

Chapter 2

In Utero Exposure to Environmental Chemicals: Lessons from Maternal Cigarette Smoking and Its Effects on Gonad Development and Puberty

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Abstract Although tobacco use has declined in recent years, a significant minority of pregnant women still smoke and cessation during gestation is rare. Substantial passive exposure to tobacco smoke is an unavoidable hazard to many more. This is of great concern, since tobacco smoke contains a large number of toxins known to be hazardous to foetal development. These include carbon monoxide, nicotine and its breakdown products, polycyclic aromatic hydrocarbons, thiocyanate and many more. Some are known or suspected endocrine-disrupting chemicals (EDCs). In addition to providing further evidence to dissuade pregnant women and those around them from smoking, studying the effects of tobacco smoke on human development gives insight into effects of other EDCs and EDC mixtures which affect the same developmental processes and end points.

In utero development of the reproductive tract is a poorly understood process that is entirely dependent on the proper functioning of the maternal and foetal endocrine systems. It is vulnerable to disruption in both genders. Thus far, only the physiological effects of the disruption of oestrogen and androgen-mediated signalling and the hypothalamic-pituitary-gonadal (HPG) axis have been investigated in detail. Our knowledge of the process in both animals and humans is far from complete. Exposure of animal models to EDCs affecting sex steroid-mediated processes can cause a

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wide range of adverse reproductive outcomes. These include impaired gametogenesis and later subfertility, early reproductive senescence, neuroendocrine dysregulation affecting fertility and sexual behaviour, genital deformities and increased susceptibility to certain cancers. Effects on humans are thus far uncertain, but many epidemiological studies have associated EDC exposure with decreasing fertility, reductions in the age of pubertal onset and increases in susceptibility to certain cancers and deformities of the reproductive tract. Studies of the physiological and cellular effects of individual EDCs and EDC mixtures in vitro and in vivo have added plausibility to these associations.

Prenatal human tobacco smoke exposure has multiple known adverse effects on reproductive development and may affect the age of puberty in females at least. Overall, maternal and foetal oestrogen and androgen synthesis are suppressed centrally by suppression of the HPG axis by the activation of the hypothalamic-pituitary-adrenal (HPA) axis and peripherally by the direct inhibition of their synthesis. The hypothalamic-pituitary-thyroid (HPT) axis and the peripheral transformation and utilization of thyroid hormones are also suppressed by activation of the HPA axis. Neuroendocrine programming of the HPA, HPT and HPG is perturbed. Decreased fertility and fecundity have been noted in both genders, and exposed females are more likely to experience early menopause. Some epidemiological studies have reported a connection with malformations of the male reproductive tract (testicular dysgenesis syndrome), the plausibility of which is supported by evidence that the production of desert hedgehog, a morphogen essential for male sexual development, is halved by maternal smoking. These outcomes are similar to those observed in epidemiological and animal studies of other EDCs and EDC mixtures, but a great deal of further research is required to determine their exact physiological effects and properly quantify the risks they pose. The effects of tobacco smoke and other EDC mixtures and EDCs on systems other than those directly mediated by the oestrogen receptors and androgen receptor have barely begun to be elucidated.

Keywords Endocrine disruption • Human • Pregnancy • Puberty • Reproductive development • Smoking • Tobacco

Introduction

Although smoking has become less popular in recent years, the direct and indirect exposure of pregnant women to tobacco smoke is still a major health issue and is likely to remain so for some time. In 2005, 33% of pregnant women in the UK were active smokers immediately before or during their pregnancy, and one in six (17%) smoked throughout [1]. In some subpopulations, this percentage is higher. A recent survey of pregnant women in Scotland, for example, found that 25% smoke at maternity book in, but only 3.5% of these quit before delivery, despite the presence of interventions intended to help them stop [2]. There has been a downward trend in

the number of pregnant women smoking, but it has been at best modest. The number of pregnant smokers in the UK decreased by only 2% between 2000 and 2005, and there was no change in Scotland [1]. In the USA, the downward trend in the number of smokers stopped in 2005 and has remained steady since then. Currently, 20% of adults in the USA smoke, and about half of children aged 3–11 are exposed regularly to second-hand smoke [3]. In developing nations, tobacco use is still rising, most notably in Africa, Southeast Asia and certain parts of Latin America [4].

