I

HISTORICAL BACKGROUND AND FUNCTIONAL ANATOMY

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HUMAN REPRODUCTIVE TRACT

Introduction

Fetal sexual differentiation is a complicated series of events, actively programmed at the appropriate critical periods of fetal life (Table 1.1). Sex chromosomes promote the development and differentiation of the primary gonad, but the actual influences are the presence or absence of testosterone and anti-Müllerian hormone production by the testis. Brain and hypothalamic sexual identities are mainly acquired during postnatal life. Testicular Müllerian-inhibiting substance secretion causes degeneration of the Müllerian ducts. In the absence of these hormones, a female phenotype develops regardless of whether an ovary is present. The development of a female phenotype involves degeneration of the Wolffian (male) duct system, retention of the Müllerian duct system, and differentiation of female-like external genitalia.

Gonadal Differentiation

The gonad arises as an identical primordium in all embryos, poised in a precarious balance between male and female developmental pathways. Depending on whether or not a Y chromosome is present, this primordium follows a testis or ovarian fate. In vertebrates, the gonads arise as paired structures within the intermediate mesoderm, which lies on either side of the embryo, filling much of the coelomic cavity between the limb buds during the first half of development. Within this region, three segments comprising the urogenital ridge are distinguished from anterior to posterior: (1) the pronephros, which includes the adrenal primordium near its caudal end; (2) the mesonephros, the central region from which the gonad arises; and (3) the metanephros, the most posterior region in which the kidney forms.[1]

Development of the Female Reproductive Tract

Müllerian ducts appear in the human embryo at 6 weeks of gestation. In the female embryo, Müllerian ducts differentiate into Fallopian tubes, the uterus, and the upper part of the vagina. In the male fetus, Müllerian ducts begin to regress at the 8th week of gestation and have disappeared by the 9th week. This regression is because of the presence of the anti-Müllerian hormone (AMH). The Müllerian duct is sensitive to AMH during a limited period of fetal development (up to

Table 1.1 Fetal Age in Weeks After the Last Menstrual Period[2]

Fetal Age (Weeks)*	*Sex-differentiating Events*
	Inactivation of one X chromosome.
4	Development of Wolffian ducts.
5	Migration of primordial germ cells in the undifferentiated gonad.
6	Development of Müllerian ducts.
7	Differentiation of seminiferous tubules.
8	Regression of Müllerian ducts in male fetus.
8	Appearance of Leydig cells. First synthesis of testosterone.
9	Total regression of Müllerian ducts. Loss of sensitivity of Müllerian ducts in the female fetus.
9	First meiotic prophase in oogonia.
10	Beginning of masculinization of external genitalia.
10	Beginning of regression of Wolffian ducts in the female fetus.
12	Fetal testis is in the internal inguinal ring.
12–14	Male penile urethra is completed.
14	Appearance of first spermatogonia.
16	Appearance of first ovarian follicles.
17	Numerous Leydig cells. Peak of testosterone secretion.
20	Regression of Leydig cells. Diminished testosterone secretion.
24	First multilayered ovarian follicles. Canalization of the vagina.
28	Cessation of oogonia multiplication.
28	Descent of testis.

8 weeks in the human fetus). The mesonephric ducts require testosterone for their development; therefore, in the female they rapidly disappear. The Fallopian tubes develop from the cranial parts of the paramesonephric ducts.[5]

DEVELOPMENT OF THE FALLOPIAN TUBE

Embryology

Between the 5th and 6th week after oocyte fertilization, a longitudinal groove called Müller's groove arises from the coelomic epithelium on each side lateral to the mesonephric duct. The edges of this groove fuse to form a canal called the Müllerian or paramesonephric duct. The Fallopian tubes develop from the cranial parts of the paramesonephric ducts, with their cranial ends remaining open and connecting the duct with the coelomic (peritoneal) cavity and the caudal end communicating with the uterine cornua. Congenital anomalies of the tube include aplasia, in which the tube fails to form; hypoplasia, in which the tube is long, narrow, and tortuous; accessory ostia; and congenital diverticula.[3,4,5]

Anatomy

The Fallopian tubes are paired, tubular, seromuscular organs whose course runs medially from the cornua of the uterus toward the ovary laterally. The tubes are situated in the upper margins of the broad ligaments between the round and utero-ovarian ligaments (Fig. 1.1). Each tube is about 10 cm long with variations

