variety of roles in oogenesis and are produced by a variety of cells. While little is known about specific BMP function in the human ovary, results from numerous animal studies have contributed to our understanding of the biological activities of BMPs during follicular development. Evidence from the rat model proposes that BMPs influence the primordial-primary transition as well as the subsequent transition to a secondary follicle. It has also been suggested that a drop in BMP levels may be indicative of dominant follicle selection [70, 71]. In the rat, thecal cells of secondary follicles have expressed BMP-4 and BMP-7, while their respective receptors expressed on granulosa cells [71]. They

serve to modulate the actions of FSH on the synthesis of the essential steroids estradiol and progesterone [72].

Oocyte paracrine signaling is responsible for activating pathways involved in regulating cumulus cell differentiation. BMP-15 and GDF-9 are two factors secreted by the oocyte to control its local microenvironment and ultimately oocyte quality [45]. BMP-15 works alongside GDF-9 to activate signaling pathways responsible for the regulation of cumulus cell differentiation and maintenance of their phenotype [45]. The absence of these oocyte-specific factors has been demonstrated to result in sterility [73, 74]. During *in vitro* culture of ovarian cortical slices, GDF-9 supplementation was observed to increase the number of secondary oocytes present after 7 days of growth and also enhanced follicle density after 14 days of culture. GDF-9 may also serve to prevent atresia [75].

Activin and Inhibin

Activin and inhibin are produced by granulosa cells in the follicle and work in an antagonistic relationship. Activin, produced by many different tissues, including the gonad and anterior pituitary, stimulates the release of FSH by acting as a transcription factor activator of the FSH β -subunit gene [76]. Meanwhile, inhibin hinders the secretion of FSH from the pituitary, balancing the actions of activin. While it is believed that inhibin plays an important role in the production of steroids and gonadotropins, little else is known about its role in the recruitment and differentiation of thecal cells [77].

c-Kit and Kit Ligand

Another important signaling pathway involved in the regulation of primordial follicles involves the c-Kit receptor and its granulosa cell produced ligand (frequently referred to as KL, stem cell factor or SCF) [78]. Little is known about the specific role of this receptor/ligand combination in the early development of follicles. The presence of the mRNA encoding for c-Kit and KL has been detected in early antral follicles present in human ovarian tissue, oocytes, and granulosa cells [78]. Much of what is known about c-Kit comes from research in the mouse model. Here, c-Kit and KL play a role in PGC survival, activation, migration, proliferation of granulosa cells, recruitment of theca cells, maintenance of meiotic competence, and the development of follicles [79].

Future Directions and Challenges

Increasing our understanding of the complex regulation of ovarian folliculogenesis and the interactions between the oocyte and granulosa-thecal cell layers has contributed

significantly to the advancement of infertility treatment. Medications and ovarian stimulation regimens have been designed which permit the manipulation of a woman's menstrual cycle, resulting in the production of multiple mature competent oocytes. This, combined with advances in our ability to treat male factor infertility, has dramatically altered pregnancy outcomes with in vitro fertilization over the last 2 decades.

One hurdle that has been impossible to overcome has been poor ovarian reserve. The continuous loss and eventual elimination of a woman's follicle pool through accelerated atresia is considered to be the impetus leading to menopause. This is based on the belief set forth over 50 years ago that oogenesis cannot occur in the adult ovary and so, at birth, the female ovary contains a finite oocyte pool [80]. Recently, exciting data have emerged that question this basic established dogma [81, 82]. The intriguing study, first presented in 2004 by Johnson and colleagues, questions the concept of a "non-renewing oocyte pool" [83]. An increasing body of evidence indicates that oogenesis may in fact occur in the adult mammalian ovary [82, 84, 85]. The potential existence of germ cells in the adult ovary and the development of techniques to manipulate such cells may open up new avenues for fertility treatment [86].

Another challenge in reproductive medicine has been fertility preservation for young women diagnosed with cancer. Radiation and chemotherapy during cancer treatment can result in fertility loss through damage to the ovarian follicle reserve. Cryopreservation of ovarian tissue offers hope to patients, but how best to use this tissue to restore fertility is still problematic. Ovarian tissue transplant after the patient is in remission has been met with limited success. Additionally, the possibility of reintroducing the cancer always remains. An alternate solution is the isolation of ovarian follicles from cryopreserved tissue, followed by in vitro maturation of the follicles [87]. The long time interval necessary for human follicle growth from the primordial to the preovulatory stage (~120 days) and the intricate signaling mechanisms necessary for proper follicle growth are quite difficult to mimic in vitro. Maintenance of the spheroid follicle architecture during prolonged growth in conventional 2D culture systems is not possible. Moreover, as the follicle flattens, oocyte:granulosa cell connections are disrupted and critical bi-directional communications between the oocyte and its surrounding somatic cells are lost. Design of 3D follicle culture models has therefore been the focus of much research [87–94].

The challenge of maturing human follicles in vitro and creating competent oocytes may take years to accomplish and will be fueled by our growing understanding of the complex interactions between the oocyte and its supporting granulosa and thecal cell components. The successful culture of human follicles in vitro will ultimately herald a new age in reproductive medicine and the treatment of infertility.

Organization of the Testis

The testes are ellipsoid in shape, measuring 2.5×4 cm in diameter and engulfed by a capsule (tunica albuginea) of strong connective tissue [95]. Along its posterior border, the testis is loosely connected to the epididymis, which gives rise to the vas deferens at its lower pole [96]. The testis has two main functions: it produces hormones, in particular testosterone, and it produces the male gamete—the spermatozoa. The spermatozoa express unique antigens that are not formed until puberty. The blood–testis barrier develops as these autoantigens develop. The blood–testis barrier makes the testis an immunologically privileged site. The testis is incompletely divided into a series of lobules. Most of the volume of the testis is made up of seminiferous tubules, which are looped or blind-ended and packed in connective tissue within the confines of the fibrous septae (see Fig. 3.5). The fibrous septae divide the parenchyma into about 370 conical lobules consisting of the seminiferous tubules and the intertubular tissue. The seminiferous tubules are separated by groups of Leydig cells, blood vessels, lymphatics, and nerves. The seminiferous tubules are the site of sperm production. The wall of each tubule is made up of myoid cells of limited contractility and also of fibrous tissue. Each seminiferous tubule is about 180 μm in diameter, the height of the germinal epithelium measures 80 μm, and the thickness of the peritubular tissue is about 8 μm. The germinal epithelium consists of cells at different stages of development located within the invaginations of Sertoli cells, namely, spermatogonia, primary and secondary spermatocytes, and spermatids. Both ends of the seminiferous tubules open into the spaces of the rete testis. The fluid secreted by the seminiferous tubules is collected in the rete testis and delivered in the ductal system of the epididymis.

Supporting Cells

The supporting cells of the testes refer to cells that are part of the cellular development that leads to a mature sperm. They are, however, extremely important to sperm production, and spermatogenesis would be impossible without them. The two most important cells are the Leydig and Sertoli cells.

Leydig Cells

The Leydig cells are irregularly shaped cells with granular cytoplasm present individually or more often in groups within the connective tissue [97, 98]. Leydig cells are the prime source of the male sex hormone testosterone. The pituitary hormone, luteinizing hormone (LH), acts on Leydig cells to stimulate the production of testosterone. This acts as

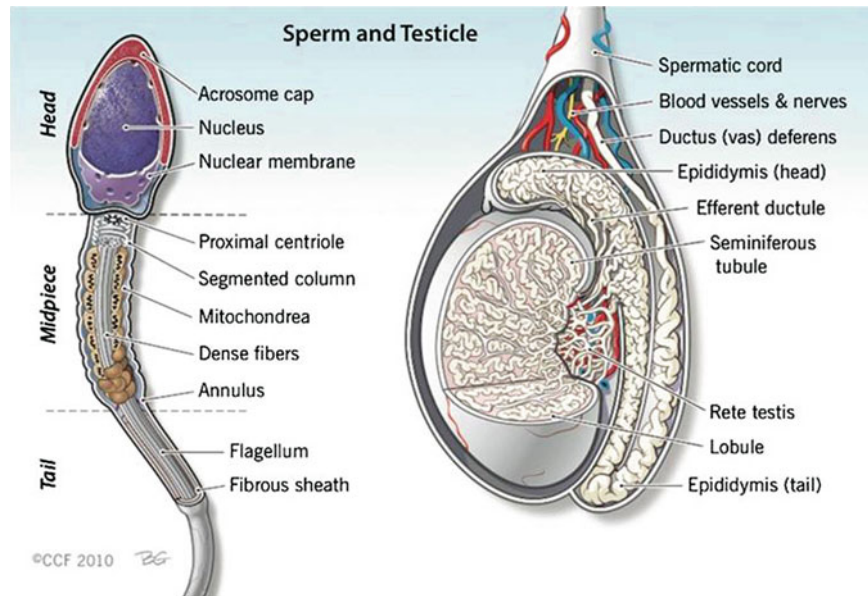


Fig. 3.5 The human testis and the epididymis. The testis shows the tunica vaginalis and tunica albuginea, seminiferous tubule septae, rete testis, and the overlying head, body, and tail of the epididymis. To the *left* is a diagrammatic representation of a fully

mature spermatozoon (All Rights Reserved Sperm Chromatin, ed. Zini A and Agarwal A, Biological and Clinical applications in Male Infertility and Assisted Reproduction, Springer Science + Business Media 2011)

a negative “feedback” on the pituitary to suppress or modulate further LH secretion. Compared with the testosterone levels in the blood, the intratesticular concentration of testosterone is many times higher, especially near the basement membrane of the seminiferous tubule.