In utero exposure to tobacco combustion products has a broad range of effects on foetal development. Thus far, much attention has been given to the effects of carbon monoxide-induced hypoxia [5, 6] and the effects of nicotine [7]. Other notable toxicants present include toxic trace elements such as cadmium, nickel and mercury [8] and polycyclic aromatic hydrocarbons (PAHs) [9]. The consequences faced by the children of smokers are diverse and worrying. Babies are lighter at birth [10], and the incidences of pre-, peri- and postnatal mortality [11] are increased. Asthma is more common amongst the children of smokers [12], as are cognitive deficits [13], and in both males and females, impaired fertility and fecundity [14–17].

Endocrine-disrupting compounds (EDCs) have been studied extensively in recent years, and there are great concerns about their possible effects on human development, including alterations to neuroendocrinological function; disrupted breast and prostate development and increased risks of breast and prostate cancer; disruptions in the regulation of metabolism and an increasing predisposition to obesity; and the disruption of cardiovascular and reproductive endocrinology and development in both genders [18]. Both natural and man-made EDCs have been found to affect male and female reproductive development in animal experimental studies, including the processes involved in puberty [19–21]. Some of the chemicals found in tobacco smoke such as PAHs, cadmium, arsenic and nicotine are EDCs, but their contribution, if any, to the described effects of smoke exposure in utero is unknown. Environmental EDC exposures typically result from contact with mixtures of compounds, which although often present individually at concentrations lower than would be expected to cause adverse effects, act together cumulatively under most circumstances [22]. Unlike most exposures, however, prenatal tobacco smoke exposure can be studied in humans as well as in animal models. The impacts on foetal development are similar to those thought to be at least partly caused by exposure to other EDCs and EDC mixtures. Therefore, by discovering how developmental processes are affected, we can learn more about EDC actions at the molecular level. Tobacco use is voluntary, but concomitant exposure to environmental tobacco smoke may not be if an individual lives with or spends time with smokers. In the UK, roughly 80% of young women drink (40% heavily) and up to 20% use recreational drugs [23]. Cessation upon falling pregnant is not marked, with <5% of women quitting smoking while pregnant. While drug-using women tend to stop taking ecstasy and reduce their alcohol consumption when they become pregnant, 50% continue to use tobacco and cannabis [24]. By better quantifying the effects of these drugs, the information available to try to convince expectant mothers and those around them to give up can hopefully be made more persuasive.

Endocrine-Disrupting Chemicals and Reproductive Development

Development of the Reproductive Tract

Between the third and eighth weeks of gestation, the human embryo undergoes a remarkable and rapid transformation, beginning as a flat, relatively undifferentiated plate of cells roughly 0.4 mm in diameter and by the end is recognizably human (Fig. 2.1), bearing recognizable structures both internally and externally. The foundations of the human reproductive tract are laid during this time, and its development carries on throughout gestation, childhood and puberty. A timeline of the events involved is shown in Fig. 2.2. Development is the same in both sexes until the seventh week. The primordial germ cells differentiate and migrate into the developing gonads. The urogenital folds and tubercle, which will become the external genitalia, appear. After this point, the sexes diverge dramatically, and three distinct phases of sexual development occur. The first occurs in utero as the basic structures of the reproductive organs develop and assume their position in the body. The second occurs perinatally as surges of gonadotropins trigger gamete maturation in both sexes and the proliferation of the cell types supporting spermatogenesis in males. The third and final stage is the process of puberty, which marks the attainment of reproductive capacity and sexually dimorphic secondary adult sexual characteristics.