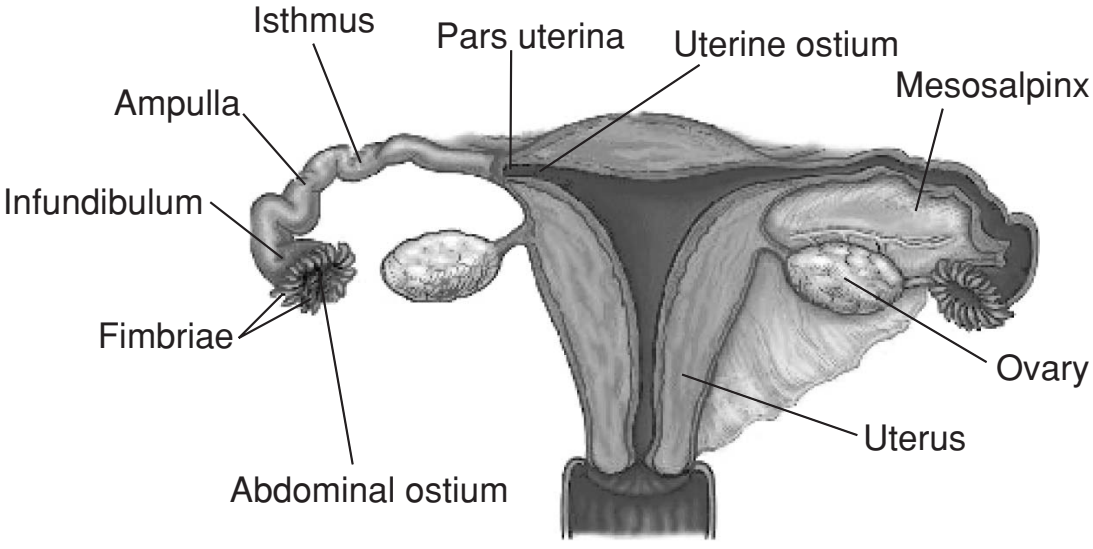

Fig. 1.1: Anatomy of the Fallopian tubes. (See Color Plate 1.)

in length from 7 to 14 cm. The abdominal ostium is situated at the base of a funnel-shaped expansion of the tube, the infundibulum, the circumference of which is enhanced by irregular processes called fimbriae. The ovarian fimbria is longer and more deeply grooved than the others and is closely applied to the tubal pole of the ovary. Passing medially, the infundibulum opens into the thin-walled ampulla, which forms more than half the length of the tube and is 1 or 2 cm in outer diameter. The isthmus is a round and cordlike structure constituting the medial one-third of the tube and 0.5–1 cm in outer diameter. The interstitial or conual portion of the tube continues from the isthmus through the uterine wall to empty into the uterine cavity.

Structure of the Fallopian Tube

The Fallopian tube or uterine tubes (Fig. 1.2) are derived from the Müllerian ducts and are paired, tubular, seromuscular organs attached laterally to the ovary and medially to the uterus fundus. They are about 7–14 cm long and are covered by peritoneum, which duplicates to form one of its loose attachments (the mesosalpinx) to the broad ligament. Their narrow proximal portion, the isthmus, begins at the uterotubal junction and extends distally to the ampulla. It is 2–3 cm long with a narrow lumen ranging from 0.1–1.0 mm in diameter.[6] The isthmic mucosa is arranged into four primary mucosal folds, with fewer ciliated cells than the other regions of the tube.

The next part is the ampullary part, which has an expanding lumen and a convoluted endosalpingeal mucosa. The ampulla is the longest portion of the human tube, ranging from 5–8 cm in length.[6] The luminal diameter varies from 1–2 mm at its junction with the isthmus to a maximum of about 1 cm near its