The Sertoli Cell

The seminiferous tubules are lined with highly specialized Sertoli cells that rest on the tubular basement membrane and extend into the lumen with a complex ramification of cytoplasm (see Fig. 3.6). Spermatozoa are produced at puberty, but are not recognized by the immune system that develops during the first year of life. The seminiferous tubule space is divided into basal (basement membrane) and luminal (lumen) compartments by strong intercellular junctional complexes called “tight junctions.” These anatomic arrangements, complemented by closely aligned myoid cells that surround the seminiferous tubule, form the basis for the blood–testis barrier. The blood–testis barrier provides a microenvironment for spermatogenesis to occur in an immunologically privileged site. Sertoli cells serve as “nurse” cells for spermatogenesis, nourishing germ cells as they develop. These also participate in germ cell phagocytosis. Multiple sites of communication exist between Sertoli cells and developing germ cells for the maintenance of spermatogenesis within an appropriate hormonal milieu. FSH binds to the high-affinity FSH receptors found on Sertoli cells signaling the secretion

of androgen-binding protein. High levels of androgens are also present within the seminiferous tubule.

The two most important hormones secreted by the Sertoli cells are AMH and inhibin. AMH is a critical component of embryonic development and is involved in the regression of the Müllerian ducts. Inhibin is a key macromolecule in pituitary FSH regulation. Some of the functions of the Sertoli cell are (1) maintenance of integrity of seminiferous epithelium, (2) compartmentalization of seminiferous epithelium, (3) secretion of fluid to form tubular lumen to transport sperm within the duct, (4) participation in spermiation, (5) phagocytosis and elimination of cytoplasm, (6) delivery of nutrients to germ cells, (7) steroidogenesis and steroid metabolism, (8) movement of cells within the epithelium, (9) secretion of inhibin and androgen-binding protein, (10) regulation of spermatogenic cycle, and (11) providing a target for hormones LH, FSH, and testosterone receptors present on Sertoli cells.

Spermatogenesis

The process of differentiation of a spermatogonium into a spermatid is known as spermatogenesis. It is a complex, temporal event whereby primitive, totipotent stem cells divide to either renew them or produce daughter cells that become into a specialized testicular spermatozoa over a span of weeks (see Fig. 3.7). Spermatogenesis involves both mitotic and meiotic proliferation as well as extensive cell remodeling. Spermatogenesis can be divided into three major phases:

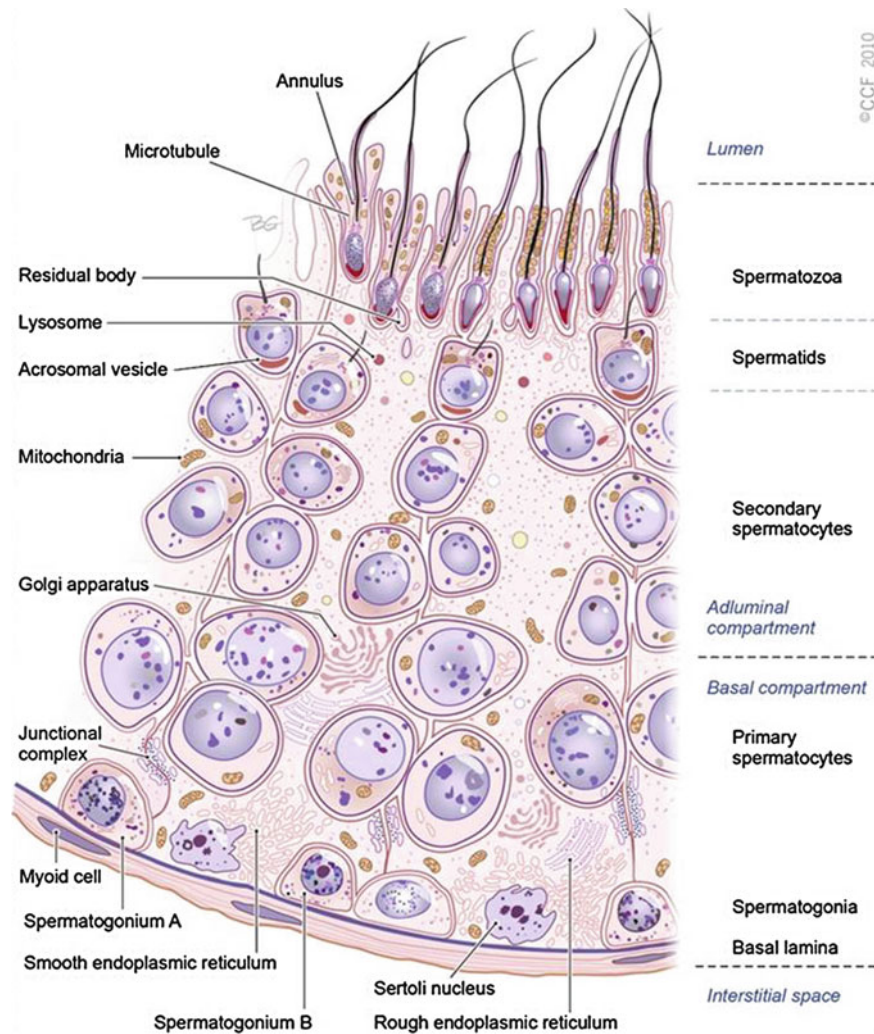


Fig. 3.6 Section of the germinal epithelium in the seminiferous tubule. Sertoli cells divide the germinal epithelium into a basal and adluminal compartment, via the Sertoli cell. Spermatozoa are released

into the lumen (All Rights Reserved Sperm Chromatin, ed. Zini A and Agarwal A, Biological and Clinical applications in Male Infertility and Assisted Reproduction, Springer Science + Business Media 2011)

(1) proliferation and differentiation of spermatogonia, (2) meiosis, and (3) spermiogenesis, a complex metamorphosis that transforms round spermatids arising from the final division of meiosis into a complex structure called the *spermatozoon*. In humans, the process of spermatogenesis starts at puberty and continues throughout the entire life span of the individual. It takes place in the lumen of seminiferous tubules. In fact, 90 % of the testis volume is determined by the seminiferous tubules and their constituent germ cells at various stages of development. Once the gonocytes have differentiated into fetal spermatogonia, an active process of mitotic replication is initiated very early in the embryonic development. This appears to be under FSH control and develops the baseline number of precursor cells of the testicle.

Within the seminiferous tubule, germ cells are arranged in a highly ordered sequence from the basement membrane to the lumen. Spermatogonia lie directly on the basement membrane, followed by primary spermatocytes, secondary spermatocytes,

and spermatids as they progress toward the tubule lumen. The tight junction barrier supports spermatogonia and early spermatocytes within the basal compartment and all subsequent germ cells within the luminal compartment.

Spermatogenesis can also be disturbed by a number of external factors, including nutrition, therapeutic drugs, increased scrotal temperature, and X-radiation.

Types of Spermatogonia

Spermatogonia represent a population of cells that divide by mitosis, providing a renewing stem cell population as well as spermatogonia that are committed to enter the meiotic process. Germ cells are staged by their morphologic appearance; there are dark type A (A_{dark}), pale type A (A_{pale}), and type B spermatogonia; primary spermatocytes (preleptotene, leptotene, zygotene, and pachytene); secondary spermatocytes;

Major Events in the Life of a Sperm

- Spermatogenesis
- Mitosis
- Meiosis
- Spermiogenesis
 - » Head
 - » Midpiece
 - » Tail
- Capacitation
- Lifespan of a spermatozoa
 - » Puberty through life
 - » 30×10^6 per day
 - » 60 to 75 days for sperm production
 - » 10 to 14 days transport (epididymis)
 - » 20 to 100 million per milliliter of ejaculate