Male

During the fifth week of human gestation, a population of cells from the yolk sac migrates through the gut mesentery and into the body of the embryo. They invade an area of coelomic epithelium, medial and ventral to the developing kidney. This area is stimulated to proliferate and thicken, forming a pair of genital ridges which develop in a bipotential manner until the end of the sixth week. In normal male foetuses, a subtle cellular change during the sixth week triggers the pattern of gene expression which ultimately leads to the male phenotype [26]. Several comprehensive reviews of this can be found in the literature [27–30]. The *SRY* gene is the key genetic switch, and its expression triggers the earliest morphological signs of sexual differentiation: the beginning of testis cord formation. *SRY* expression is Sertoli cell specific, and the Sertoli cells are central to the process of testicular morphogenesis. During the seventh week, the pre-Sertoli cells mature, polarizing and aggregating around the germ cells to form the seminiferous cords. This process divides the gonad into tubular testis cords, which are composed of Sertoli and germ cells, and the interstitial spaces between the cords. These spaces are not empty, but contain several important cell types. This process is dependent on the migration of mesonephric endothelial cells into the gonad during vascularization [29]. As vascularization commences, peritubular myoid cells, which are resident in the testis from the

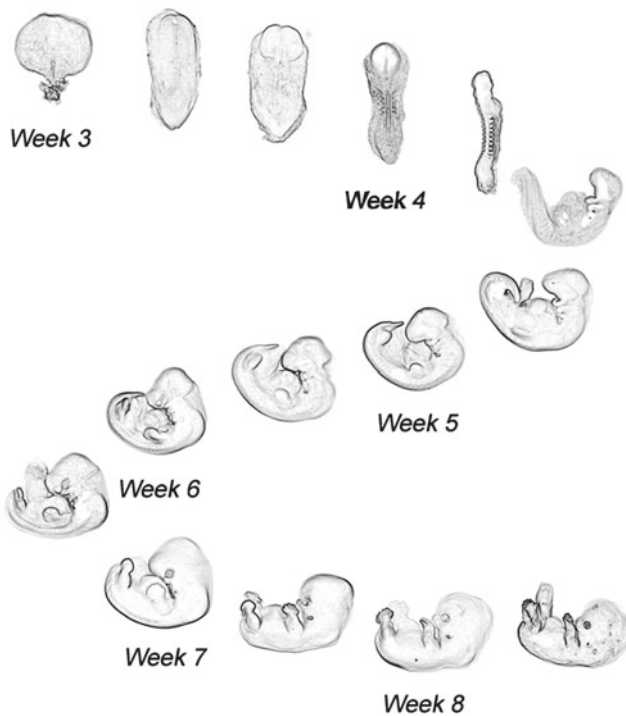


Fig. 2.1 External morphology of the human embryo between the 3rd and 8th weeks of gestation. The embryo begins as a disc of cells. In the 3rd week, this folds in on itself to form the notochord, the precursor of the vertebral column, and the neural plate, which will become the brain and spinal cord begins to form on the embryos surface. A portion of the yolk sac is incorporated into the embryo, which will become the lining of the gastrointestinal tract. The neural plate, which will become the brain and spinal cord also begins to form at this time, and the notochord begins to form somites, projections in the middle of the embryo which eventually form vertebrae and ribs. They continue to appear until midway through the 5th week. The neural plate folds in on itself, closing in the 4th week. The optic and lens placodes, which will form the eyes, form during week 4, as does the heart prominence and the pharyngeal arches which will form the pharynx. During the 5th week, the nasal pit, otic placode and otocysts appear, and the limb buds begin to develop. Retinal pigments appear during the 6th week, and at the same time, the nasal pits migrate to the ventral side of the embryo, and the limb buds form hand plates as the arms begin to develop. Digital rays appear on the hand plates during week 6, and footplates appear as the legs begin to form. The liver prominence begins to grow during this week, and the eyes and acoustic meatus become visible. The skeleton begins to ossify during the 7th week, the eyelids form and the body of the embryo begins to straighten, which together with its developing fingers and rounding head begins to give it a distinctly human appearance. This continues during the 8th week, and as the embryo uncurls, the limbs lengthen, the hands and feet turn inwards and the nose, ears and mouth become distinct[25]

second week of gestation, form a single layer of flattened cells around the Sertoli cells. These smooth muscle-like cells cooperate with the Sertoli cells to deposit the basal lamina, enclosing the seminiferous cords. Undifferentiated fibroblast-like cells then form the connective tissue in the interstitial spaces, which are also penetrated by cells for the gonadal vasculature migrating in from the mesonephros.