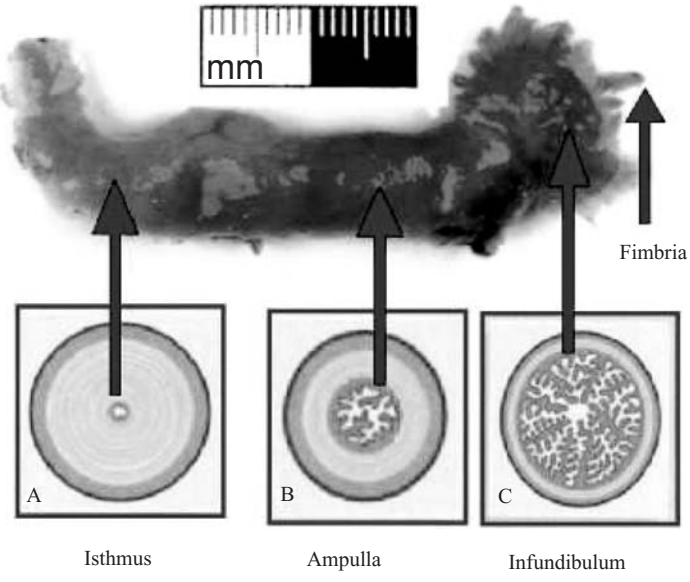

Fig. 1.2: Diagram of Fallopian tube showing different parts in cross-section: the proximal region (a) isthmus, the middle region (b) ampulla and the distal region, and (c) infundibulum. (See Color Plate 2.)

distal end. The myosalpinx in the ampulla is not organized into clear-cut muscular layers.[7] The mucosa is abundant in ciliated cells.

The distal part, the infundibulum, is the part of the tube ending in the fimbria, which is found close to the ovary. The ovarian fimbria is longer and more deeply grooved than the others and is closely applied to the tubal pole of the ovary.

The blood supply of the uterine tube comes from the vascular arch of the ovarian and uterine arteries, from which branches pass through the mesosalpinx to reach the muscular wall.

Histological Organization

The tubal wall consists of three layers: the internal mucosa (endosalpinx), the intermediate muscular layer (myosalpinx), and the outer serosa, which is continuous with the peritoneum of the broad ligament and uterus, the upper margin of which is the mesosalpinx. The endosalpinx is thrown into longitudinal folds called primary folds, which increase in number toward the fimbria and are lined by columnar epithelium of three types: ciliated, secretory, and peg cells. In the ampullary and infundibular regions, secondary folds of the tubal mucosa also exist, markedly increasing the surface areas of these segments of the tube. The myosalpinx actually consists of an inner circular and an outer longitudinal layer to which a third layer is added in the interstitial portion of the tube.[8]

Cell Form and Function of the Tubal Mucosa

The entire tubal mucosa has a columnar epithelial lining resting on connective tissue. The epithelium has two different cell types: ciliated and secretory cells. Ciliated cells are relatively square in profile, and the cilia are about 7 μm long.[6]

The percentage of ciliated cells dramatically increases from the isthmus to the fimbria.[9–11] The ciliated cells are most common in the fimbriated infundibulum. They carry upright cilia, and their primary role is to mobilize the gametes and embryo within the tube. The beat rate of the cilia is directed by the levels of estrogen and progesterone, with highest levels of activity at the time of ovulation.

The secretory cells are most abundant in the ampullary region. Secretory cells contain a granular cytoplasm characterized by fine granules, with the endoplasmic reticulum spread out irregularly.[6] In the follicular phase, the nucleus is elongated and placed with its long axis parallel to the long axis of the cell; later on in this stage the nucleus becomes more rounded in the apical of the cells.[8] These cells have several varieties of microvilli and contain high amounts of endoplasmic reticulum. Secretory material is accumulated in these cells and then released into the lumen for nourishment of the oocyte and embryo, with a possible role in the process of fertilization.

The Uterine Tube Cycle

The first description of a distinct cyclicity in the histologial appearance of the tubal epithelium in women was by Novak and Everett in 1928. They recorded that the ciliated and secretory cells underwent cyclical changes under the effect of estrogen-progesterone during the menstrual cycle. The epithelial cells reached their maximum height and degree of ciliation during the late follicular phase in the ampulla and fimbria. In the late luteal phase, atrophy and deciliation occurred, especially in the fimbrial region. Hypertrophy and reciliation started in the early follicular phase. During pregnancy and throughout the postpartum period, further atrophy and deciliation occurred.[8]

Tubal Motility

Peristaltic contraction of the smooth muscle fibers in the tubal wall allows the gametes (the sperm and egg) to be brought together, thus allowing fertilization and subsequent transport of the fertilized ovum from the normal site of fertilization in the ampulla to the normal site of implantation in the uterus. This movement is primarily regulated by three intrinsic systems: the estrogen-progesterone hormonal milieu, the adrenergic-nonadrenergic system, and prostaglandins.