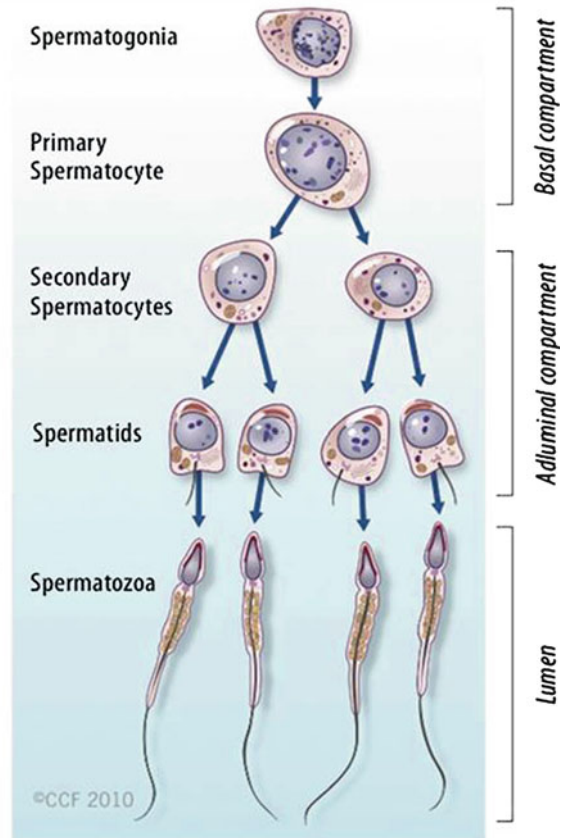


Fig. 3.7 A diagrammatic representation of major events in the life of a sperm involving spermatogenesis, spermiogenesis, and spermiation during which the developing germ cells undergo mitotic and meiotic division to reduce the chromosome content (All Rights Reserved Sperm

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and spermatids (Sa, Sb, Sc, Sd₁, and Sd₂). Other proliferative spermatogonia include A_{paired} (A_{pr}), resulting from dividing A_{isolated} (A_{is}), and subsequently dividing to form A_{aligned} (A_{al}). Differentiated spermatogonia include type A1, A2, A3, A4, intermediate, and type B, each a result of the cellular division of the previous type. In humans, four spermatogonial cell types have been identified; these are A_{long} , A_{dark} , A_{pale} , and B [99, 100]. In the rat, type A_{isolated} (A_{is}) is believed to be the stem cell [101]; however, it is not clear which human type A spermatogonia is the stem cell. Some investigators have proposed that the type A_{dark} spermatogonia represent the reserve or nonproliferative spermatogonial population that gives rise to A_{pale} [100]. Type B spermatogonia possess considerably more chromatin within the inner nuclear envelope than intermediate or type A spermatogonia (see Fig. 3.8). Type B spermatogonia represent the cells that differentiate and enter into the process of meiosis, where they are called primary spermatocytes [100]. They are the differential precursors to preleptotene spermatocytes. This last mitotic division helps maintain a pool of stem cells so that the process can continue indefinitely.

Spermatogonia do not separate completely after meiosis but remain joined by intercellular bridges, which persist throughout all stages of spermatogenesis and are thought to

facilitate biochemical interactions, allowing synchrony of germ cell maturation [102].

Spermatocytogenesis

The purpose of spermatogenesis is to produce genetic material necessary for the replication of the species through mitosis and meiosis. Spermatocytogenesis takes place in the basal compartment. Primary spermatocytes enter the first meiotic division to form secondary spermatocytes. The prophase of the first meiotic division is very long; thus, the primary spermatocyte has the longest life span. Secondary spermatocytes undergo the second meiotic division to produce spermatids. Secondary spermatocytes are short-lived (1.1–1.7 days).

Mitosis

Mitosis involves proliferation and maintenance of spermatogonia. It is a precise, well-orchestrated sequence of events involving duplication of the genetic material (chromosomes), breakdown of the nuclear envelope, and equal

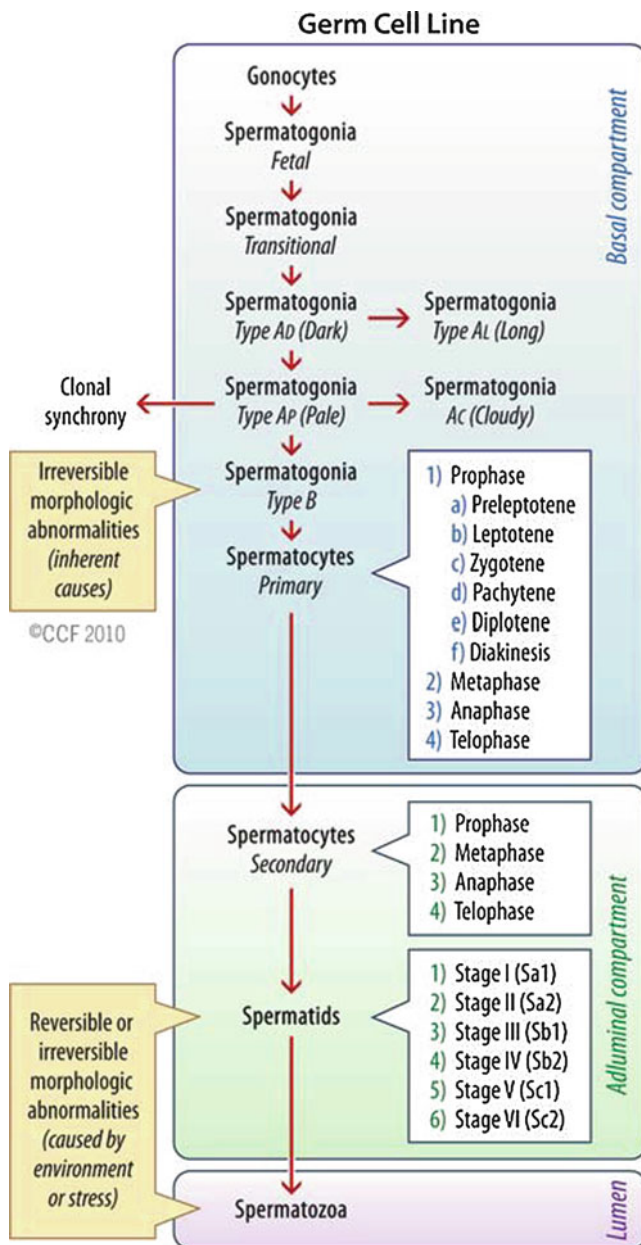


Fig. 3.8 Schematic representation of the development of a diploid undifferentiated germ cell into a fully functional haploid spermatozoon along the basal to the adluminal compartment and final release into the lumen. Different steps in the development of primary, secondary, and spermatid stages are also shown and the irreversible and reversible morphological abnormalities that may occur during various stages of spermatogenesis (All Rights Reserved Sperm Chromatin, ed. Zini A and Agarwal A, Biological and Clinical applications in Male Infertility and Assisted Reproduction, Springer Science + Business Media 2011)

division of the chromosomes and cytoplasm into two daughter cells [103]. DNA is also spatially organized into loop domains on which specific regulatory proteins interact during cellular replication [103–105]. The mitotic phase involves spermatogonia (types A and B) and primary spermatocytes (spermato-

cytes I). Developing germ cells interconnected by intracellular bridges produce the primary spermatocyte through a series of mitotic divisions. Once the baseline number of spermatogonia is established after puberty, the mitotic component will proceed in order to continue to provide precursor cells and to start the process of differentiation and maturation.

Meiosis

Meiosis is a complex process with specific regulatory mechanisms of its own [106]. The process commences when type B spermatogonia lose their contact with the basement membrane to form preleptotene primary spermatocytes. Thus, each primary spermatocyte can theoretically yield four spermatids, although fewer actually result, because some germ cells are lost due to the complexity of meiosis. The primary spermatocytes are the largest germ cells of the germinal epithelium. Meiosis is characterized by prophase, metaphase, anaphase, and telophase. In this, two successive cell divisions yield four haploid spermatids from one diploid primary spermatocyte. As a consequence, the daughter cells contain only half of the chromosome content of the parent cell. After the first meiotic division (reduction division), each daughter cell contains one partner of the homologous chromosome pair, and they are called *secondary spermatocytes*. These cells rapidly enter the second meiotic division (equational division), in which the chromatids then separate at the centromere to yield haploid early round spermatids. Meiosis assures genetic diversity and involves primary and secondary spermatocytes, which give rise to spermatids.

The meiotic phase involves primary spermatocytes until spermatids are formed; during this process, chromosome pairing, crossover, and genetic exchange are accomplished until a new genome is determined. In turn, a post-meiotic phase involving spermatids all the way up to spermatozoa develops, ending in the formation of the specialized cell.