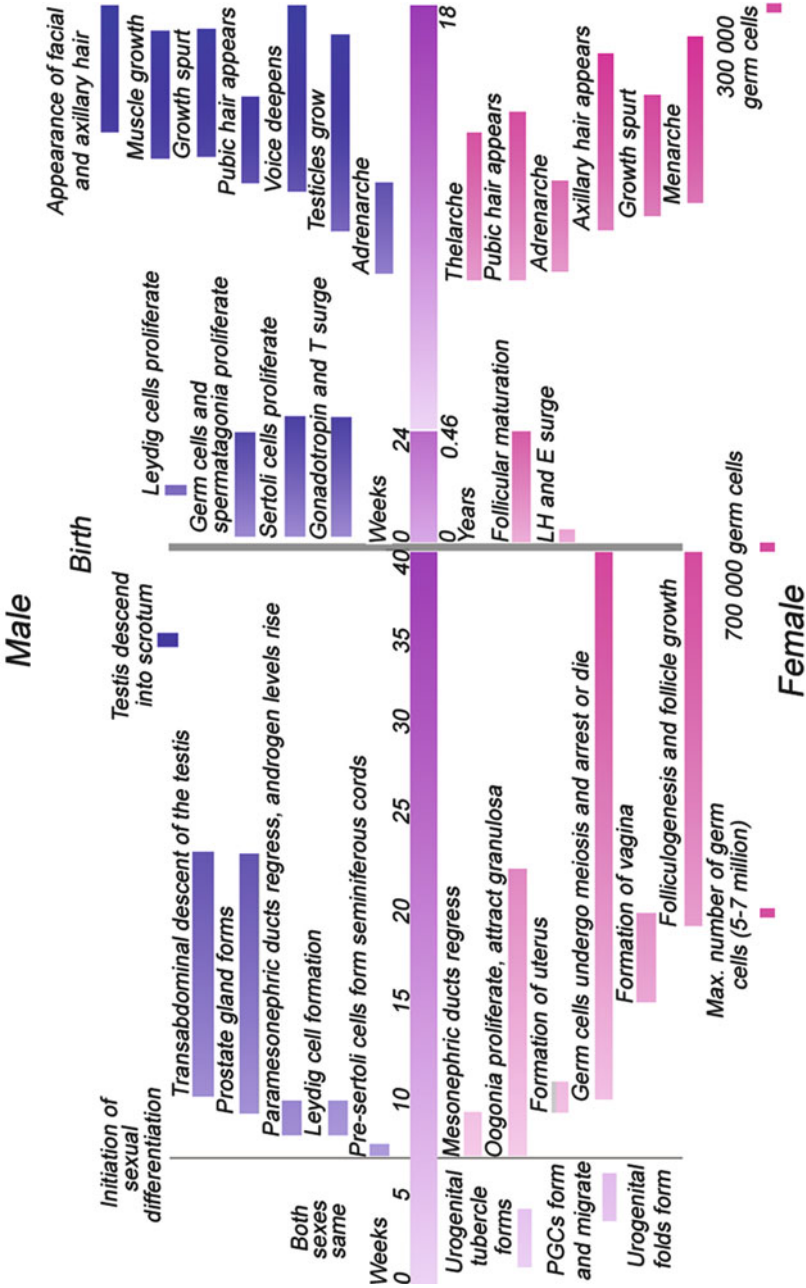


Fig. 2.2 Timeline of the development of the human reproductive tract from conception until the end of puberty in the male and female. PGCs primordial germ cells, LH luteinizing hormone, E oestrogen, T testosterone