Estrogens acting via their nuclear receptor stimulate tubal motility, whereas progesterone inhibits tubal motility. Before ovulation, contractions are gentle, with some individual variations in rate and pattern. At ovulation, tubal contractions become vigorous, and the mesosalpinx contracts to bring the tube in more contact with the ovary, while the fimbria contracts rhythmically to sweep over the ovarian surface. As the progesterone level rises 4–6 days after ovulation, tubal motility slows. This may lead to relaxation of the tubal musculature to allow passage of the ovum into the uterus by the action of the tubal cilia. The effects of estrogen and progesterone on oviductal motility and morphology are mediated

through these steroids' receptors. The changes in receptor levels are critical in determining the functional state of the Fallopian tube.

Adrenergic innervation is thought to be involved in regulations of tubal motility, particularly isthmic motility changes. During menstruation and the proliferative (preovulatory) phase, the human tube is very sensitive to α-adrenergic compounds such as norepinephrine. After ovulation and during the luteal phase, the response to norepinephrine is decreased and the inhibitory effect of β-adrenergic compounds is more evident. Activation of the receptors by raised progesterone levels in the luteal phase leads to relaxation of the circular muscles; thus, the isthmic luminal diameter is increased and trans-isthmic passage of the fertilized ovum is facilitated.[12]

Although there is some controversy regarding the role of prostaglandins in the regulation of spontaneous tubal motility, it is generally accepted that prostaglandin $F_{2\,a}$ ($PGF_{2\,a}$) stimulates, whereas PGE_1 and PGE_2 inhibit Fallopian tube contractions. Contrary to their differential activity on tubal motility, all three natural prostaglandins ($PGF_{2\,a}$, PGE_1, and PGE_2) stimulate ciliary activity in vitro.[13,14]

In summary, the initial rise in progesterone after ovulation causes contractions of the two inner layers of the uterotubal junction (UTJ), thus causing tubal locking of the ovum. After a few days, sensitivity of the muscles to adrenergic stimulation diminishes, whereas other factors, such as prostaglandins, dominate, leading to relaxation of the uterotubal junction and release of the fertilized ovum into the uterine cavity.

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II

COCULTURE AND ASSISTED REPRODUCTION

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INTRODUCTION

Coculture in assisted reproduction refers to the culture of embryos with somatic cells that aim to enhance the development of the cocultured embryos. In the early 1990s, human embryo culture medium consisted essentially of inorganic salt; nutrient components such as glucose, pyruvate, and lactate; and serum albumin or serum. These culture media were suboptimal for embryo development in vitro as indicated by the low blastulation rate and implantation rate in assisted reproduction treatment. Hence, coculture was used to improve the embryo culture environment based on the assumption that the somatic cells would produce growth factors promoting the development of the embryo in culture. The use of coculture in assisted reproduction declined after the development of sequential culture,[1] which has rapidly become the method of choice for culturing human embryos because of the simplicity in the use of the sequential culture system. This chapter discusses the role of coculture in modern-day assisted reproduction. Emphasis will be on coculture using oviductal cells, which is the theme of this book.

OVIDUCT AND EARLY EMBRYO DEVELOPMENT

The oviduct has long been thought to be a passive conduit for the passage of the gamete and embryo in the early reproductive events. Although direct evidence is lacking, there are three lines of circumstantial evidence (mainly from animal studies) that indicate the oviduct has an additional function in enhancing the development of preimplantation embryos. The first line of evidence comes from observations of the cyclical changes in the development and activity of the ciliated cells and secretory cells of primate oviduct in the reproductive cycle. The second line of evidence derives from reports showing expression of growth factors in the oviduct and the corresponding receptors in the preimplantation embryo or vice versa. In addition, some of the oviductal growth factors are expressed in a cyclical manner. The third line of evidence includes studies reporting embryotrophic activity of oviductal cell coculture and growth factors that are found to be expressed in the oviduct. These observations highlight the possible biochemical communication between the oviductal cells and the embryos in regulating preimplantation embryo development.

COCULTURE IN ASSISTED REPRODUCTION

Oviductal Coculture in Clinical-Assisted Reproduction

The oviduct is the natural site of early embryo development. The period when we culture the embryos in assisted reproduction is mainly the time when they should be in the oviduct in vivo. Based on the belief that the oviduct should provide the best microenvironment for embryo development, several groups used oviductal cells as the helper cells for coculture in assisted reproduction. In experimental condition, oviductal cells decrease embryo fragmentation,[2] increase the rate of blastulation,[2,3] hatching,[4] and the number of cells per blastocyst[3] of human embryos.