Spermiogenesis

Spermiogenesis is a process during which the morphologic changes occur during the differentiation of the spermatid into the spermatozoon. It begins once the process of meiosis is completed. Six different stages have been described in the process of spermatid maturation in humans, as S_{a-1} and S_{a-2} , S_{b-1} and S_{b-2} , and S_{c-1} and S_{c-2} (see Fig. 3.9). Each of these stages can be identified by the morphologic characteristics. During the S_{a-1} stage, both the Golgi complex and mitochondria are well developed and differentiated, the acrosomal vesicle appears, the chromatid body develops in one pole of the cell opposite from the acrosomal vesicle, and the proximal centriole and the axial filament appear. During S_{b-1} and

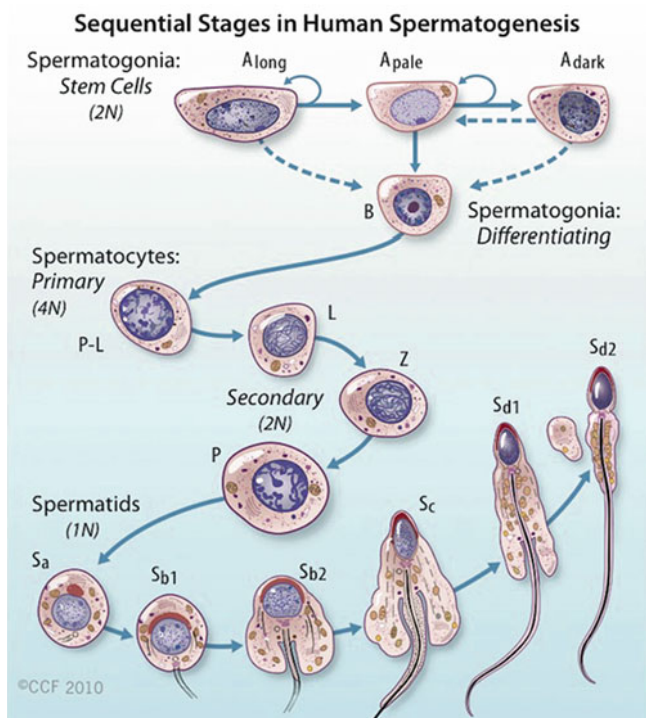


Fig. 3.9 Differentiation of a human diploid germ cell into a fully functional spermatozoon (All Rights Reserved Sperm Chromatin, ed. Zini A and Agarwal A, Biological and Clinical applications in Male Infertility and Assisted Reproduction, Springer Science + Business Media 2011)

S_{b-2} stages, acrosome formation is completed, the intermediate piece is formed, and the tail develops. This process is completed during the S_c stages.

Nuclear Development

During spermatogenesis, a number of changes occur to the nucleus and its contents. The nucleus of the spermatozoa elongates and flattens from step 1 to step 8 of spermiogenesis [107], giving the head its characteristic oval shape. The necessity of this compaction has been debated, but it is widely believed to facilitate the penetration of the oocyte and to help optimize the swimming capability of the spermatozoa [108].

The compaction of the nucleus includes modification of the chromatin material. During the last post-meiotic phase of spermiogenesis, the histone molecules, around which the DNA is organized, are converted into transitional proteins, which are then converted to protamines [109]. Protamines contain large amounts of cysteine, which aid in the formation of protamine disulfide bonds as the sperm matures in the epididymis [110–112]. Within 2–4 h of fertilization, the protamines in the chromatin of the spermatozoa are replaced by histones from the oocyte.

Spermiation

The process whereby a mature spermatid frees itself from the Sertoli cell and enters the lumen of the tubule as a spermatozoon is known as *spermiation*. The spermatids originating from the same spermatogonia remain connected by bridges to facilitate the transport of cytoplasmic products. Spermiation involves the active participation of the Sertoli cell. This may also involve actual cell movement as the spermatids advance towards the lumen of the seminiferous tubules [112]. The mature spermatids close their intracellular bridges, disconnect their contact to the germinal epithelium, and become free cells called *spermatozoa*. Portions of the cytoplasm of the Sertoli cell known as *cytoplasmic droplet* may remain as part of the spermatozoon during the process of spermiation. This is a morphological feature present on the immature sperm in semen [113].

The Cycle or Wave of Seminiferous Epithelium

A cycle of spermatogenesis involves the division of primitive spermatogonial stem cells into subsequent germ cell types through the process of meiosis. Type A spermatogonial divisions occur at a shorter time interval than the entire process of spermatogenesis. Therefore, at any given time, several cycles of spermatogenesis coexist within the germinal epithelium. In humans, spermatocyte maturation takes 25.3 days, spermiogenesis 21.6 days, and the total estimated time for spermatogenesis is 74 days. Spermatogenesis is not random throughout the seminiferous epithelium. Germ cells are localized in spatial units referred to as *stages* and represent consistent associations of germ cell steps [114, 115]. In rodent spermatogenesis, one stage can be found in a cross-section of seminiferous tubule.

Each stage is recognized by development of the acrosome, meiotic divisions and shape of the nucleus, and release of the sperm into lumen of the seminiferous tubule. The cycle of spermatogenesis can be identified for each species, but the duration of the cycle varies for each species [100]. The stages of spermatogenesis are sequentially arranged along the length of the tubule. This arrangement of the stages of spermatogenesis is such that it results in a “wave of spermatogenesis” along the tubule. This wave is in space but the cycle is in time [115]. Along the length of the seminiferous tubule there are only certain cross-sections where spermatozoa are released. In the rat, all stages are involved in spermatogenesis, but spermatozoa are released only in stage VIII.

The stages of spermatogenesis are organized spatially as well as in time [115]. Thus, a position in the tubule that is occupied by cells comprising stage I will become stage II, followed by stage III, until the cycle repeats. In humans, the

duration of the cycle is 16 days, and the progression from spermatogonia to spermatozoa takes 70 days, or four and a half cycles of the seminiferous cycle. During spermatogenesis, cytoplasmic bridges link cohorts of germ cells that are at the same point in development, and these cells pass through the process together. Groups of such cells at different stages can be observed histologically on cross-section, and many germ cell cohorts are seen only in association with certain other germ cells. This has led to the description of six stages of the seminiferous tubule epithelium in men. To add another level of complexity, the steps of the spermatogenic cycle within the space of seminiferous tubules demonstrate a specific spatial organization, termed *spermatogenic waves*. In humans, this wave appears to describe a spiral cellular arrangement as one progresses down the tubule. This spatial arrangement probably exists to ensure that sperm production is a continuous rather than a pulsatile process.

Efficiency of Spermatogenesis

Spermatogenic efficiency varies between different species but appears to be relatively constant in man. The time for the differentiation of a spermatogonium into a mature spermatid is estimated to be 70 ± 4 days [116]. In comparison to animals, the spermatogenic efficiency in man is poor. The daily rate of spermatozoa production is calculated at 3–4 million per gram of testicular tissue [117]. A higher number of spermatozoa should be expected in the ejaculate than the 20 million/mL described by the World Health Organization manual in 1999 [118] and the 15 million/mL in 2010 [119]. A majority of the cells developed (>75 %) are lost as a result of apoptosis or degeneration; of the remaining, more than half are abnormal. Therefore, only about 12 % of the spermatogenic potential is available for reproduction [120]. An age-related reduction in daily sperm production in men, which is associated with a loss of Sertoli cells, is also seen. This may result from an increase in germ cell degeneration during prophase of meiosis or from loss of primary spermatocytes. There is also a reduction in the number of Leydig cells, non-Leydig interstitial cells, myoid cells, and Sertoli cells.

Structure of the Spermatozoa

Spermatozoa are highly specialized and condensed cells that neither grow nor divide. A spermatozoon consists of the head, which contains the paternal material (DNA) and the acrosome, the neck, and the tail, which provides motility. The spermatozoon is endowed with a large nucleus but lacks the large cytoplasm characteristic of most body cells. Men are unique in the morphologic heterogeneity of the ejaculate.

Head

The head of the spermatozoa is ovular, measuring about 4.0–5.5 μm in length and 2.5–3.5 μm in width. The normal length-to-width ratio is about 1.50–1.70 [118]. Under bright field illumination, the most commonly observed aberrations include head shape/size defects, including large, small, tapering, piriform, amorphous, vacuolated (>20 % of the head surface occupied by unstained vacuolar areas), and double heads, or any combination [118].

The head also contains the acrosome, which is a cap-like structure represented by the Golgi complex and covers about two thirds of the anterior head area [118]. The apical thickening seen in many other species is missing; however, the acrosome shows a uniform thickness/thinning toward the equatorial segment and covers about 40–70 % of the sperm head. When observed under the scanning electron microscope, a furrow that completely encircles the head (i.e., acrosomal and post-acrosomal regions) divides the sperm head unequally. An equatorial segment that is followed by the post-acrosomal region is not very clearly visible on scanning electron microscope. The maximal thickness and width of the spermatozoon is seen in the post-acrosomal region.

During fertilization of the egg, the fusion of the outer acrosomal membrane with the plasma membrane at multiple sites releases the acrosomal enzymes at the time of the acrosome reaction. The anterior half of the head is devoid of the plasma and outer acrosomal membrane and is covered only by the inner acrosomal membrane. The posterior region of the sperm head is covered by a single membrane called the *postnuclear cap*. The overlap of the acrosome and the post-nuclear cap results in an equatorial segment which does not participate in the acrosome reaction.

Neck

This forms a junction between head and tail. It is fragile and the presence of decapitated spermatozoa is a common abnormality.