Between the eighth and the tenth week, another important cell type follows this path and differentiates in these spaces. Those remaining in the mesonephros form the steroid-secreting cells in the adrenal cortex, and those that enter the gonad form the Leydig cells in males. These are the cells that are stimulated to secrete androgens and drive the further masculinization of the embryo. Initially, Leydig cell development occurs independently of the influence of gonadotropins in rodents, and possibly in humans [31]. This is a relatively short phase, and in humans, early androgen secretion rapidly comes under the control of human chorionic gonadotropin (hCG), levels of which peak during the tenth week [32]. Later in gestation, the foetal HPG axis plays a more dominating role [31–34]. The development of the male gonads can occur without the presence of the germ cells, although there is some evidence that they may support Sertoli cell differentiation [35].

In addition to the gonads, several internal and external structures in the sexually undifferentiated embryo must successfully develop and differentiate [29]. Two pairs of genital ducts, the mesonephric (Wolffian) and paramesonephric (Müllerian) ducts, exist in the undifferentiated embryo. In the male, the secretion of anti-Müllerian hormone (AMH) by the Sertoli cells causes the paramesonephric ducts to actively degenerate in the eighth–tenth weeks of gestation. They leave behind the appendix testis, a vestigial remnant usually present on the top of the testis attached to its covering membrane (the tunica vaginalis), and a small pouch in the area of the prostatic urethra which eventually forms the prostatic utricle. The mesonephric duct differentiates into the epididymis, vas deferens and seminal vesicles, which together form a ductal system connecting the testis to the urethra. The development of this system is stimulated by testosterone and is dependent on region-specific interactions between the mesenchyme and the mesonephric duct epithelium.

Early in development, an anterior-posterior androgen gradient forms, and the mesenchymal cells alone express androgen receptors. The anterior portion of the mesonephric duct begins to elongate and convolute to form the structure which will become the epididymis, the posterior forms the vas deferens and outgrowths from the posterior end of the vas deferens form the seminal vesicles [36, 37]. Rising androgen levels between the eighth and tenth weeks of gestation also stimulate prostate gland formation. Although some tissues within the prostate remain oestrogen responsive, embryonic, foetal and postnatal prostate growth and development is dependent on the presence of testosterone. In the embryo, it triggers the epithelium of the urogenital sinus to form an outgrowth into its mesenchyme, where it forms numerous solid buds which go on to become smooth muscle-lined prostatic ducts. During the regression of the paramesonephric duct, some of its epithelial cells undergo epithelial-mesenchymal transition, and a portion of these cells make up part of the mesenchyme that participates in the formation of the prostate [38]. This tissue remains responsive to oestrogens and starts to proliferate under the influence of maternal oestrogen during week 12 [38, 39]. Maternal oestrogens drive the proliferation of the prostatic stroma until week 23.

The genital tubercle is a small outgrowth which forms during the first month of gestation from tissue associated with the urogenital sinus. Its early development is guided by similar processes to limb development and is sex hormone independent.

Early in the fifth week, it is joined by the urogenital folds. These consist of an inner pair of folds which arise as an enlargement of the underlying ectoderm and extend along the edge of the developing cloaca, and an outer pair arising from the mesoderm form shortly after. These are the labioscrotal swellings. Although these structures are fully formed by the seventh week of gestation, the external genitalia of both genders remain relatively undifferentiated until about the 12th. Testosterone secretion masculinizes the external genitalia of the male, a process that becomes obvious at around weeks 14–16. However, recent data from the rat suggest that differentiation of a masculine reproductive system is programmed by the action of Leydig cell-derived androgens prior to morphologically apparent differentiation, and this time window in humans is estimated to be in the period 8–14 weeks [31, 40]. The anogenital distance increases, and the genital tubercle enlarges and elongates to form the phallus. This is accompanied by the closure of the urogenital folds, extending the urethra to the tip of the fully formed penis, and the fusion of the labioscrotal swellings to form the scrotum [41, 42]. This is an exceptionally delicate androgen-mediated developmental process. Its disruption results in the birth of boys with a spectrum of genital deformities, ranging from mild hypospadias to full pseudohermaphroditism.