Clinical studies including prospective randomized trials using oviductal cell coculture[5–12] had been reported. All except one[10] demonstrated either an improvement in embryo quality or pregnancy/implantation rate after coculture with oviductal cells of human or animal origin. The indications for coculture were advanced age,[9] multiple implantation failures in assisted reproduction,[6,9] and high basal follicle-stimulating hormone (FSH) level,[13] whereas coculture was inefficient in patients with good prognosis, such as young age (<age 35) and attending the first assisted reproduction cycle.[7]

Despite the positive findings on oviductal cell coculture, it is not used in assisted reproduction programs because of the extra resources required for its implementation. These resources include additional incubators, cryopreservation facilities, and manpower for the culture and storage of the donated oviductal cells and the need for screening of donors for infectious disease. Moreover, the donors of oviduct are limited. The use of oviduct of animal origin is not advisable because of the risk of transferring unknown disease from the donor animals to the human.

Coculture Studies with Other Somatic Cells in Clinical IVF

Cells other than oviductal cells, including granulosa cells, Vero cells, and endometrial cells, have been used for coculture. Currently, only homologous endometrial cell coculture is still practicing in assisted reproduction.[14,15] This is because an endometrial sample is easy to obtain from the patient for her own use, thus eliminating the need for screening donated endometrial samples for infectious disease. Endometrial cells are also easy to grow in culture. Besides, blastocyst encounters the endometrial cells in vivo providing a physiological basis for the use of these cells.

Efficacy of Coculture

The efficacy of coculture varies with the helper cells used. Although different helper cells can stimulate development of embryos in coculture, oviductal epithelial cells seem to provide a better support to the cocultured embryos, consistent with the assumption that the in vivo oviductal microenvironment is best for preimplantation embryo development. Oviductal epithelial cells are better than granulosa cells,[16] oviductal stromal cells or endometrial epithelial cells,[17] and fetal

uterine fibroblast[7] in producing high-quality bovine embryos. The posttransfer development of sheep embryos after oviductal coculture is superior to those after fibroblast coculture, although both cell types stimulate embryo development in culture.[18] Human Fallopian tube epithelial cells are more efficient than oviductal fibroblasts in supporting mouse embryo development.[19] Human fibroblasts did not enhance the success rate of assisted reproduction when compared to medium alone culture in a prospectively randomized study.[20] The differential efficacy of the various helper or supporting cells in enhancing embryo development is probably because of their varied ability in the production of embryotrophic factors such as growth factors. For instance, human Fallopian tube cells but not buffalo rat liver cells express leukemia inhibitory factor (LIF) and interleukin-6 (IL-6) and possibly also activins.[21]

COCULTURE AND SEQUENTIAL CULTURE

Sequential culture refers to the use of more than one media for culturing embryos at different stages of development. Usually two media are used; one for the precompaction stages and the other for the postcompaction stages. The rationale of sequential culture is based on the knowledge of the changing metabolic needs of preimplantation embryos before and after compaction. This culture method is currently the method of choice for culturing embryos to the blastocyst stage in most assisted reproduction programs, because it requires minimal additional resources that most assisted reproduction centers can afford and it also does not have the limitations associated with coculture mentioned earlier.

The culture condition in sequential culture is not yet fully optimal because the development of embryos in such a system can still be improved under certain conditions. The development of human embryo in sequential culture systems is enhanced when the media are supplemented with granulocyte-macrophage colony-stimulating factor (GM-CSF)[22] and insulin-like growth factor-1 (IGF-1).[23] Human Fallopian tube cell coculture in sequential culture medium also improves the hatching rate of mouse embryos when compared to sequential culture alone.[24] The latter study suggests that oviductal cell coculture and sequential culture are not mutually exclusive.

It would be ideal if one can have the oviductal embryotrophic activity in the sequential culture system without the use of oviductal cells. This can be accomplished provided that the components and their composition in the oviductal microenvironment or in the oviductal coculture system are known. These data would allow us to simulate the oviductal microenvironment in vitro and to eliminate the complication involved in the use of donor oviductal tissue.

SIMULATION OF OVIDUCTAL MICROENVIRONMENT

The idea of simulating the oviductal microenvironment to improve embryo development has been practiced for many years. Early preimplantation embryos