Tail

The sperm tail is 40–50 μm long and arises at the spermatid stage. The tail contains the motility apparatus of the spermatozoa and propels by waves generated in the neck region and pass along the tail like a whiplash. It is formed during spermiogenesis due to the differentiation of the centriole into three parts, which can be clearly observed under scanning electron microscopy: the midpiece, the main or principle piece, and the endpiece. The mitochondria are organized helically around the midpiece. The mitochondrial sheath of the midpiece is relatively short but slightly longer than the

combined length of the head and neck. An axial core comprising of two central fibrils is surrounded by a concentric ring of nine double fibrils, which continue to the end of the tail. The additional outer ring comprises nine coarse fibrils. The principle piece, the longest part of the tail, provides most of the propellant machinery. The coarse nine fibrils of the outer ring diminish in thickness and finally disappear, leaving only the inner fibrils in the axial core for most of the length of the principle piece. The fibrils of the principle piece are surrounded by a fibrous tail sheath, which consist of branching and anastomosing semicircular strands or ribs held together by their attachment to two bands that run lengthwise along opposite sides of the tail. The tail terminates in the endpiece with a length of 4–10 μm and a diameter of less than 1 μm . The small diameter is due to the absence of the outer fibrous sheath and a distal fading of the microtubules.

The tail can be clearly observed under bright field illumination. Common tail abnormalities include tail absence, bent tails, distended or irregular/bent midpiece, abnormally thin midpiece (no mitochondrial sheath), and short, multiple, hairpin, and broken tails, tails of irregular width, coiling tails with terminal droplets, or any of these combinations [118].

Regulation of Spermatogenesis

The spermatogenic process is maintained by different intrinsic and extrinsic influences.

Intrinsic Regulation

Leydig cells secrete hormone (testosterone), neurotransmitters (neuroendocrine substances) and growth factors to neighboring Leydig cells, blood vessels, lamina propria of the seminiferous tubules, and Sertoli cells [97, 120, 121]. They help maintain the nutrition of the Sertoli cells and the cells of the peritubular tissue and influence the contractility of myofibroblasts, thereby regulating the peristaltic movements of seminiferous tubules and transportation of the spermatozoa. Leydig cells also help in the regulation of blood flow in the intertubular microvasculature [95]. In addition, different growth factors are delivered from Sertoli cells and various germ cells participating in a complicated regulation of cell functions and developmental processes of germ cells. Altogether these factors represent an independent intratesticular regulation of spermatogenesis.

Extrinsic Influences

The local regulation of spermatogenesis is controlled by the hypothalamus and hypophysis. Pulsatile secretion of

gonadotropin-releasing hormone of the hypothalamus initiates the release of LH from the hypophysis; in response, the Leydig cells produce testosterone. Testosterone not only influences spermatogenesis, but is also distributed throughout the body. It thus provides feedback to the hypophysis that regulates the secretory activity of Leydig cells. Stimulation of Sertoli cells by FSH is necessary for maturation of the germ cells. Complete qualitative spermatogenesis requires both FSH and LH. The interaction between the endocrine and paracrine mechanisms determines the functions within the testis [122]. Inhibin secreted by Sertoli cells functions in the feedback mechanism directed to the hypophysis. These extratesticular influences are necessary for the regulation of intratesticular functions. Thus, both growth and differentiation of testicular germ cells involves a series of complex interactions both between somatic and germinal elements [123, 124].

The Epididymis

The epididymis lies along the dorsolateral border of each testis. It is made up of the efferent ductules, which emanate from the rete testis, and the epididymal ducts. The epididymis opens into the vas deferens, which then passes through the inguinal canal into the peritoneal cavity and opens into the urethra adjacent to the prostate. It is divided into three functionally distinct regions: head, body, and tail or caput epididymis, corpus epididymis, and cauda epididymis. Their functions can be described simplistically and increasing the concentration, maturation, and storage of the spermatozoa.

Much of the testicular fluid that transports spermatozoa from the seminiferous tubules is resorbed in the caput, increasing the concentration of the spermatozoa by 10- to 100-fold. The epididymal epithelium secretes the epididymal plasma in which the spermatozoa are suspended. As the newly developed spermatozoa pass through these regions of the epididymis, many changes occur, including alterations in net surface charge, membrane protein composition, immunoreactivity, phospholipid and fatty acid content, and adenylate cyclase activity. Many of these changes are thought to improve the structural integrity of the sperm membrane and also increase the fertilization ability of the spermatozoa. The capacities for protein secretion and storage within the epididymis are known to be extremely sensitive to temperature and reproductive hormone levels, including estrogens.

As many as half of the spermatozoa released from the testis die and disintegrate within the epididymis and are resorbed by the epididymal epithelium. The remaining mature spermatozoa are stored in the cauda epididymis, which contain about 70 % of all spermatozoa present in the male tract. The capacity for sperm storage decreases distally; spermatozoa in the vas deferens may only be motile for a few days. The environment of the cauda epididymis is adapted for storage in

laboratory animals. In humans, it is not a perfect storage organ, and the spermatozoa do not remain in a viable state indefinitely. After prolonged sexual activity, caudal spermatozoa first lose their fertilizing ability, followed by their motility and then their vitality; they finally disintegrate. Unless these older, senescent spermatozoa are eliminated from the male tract at regular intervals, their relative contribution to the next ejaculate(s) increases, thus reducing semen quality, even though such ejaculates do have a high sperm concentration. The transit time of sperm through the fine tubules of the epididymis is thought to be 10–15 days in humans.

As spermatozoa traverse the epididymis, they are exposed to a continuously changing milieu of the luminal fluid derived from the rete testis and modified by the secretory and absorptive activity of the epididymal epithelium. In nonhuman mammals, there is compelling evidence that the epididymal epithelium does provide essential factors for sperm maturation [125, 126]. In humans, most of the information is obtained from treatment of pathologic cases rather than from normal fertile men. Both epididymal maturation and capacitation are necessary before fertilization. The epididymis is limited to a storage role because spermatozoa that have never passed through the epididymis and that have been obtained from the efferent ductules in men with congenital absence of vas deferens can fertilize the human oocyte *in vitro* and result in pregnancy with live birth (as well as with intracytoplasmic sperm injection with sperm obtained after testicular biopsy). A direct involvement of the epididymis comes from the results of epididymovasostomy bypass operations for epididymal obstruction. However, these results are not consistent, whereas significantly reduced fertility was reported in anastomosis of vas deferens to the proximal 10 mm of the duct compared to anastomosis of the more distal region of the tract [127]; others have reported pregnancies in anastomosis of the vas deferens to the efferent ductules [128] or from spermatozoa retrieved from the efferent ductules and proximal caput regions of men with congenital absence of vas deferens and used for successful *in vitro* fertilization [129]. More direct evidence of epididymal involvement comes from experiments in which immature epididymal sperm recovered from fertile men were incubated in the presence of human epithelial cell cultures [130]. These spermatozoa showed improved motility and a significant increase in their capacity to attach to human zona *in vitro*.

Sperm Entry into Cervical Mucus

At the moment of ejaculation, spermatozoa from the cauda epididymis are mixed with secretions of the various accessory glands in a specific sequence and deposited around the external cervical os and in the posterior fornix of the vagina. Spermatozoa in the first fraction of the ejaculate have

significantly better motility and survival than the later fractions. The majority of the spermatozoa penetrate cervical mucus within 15–20 min of ejaculation [130]. The ability to migrate across the semen–mucus interphase is highly dependent on the specific movement pattern of the spermatozoa [131]. At the time of sperm penetration into the cervical mucus, further selection of the spermatozoa occurs based on the differential motility of the normal vs. abnormal spermatozoa. This is further modified once the “vanguard” spermatozoon is within the cervical mucus [132]. The receptivity of the cervical mucus to the penetration by the spermatozoa is cyclic, increasing over a period of about 4 days before ovulation and decreasing rapidly after ovulation. Maximum receptivity is seen the day before and on the day of the LH peak [133]. Spermatozoa enter the uterine cavity from the internal cervical os by virtue of their own motility. From here the spermatozoa traverse to the site of fertilization in the ampulla of the fallopian tube or the oviduct.

Capacitation and Acrosome Reaction

Animal studies in rats and rabbits indicate that spermatozoa that are stored in the female tract are unable to penetrate the ova. They have to spend time in the female tract before they acquire this ability. Capacitation is a series of cellular or physiological changes that spermatozoa must undergo in order to fertilize [134]. It represents a change in the molecular organization of the intact sperm plasma membrane that is characterized by the ability to undergo the acrosome reaction, to bind to the zona pellucida, and to acquire hypermotility.

Capacitation involves the removal of seminal plasma factors that coat the surface of the sperm; modification of the surface charge; modification of the sperm membrane and of the sterols, lipids, and glycoproteins and the outer acrosomal membrane lying immediately under it. It also involves an increase in intracellular-free calcium [135]. Changes in sperm metabolism, increase in 3',5'-cyclic monophosphate, and activation of acrosomal enzymes are believed to be components of capacitation. Sperm capacitation may be initiated *in vivo* during migration through cervical mucus [136]. Capacitation may be an evolutionary consequence of the development of a storage system for inactive sperm in the caudal epididymis.

Capacitation can also be achieved by culture medium supplemented with appropriate substrates for energy and in the presence of protein or biological fluid such as serum or follicular fluid. Usually it takes about 2 h for sperm to undergo capacitation *in vitro*. Further modifications occur when capacitated sperm reach the vicinity of the oocyte.

The acrosome reaction confers the ability to penetrate the zona pellucida and also confers the fusogenic state in the plasmalemma overlying the nonreactive equatorial segment,

which is needed for interaction with the oolemma. There are distinct fusion points between the outer acrosomal membrane and the plasma membrane. The fusion begins posteriorly around the anterior border of the equatorial segment, which is always excluded from the reaction. The changes, termed *acrosome reaction*, prepare the sperm to fuse with the egg membrane. The removal of cholesterol from the surface membrane prepares the sperm membrane for the acrosome reaction [137]. In addition, D-mannose-binding lectins are also involved in the binding of human sperm to the zona pellucida [138].

Thus, these series of changes are necessary to transform the stem cells into fully mature, functional spermatozoa equipped to fertilize the egg.

References

1. Yao MWM, Batchu K. Oogenesis. In: Falcone T, Hurd WW, editors. *Clinical reproductive medicine and surgery*. 1st ed. Philadelphia: Mosby/Elsevier; 2007. p. 51–67.
2. Coutsoukis P. The ovaries 2007 [cited 31 Aug 2012]. http://www.theodora.com/anatomy/the_ovaries.html.
3. Body GS, Aot H. The ovaries [Online Webpage]. 2009 [cited 31 Aug 2012]. <http://education.yahoo.com/reference/gray/subjects/subject/266>.
4. Heffner LJ, Schust DJ. *The reproductive system at a glance*. 3rd ed. New York: Wiley; 2010.
5. Histology test atlas book. Chapter 18: The female reproductive system. http://www.visualhistology.com/products/atlas/VHA_Chpt18_The_Female_Reproductive_System.html.
6. Fujimoto T, Miyayama Y, Fuyuta M. The origin, migration and fine morphology of human primordial germ cells. *Anat Rec*. 1977;188(3):315–30.
7. Gondos B, Bhiraleus P, Hobel CJ. Ultrastructural observations on germ cells in human fetal ovaries. *Am J Obstet Gynecol*. 1971;110(5):644–52.
8. Gondos B, Westergaard L, Byskov AG. Initiation of oogenesis in the human fetal ovary: ultrastructural and squash preparation study. *Am J Obstet Gynecol*. 1986;155(1):189–95.
9. Witschi E. Migration of the germ cells of human embryo from the yolk sac to the primitive gonadal folds. *Contrib Embryol Carnegie Inst*. 1948;32:69–80.
10. Byskov AG. Differentiation of mammalian embryonic gonad. *Physiol Rev*. 1986;66(1):71–117.
11. Goto T, Adjaye J, Rodeck CH, Monk M. Identification of genes expressed in human primordial germ cells at the time of entry of the female germ line into meiosis. *Mol Hum Reprod*. 1999;5(9):851–60.
12. Baker TG. A quantitative and cytological study of germ cells in human ovaries. *Proc R Soc Lond B Biol Sci*. 1963;158:417–33.
13. Gougeon A. Regulation of ovarian follicular development in primates: facts and hypotheses. *Endocr Rev*. 1996;17(2):121–55.
14. Hardy K, Wright CS, Franks S, Winston RM. In vitro maturation of oocytes. *Br Med Bull*. 2000;56(3):588–602.
15. Hansen KR, Knowlton NS, Thyer AC, Charleston JS, Soules MR, Klein NA. A new model of reproductive aging: the decline in ovarian non-growing follicle number from birth to menopause. *Hum Reprod*. 2008;23(3):699–708.
16. Paulson RJ. *Oocytes from development to fertilization*. 3rd ed. Boston, MA: Blackwell; 1991.
17. University of Alabama at Birmingham. Oogenesis 2012 [cited 21 Aug 2012]. <http://main.uab.edu/show.asp?durki=19786>.
18. Gilbert S. *Developmental biology*. 6th ed. Sunderland, MA: Sinauer; 2000.
19. Canipari R. Oocyte–granulosa cell interactions. *Hum Reprod Update*. 2000;6(3):279–89.
20. Durinzi K, Saniga E, Lanzendorf S. The relationship between size and maturation in vitro in the unstimulated human oocyte. *Fertil Steril*. 1995;63:404–6.
21. Eppig J, Wigglesworth K, Pendola F. The mammalian oocyte orchestrates the rate of ovarian follicular development. *Proc Natl Acad Sci USA*. 2002;99:2890.
22. Matzuk M, Burns K, Viveiros M, Eppig JJ. Intercellular communication in the mammalian ovary: oocytes carry the conversation. *Science*. 2002;296:2178–80.
23. McGee EA, Hsueh AJ. Initial and cyclic recruitment of ovarian follicles. *Endocr Rev*. 2000;21(2):200–14.
24. Zeleznik AJ. The physiology of follicle selection. *Reprod Biol Endocrinol*. 2004;2:31.
25. Gougeon A. Dynamics of follicular growth in the human: a model from preliminary results. *Hum Reprod*. 1986;1(2):81–7.
26. Vegetti W. FSH and folliculogenesis: from physiology to ovarian stimulation. *Reprod Biomed Online*. 2006;12(6):684–94.
27. Van Fauser BC, Heusden AM. Manipulation of human ovarian function: physiological concepts and clinical consequences. *Endocr Rev*. 1997;18(1):71–106.
28. Sullivan MW, Stewart-Akers A, Krasnow JS, Berga SL, Zeleznik AJ. Ovarian responses in women to recombinant follicle-stimulating hormone and luteinizing hormone (LH): a role for LH in the final stages of follicular maturation. *J Clin Endocrinol Metab*. 1999;84(1):228–32.
29. Zeleznik AJ, Hillier SG. The role of gonadotropins in the selection of the preovulatory follicle. *Clin Obstet Gynecol*. 1984;27(4):927–40.
30. Zelinski-Wooten MB, Hess DL, Wolf DP, Stouffer RL. Steroid reduction during ovarian stimulation impairs oocyte fertilization, but not folliculogenesis, in rhesus monkeys. *Fertil Steril*. 1994;61(6):1147–55.
31. Griffin J, Emery BR, Huang I, Peterson CM, Carrell DT. Comparative analysis of follicle morphology and oocyte diameter in four mammalian species (mouse, hamster, pig, and human). *J Exp Clin Assist Reprod*. 2006;3:2.
32. Sugiura K, Pendola FL, Eppig JJ. Oocyte control of metabolic cooperativity between oocytes and companion granulosa cells: energy metabolism. *Dev Biol*. 2005;279(1):20–30.
33. Kidder GM, Vanderhyden BC. Bidirectional communication between oocytes and follicle cells: ensuring oocyte developmental competence. *Can J Physiol Pharmacol*. 2010;88(4):399–413.
34. Buccione R, Schroeder AC, Eppig JJ. Interactions between somatic cells and germ cells throughout mammalian oogenesis. *Biol Reprod*. 1990;43(4):543–7.
35. Buccione R, Vanderhyden BC, Caron PJ, Eppig JJ. FSH-induced expansion of the mouse cumulus oophorus in vitro is dependent upon a specific factor(s) secreted by the oocyte. *Dev Biol*. 1990;138(1):16–25.
36. Eppig JJ, Schroeder AC. Capacity of mouse oocytes from preantral follicles to undergo embryogenesis and development to live young after growth, maturation, and fertilization in vitro. *Biol Reprod*. 1989;41(2):268–76.
37. Kidder GM, Mhawi AA. Gap junctions and ovarian folliculogenesis. *Reproduction*. 2002;123(5):613–20.
38. Murray A, Spears N. Follicular development in vitro. *Semin Reprod Med*. 2000;18(2):109–22.
39. Eppig JJ. Intercellular communication between mammalian oocytes and companion somatic cells. *Bioessays*. 1991;13(11):569–74.
40. Downs SM, Hunzicker-Dunn M. Differential regulation of oocyte maturation and cumulus expansion in the mouse oocyte-cumulus

- cell complex by site-selective analogs of cyclic adenosine monophosphate. *Dev Biol.* 1995;172(1):72–85.
41. Fagbohun CF, Downs SM. Metabolic coupling and ligand-stimulated meiotic maturation in the mouse oocyte-cumulus cell complex. *Biol Reprod.* 1991;45(6):851–9.
 42. Granot I, Dekel N. Phosphorylation and expression of connexin-43 ovarian gap junction protein are regulated by luteinizing hormone. *J Biol Chem.* 1994;269(48):30502–9.
 43. Eppig J. Mouse oocytes control metabolic co-operativity between oocytes and cumulus cells. *Reprod Fertil Dev.* 2005;17(1–2):1–2.
 44. Zheng P, Dean J. Oocyte-specific genes affect folliculogenesis, fertilization, and early development. *Semin Reprod Med.* 2007;25(4):243–51.
 45. Gilchrist RB, Lane M, Thompson JG. Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. *Hum Reprod Update.* 2008;14(2):159–77.
 46. Coskun S, Uzumcu M, Lin YC, Friedman CI, Alak BM. Regulation of cumulus cell steroidogenesis by the porcine oocyte and preliminary characterization of oocyte-produced factor(s). *Biol Reprod.* 1995;53(3):670–5.
 47. Eppig JJ, Pendola FL, Wigglesworth K, Pendola JK. Mouse oocytes regulate metabolic cooperativity between granulosa cells and oocytes: amino acid transport. *Biol Reprod.* 2005;73(2):351–7.
 48. Carabatsos MJ, Sellitto C, Goodenough DA, Albertini DF. Oocyte-granulosa cell heterologous gap junctions are required for the coordination of nuclear and cytoplasmic meiotic competence. *Dev Biol.* 2000;226(2):167–79.
 49. Simon AM, Goodenough DA, Li E, Paul DL. Female infertility in mice lacking connexin 37. *Nature.* 1997;385(6616):525–9.
 50. Juneja SC, Barr KJ, Enders GC, Kidder GM. Defects in the germ line and gonads of mice lacking connexin43. *Biol Reprod.* 1999;60(5):1263–70.
 51. Lampe PD, Lau AF. Regulation of gap junctions by phosphorylation of connexins. *Arch Biochem Biophys.* 2000;384(2):205–15.
 52. Lampe PD, Lau AF. The effects of connexin phosphorylation on gap junctional communication. *Int J Biochem Cell Biol.* 2004;36(7):1171–86.
 53. Rankin T, Soyal S, Dean J. The mouse zona pellucida: folliculogenesis, fertility and pre-implantation development. *Mol Cell Endocrinol.* 2000;163(1–2):21–5.
 54. Soyal SM, Amlah A, Dean J. Figalpha, a germ cell-specific transcription factor required for ovarian follicle formation. *Development.* 2000;127(21):4645–54.
 55. Heikinheimo O, Gibbons WE. The molecular mechanisms of oocyte maturation and early embryonic development are unveiling new insights into reproductive medicine. *Mol Hum Reprod.* 1998;4(8):745–56.
 56. Jammongjit M, Hammes SR. Oocyte maturation: the coming of age of a germ cell. *Semin Reprod Med.* 2005;23(3):234–41.
 57. Mehlmann LM. Stops and starts in mammalian oocytes: recent advances in understanding the regulation of meiotic arrest and oocyte maturation. *Reproduction.* 2005;130(6):791–9.
 58. Rajesh C, Pittman DL. Cell cycle regulation in mammalian germ cells. *Results Probl Cell Differ.* 2006;42:343–67.
 59. Sun QY, Miao YL, Schatten H. Towards a new understanding on the regulation of mammalian oocyte meiosis resumption. *Cell Cycle.* 2009;8(17):2741–7.
 60. Tripathi A, Kumar KV, Chaube SK. Meiotic cell cycle arrest in mammalian oocytes. *J Cell Physiol.* 2010;223(3):592–600.
 61. Zhang M, Xia G. Hormonal control of mammalian oocyte meiosis at diplotene stage. *Cell Mol Life Sci.* 2012;69(8):1279–88.
 62. Gautier J, Minshull J, Lohka M, Glotzer M, Hunt T, Maller JL. Cyclin is a component of maturation-promoting factor from *Xenopus*. *Cell.* 1990;60(3):487–94.
 63. Jones KT. Turning it on and off: M-phase promoting factor during meiotic maturation and fertilization. *Mol Hum Reprod.* 2004;10(1):1–5.
 64. Bowen R. Gonadotropins: luteinizing and follicle stimulating hormones. Colorado State University; 2006 [updated 30 April 2006; cited 29 Aug 2012]. <http://www.vivo.colostate.edu/hbooks/pathophys/endocrine/hypopit/lhfs.html>.
 65. Simoni M, Gromoll J, Nieschlag E. The follicle-stimulating hormone receptor: biochemistry, molecular biology, physiology and pathophysiology. *Endocr Rev.* 1997;18(6):739.
 66. Williams CJ, Erickson GF. Morphology and physiology of the ovary. *Endotext.org*; 2012. <http://www.endotext.org/female/female1/femaleframe1.htm>.
 67. Durlinger AL, Visser JA, Themmen AP. Regulation of ovarian function: the role of anti-mullerian hormone. *Reproduction.* 2002;124:601–9.
 68. Visser JA, de Jong FH, Laven JS, Themmen AP. Anti-Mullerian hormone: a new marker for ovarian function. *Reproduction.* 2006;131(1):1–9.
 69. Weenen C, Laven JS, Von Bergh AR, Cranfield M, Groome NP, Visser JA. Anti-Mullerian hormone expression pattern in the human ovary: potential implications for initial and cyclic follicle recruitment. *Mol Hum Reprod.* 2003;10(2):77–83.
 70. Shimasaki S, Moore RK, Otsuka F, Erickson GF. The bone morphogenetic protein system in mammalian reproduction. *Endocr Rev.* 2004;25(1):72–101.
 71. Shimasaki S, Zachow RJ, Li D, Kim H, Iemura S, Ueno N, et al. A functional bone morphogenetic protein system in the ovary. *Proc Natl Acad Sci USA.* 1999;96:7282–7.
 72. Lee WS, Otsuka F, Moore RK, Shimasaki S. The effect of bone morphogenetic protein-7 on folliculogenesis and ovulation in the rat. *Biol Reprod.* 2001;65:994–9.
 73. Dong J, Albertini DF, Nishimori K, Kumar TR, Lu N, Matzuk MM. Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature.* 1996;383(6600):531–5.
 74. Galloway SM, McNatty KP, Cambridge LM, Laitinen MP, Juengel JL, Jokiranta TS, et al. Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner. *Nat Genet.* 2000;25(3):279–83.
 75. Hreinnsson JG, Scott JE, Rasmussen C, Swahn ML, Hsueh AJ, Hovatta O. Growth differentiation factor-9 promotes the growth, development, and survival of human ovarian follicles in organ culture. *J Clin Endocrinol Metab.* 2002;1:316.
 76. Systems RD. BMPs influence FSH synthesis [Online Website]. Technical Information > Literature > Cytokine Bulletin [cited 21 Aug 2012]. http://www.rndsystems.com/cb_detail_objectname_FA01_BMPs.aspx.
 77. Young JM, McNeilly AS. Theca: the forgotten cell of the ovarian follicle. *Reproduction.* 2010;140:489–504.
 78. Carlsson IB, Laitinen MP, Scott JE, Louhio H, Velentzis L, Tuuri T, et al. Kit ligand and c-Kit are expressed during early human ovarian follicular development and their interaction is required for the survival of follicles in long-term culture. *Reproduction.* 2006;131(4):641–9.
 79. Hutt KJ, McLaughlin EA, Holland MK. Kit ligand and c-Kit have diverse roles during mammalian oogenesis and folliculogenesis. *Mol Hum Reprod.* 2006;12(2):61–9.
 80. Zuckerman S. The number of oocytes in the mature ovary. *Recent Prog Horm Res.* 1951;95(6):63–108.
 81. Tilly JL, Niikura Y, Rueda BR. The current status of evidence for and against postnatal oogenesis in mammals: a case of ovarian optimism versus pessimism? *Biol Reprod.* 2009;80(1):2–12.
 82. Virant-Klun I, Stimpfel M, Skutella T. Stem cells in adult human ovaries: from female fertility to ovarian cancer. *Curr Pharm Des.* 2012;18(3):283–92.
 83. Johnson J, Canning J, Kaneko T, Pru JK, Tilly JL. Germline stem cells and follicular renewal in the postnatal mammalian ovary. *Nature.* 2004;428(6979):145–50.
 84. White YA, Woods DC, Takai Y, Ishihara O, Seki H, Tilly JL. Oocyte formation by mitotically active germ cells purified from ovaries of reproductive-age women. *Nat Med.* 2012;18(3):413–21.

85. Virant-Klun I, Stimpfel M, Skutella T. Ovarian pluripotent/multipotent stem cells and in vitro oogenesis in mammals. *Histol Histopathol.* 2011;26(8):1071–82.
86. Tilly JL, Telfer EE. Purification of germline stem cells from adult mammalian ovaries: a step closer towards control of the female biological clock? *Mol Hum Reprod.* 2009;15(7):393–8.
87. Smits J, Dolmans MM, Donnez J, Fortune JE, Hovatta O, Jewgenow K, et al. Current achievements and future research directions in ovarian tissue culture, in vitro follicle development and transplantation: implications for fertility preservation. *Hum Reprod Update.* 2010;16(4):395–414.
88. Desai N, Alex A, AbdelHafez F, Calabro A, Goldfarb J, Fleischman A, et al. Three-dimensional in vitro follicle growth: overview of culture models, biomaterials, design parameters and future directions. *Reprod Biol Endocrinol.* 2010;8:119.
89. Hornick JE, Duncan FE, Shea LD, Woodruff TK. Isolated primate primordial follicles require a rigid physical environment to survive and grow in vitro. *Hum Reprod.* 2012;27(6):1801–10.
90. Kreeger PK, Deck JW, Woodruff TK, Shea LD. The in vitro regulation of ovarian follicle development using alginate-extracellular matrix gels. *Biomaterials.* 2006;27(5):714–23.
91. Shikanov A, Smith RM, Xu M, Woodruff TK, Shea LD. Hydrogel network design using multifunctional macromers to coordinate tissue maturation in ovarian follicle culture. *Biomaterials.* 2011;32(10):2524–31.
92. West ER, Xu M, Woodruff TK, Shea LD. Physical properties of alginate hydrogels and their effects on in vitro follicle development. *Biomaterials.* 2007;28(30):4439–48.
93. Xu M, Kreeger PK, Shea LD, Woodruff TK. Tissue-engineered follicles produce live, fertile offspring. *Tissue Eng.* 2006;12(10):2739–46.
94. Xu M, West-Farrell ER, Stouffer RL, Shea LD, Woodruff TK, Zelinski MB. Encapsulated three-dimensional culture supports development of nonhuman primate secondary follicles. *Biol Reprod.* 2009;81(3):587–94.
95. Middendorff R, Muller D, Mewe M, Mukhopadhyay AK, Holstein AF, Davidoff MS. The tunica albuginea of the human testis is characterized by complex contraction and relaxation activities regulated by cyclic GMP. *J Clin Endocrinol Metab.* 2002;87:3486–99.
96. de Kretser DM, Temple-Smith PD, Kerr JB. Anatomical and functional aspects of the male reproductive organs. In: Bandhauer K, Fricks J, editors. *Handbook of urology.* Berlin: Springer; 1982. Chapter 16, p. 1–31.
97. de Kretser DM, Kerr JB. The cytology of the testis. In: Knobil E, Neil JD, editors. *The physiology of reproduction.* New York: Raven; 1994. p. 1177–290.
98. Christensen AK. Leydig cells. In: Hamilton DW, Greep RO, editors. *Handbook of physiology.* Baltimore: Williams & Wilkins; 1975. p. 57–94.
99. Clermont Y. The cycle of the seminiferous epithelium in man. *Am J Anat.* 1963;112:35–51.
100. Clermont Y. Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. *Physiol Rev.* 1972;52:198–236.
101. Huckins C. The spermatogonial stem cell population in adult rats. I. Their morphology, proliferation and maturation. *Anat Rec.* 1971;169:533–57.
102. Dym M, Fawcett DW. Further observations on the numbers of spermatogonia, spermatocytes, and spermatids connected by intercellular bridges in the mammalian testis. *Biol Reprod.* 1971;4:195–215.
103. Paulson JR, Laemmli UK. The structure of histone-depleted metaphase chromosomes. *Cell.* 1977;12:817–28.
104. Izaurralde E, Kas E, Laemmli UK. Highly preferential nucleation of histone H1 assembly on scaffold-associated regions. *J Mol Biol.* 1989;210:573–85.
105. Adachi Y, Kas E, Laemmli UK. Preferential cooperative binding of DNA topoisomerase II to scaffold-associated regions. *EMBO J.* 1989;13:3997.
106. Giroux CN. Meiosis: components and process in nuclear differentiation. *Dev Genet.* 1992;13:387–91.
107. Auger J, Dadoune JP. Nuclear status of human sperm cells by transmission electron microscopy and image cytometry: changes in nuclear shape and chromatin texture during spermiogenesis and epididymal transit. *Biol Reprod.* 1993;49:166–75.
108. Miller D, Brinkworth M, Iles D. Paternal DNA packaging in spermatozoa: more than the sum of its parts? DNA, histones, protamines and epigenetics. *Reproduction.* 2010;139:287–301.
109. Braun RE. Packaging paternal chromosomes with protamine. *Nat Genet.* 2001;28:10–2.
110. Balhorn R. The protamine family of sperm nuclear proteins. *Genome Biol.* 2007;8:227.
111. Bedford JM, Calvin H, Cooper GW. The maturation of spermatozoa in the human epididymis. *J Reprod Fertil Suppl.* 1973;18:199–213.
112. Russell L. Morphological and functional evidence for Sertoli-germ cell relationships. In: Russell LD, Griswold MD, editors. *The Sertoli cell.* Clearwater, FL: Cache Press; 1993. p. 365–90.
113. Breucker H, Schafer E, Holstein AF. Morphogenesis and fate of the residual body in human spermiogenesis. *Cell Tissue Res.* 1985;240:303–9.
114. Clermont Y, Perey B. The stages of the cycle of the seminiferous epithelium of the rat: practical definitions in PA-Schiff-hematoxylin and hematoxylin-eosin stained sections. *Rev Can Biol.* 1957;16:451–62.
115. Perey B, Clermont Y, LeBlonde CP. The wave of seminiferous epithelium in the rat. *Am J Anat.* 1961;108:47–77.
116. Heller CH, Clermont Y. Kinetics of the germinal epithelium in man. *Recent Prog Horm Res.* 1964;20:545–75.
117. Schulze W, Rehder U. Organization and morphogenesis of the human seminiferous epithelium. *Cell Tissue Res.* 1984;237:395–407.
118. World Health Organization. *Laboratory manual for the examination of human semen and sperm-cervical mucus interaction.* 4th ed. New York: Cambridge University Press; 1999.
119. World Health Organization. *Laboratory manual for the examination of human semen and sperm-cervical mucus interaction.* 5th ed. New York: Cambridge University Press; 2010.
120. Sharpe RM. Regulation of spermatogenesis. In: Knobil E, Neil JD, editors. *The physiology of reproduction.* New York: Raven; 1994. p. 1363–434.
121. Barros C, Franklin B. Behaviour of the gamete membranes during sperm entry into the mammalian egg. *J Cell Biol.* 1968;37:13.
122. Bellve AR, Zheng W. Growth factors as autocrine and paracrine modulators of male gonadal functions. *J Reprod Fertil.* 1989;85:771–93.
123. Skinner MK. Cell-cell interactions in the testis. *Endocr Rev.* 1991;12:45–77.
124. Sharpe T. Intratesticular control of steroidogenesis. *Clin Endocrinol.* 1990;33:787–807.
125. Bedford JM. Effect of duct ligation on the fertilizing capacity of spermatozoa in the epididymis. *J Exp Zool.* 1967;166:271–81.
126. Orgebin-Crist M. Maturation of spermatozoa in the rabbit epididymis: fertilizing ability and embryonic mortality in does inseminated with epididymal spermatozoa. *Ann Biol Anim Biochim Biophys.* 1967;7:373–9.
127. Schoysman RJ, Bedford JM. The role of the human epididymis in sperm maturation and sperm storage as reflected in the consequences of epididymovasostomy. *Fertil Steril.* 1986;46:293–9.
128. Silber SJ, Balmaceda J, Borrero C, Ord T, Asch R. Pregnancy with sperm aspiration from the proximal head of the epididymis: a new treatment for congenital absence of the vas deferens. *Fertil Steril.* 1988;50:525–8.

129. Moore HD, Curry MR, Penfold LM, Pryor JP. The culture of human epididymal epithelium and in vitro maturation of epididymal spermatozoa. *Fertil Steril*. 1992;58:776–83.
130. Tredway DR, Settlege DS, Nakamura RM, Motoshima M, Umezaki CU, Mishell Jr DR. Significance of timing for the postcoital evaluation of cervical mucus. *Am J Obstet Gynecol*. 1975;121:387–93.
131. Mortimer D. Objective analysis of sperm motility and kinematics. In: Keel BA, Webster BW, editors. *Handbook of laboratory diagnosis and treatment of infertility*. Boca Raton: CRC; 1990. p. 97–133.
132. Katz DF, Drobnis E, Overstreet JW. Factors regulating mammalian sperm migration through the female reproductive tract and oocyte vestments. *Gamete Res*. 1989;22:443–69.
133. Mortimer D. Sperm transport in the human female reproductive tract. In: Finn CA, editor. *Oxford reviews of reproductive biology*. Oxford, UK: Oxford University Press; 1983. Chapter 5, p. 30–61.
134. Yanagamachi R. Mammalian fertilization. In: Knobil E, O'Brien NJ, editors. *The physiology of reproduction*. New York: Raven; 1994.
135. Thomas P, Meizel S. Phosphatidylinositol 4,5-bisphosphate hydrolysis in human sperm stimulated with follicular fluid or progesterone is dependent upon Ca^{2+} influx. *Biochem J*. 1989;264:539–46.
136. Overstreet JW, Katz DF, Yudin AI. Cervical mucus and sperm transport in reproduction. *Semin Perinatol*. 1991;15:149–55.
137. Parks JE, Ehrenwalt E. Cholesterol efflux from mammalian sperm and its potential role in capacitation. In: Bavister BD, Cummins J, Raldon E, editors. *Fertilization in mammals*. Norwell, MA: Serono Symposia; 1990.
138. Benoff S, Hurley I, Cooper GW, Mandel FS, Hershlag A, Scholl GM, et al. Fertilization potential in vitro is correlated with head-specific mannose-ligand receptor expression, acrosome status and membrane cholesterol content. *Hum Reprod*. 1993;8:2155–66.