Hypospadias and pseudohermaphroditism are the result of the incomplete closure of the urogenital folds. In mild cases of hypospadias, the end of the urethra falls a little short of the tip of the penis, resulting in an enlarged urethral opening or small slit in the glans. In more severe cases, the urethra opens further down the shaft or even at the base of the penis, and reconstructive surgery is required to ensure the penis will be functional later in life. The non-closure of both the urogenital and the labioscrotal folds results in pseudohermaphroditic genitalia, making it difficult to ascertain the baby's gender at birth [43]. Hypospadias is the most common congenital human deformity, affecting 1 in 125 live male births, and both genetic and environmental factors are implicated in its aetiology [43, 44]. Pseudohermaphroditism is less frequent and usually results from genetic mutations that affect either the androgen receptor [45–47] or testosterone/dihydrotestosterone synthesis [47]. Associations made between increasing incidences of hypospadias, cryptorchidism, and later poor semen quality and testicular cancer have led to the hypothesis that these conditions may be part of a single underlying condition, testicular dysgenesis syndrome, which arises from disruption of the androgen-dependant processes involved in the development of the male reproductive tract [48].

The descent of the testis from the abdominal cavity to the scrotum is one of the final gestational events for the human male foetus. It represents the culmination of a much longer process involving two distinct stages, transabdominal and inguinoscrotal descent [41]. These stages occur separately and are mediated by different sets of factors. Transabdominal descent starts at about weeks 10–14 and finishes at about weeks 20–23. Two pairs of gubernacula form from the cranial suspensory ligament of the testis in an androgen-dependant process. One pair is located above the testis, extending from the upper gonadal tips to the posterior abdominal wall, and degenerate to allow testicular descent. The others, which are much more robust, form between the lower poles of the testis and an area of the perineum which develops

during this process into the inner ring of the inguinal canal. During their development, the lower gubernacular segments connect with a larger extra abdominal process extending through the inguinal canal and attaching to the scrotum. Gubernacular development and transabdominal testicular descent are dependent on the production of INSL3 in rodents, an insulin-like peptide hormone structurally similar to relaxin. Its action is slightly enhanced by androgens, but androgens are not required for gubernacular formation. The inguinoscrotal stage is mediated by androgens alone and usually occurs by week 35 of gestation. Both processes can be disrupted in animal experimental studies by anti-androgens, oestrogens and oestrogen-like compounds. During the inguinoscrotal stage, the testes pass through the inguinoscrotal canal quickly and then settle within the scrotum. The gubernacula contract and their collagen content increases as they undergo involution to form the small band of ligamentous tissue that attaches the testis to the scrotal floor.

Female

In the absence of SRY, activation of the Wnt4 pathway, and possibly other factors, blocks the expression of the male gonadal phenotype in a poorly understood process that enables ovarian development [28, 29]. Gonadal development commences slightly later in the female than in the male. Upon entering the ovary, the primordial germ cells are surrounded by support cells derived from the ovarian mesenchyme. After a few divisions, the germ cells differentiate into oogonia, which unlike male germ cells continue to divide, forming 'nests' of clustered oocytes. This proliferation continues from the seventh to the end of the 22nd week by which time most of the oocytes will be in mitotic arrest. Shortly after their formation, the oocyte nests begin to break down as support cells invade the nest and differentiate into pre-granulosa cells which surround the developing oocytes [49].

Granulosa cells are essential for oocyte survival. As well as physically surrounding the oocytes, they are responsive to gonadotropins and a variety of other factors. In response, they secrete a number of paracrine and endocrine factors that both sustain the oocyte and developing follicle and participate in endocrine signalling. Oocytes that attract a sufficient number of granulosa cells will go on to develop into primordial follicles, whilst those that do not will undergo apoptosis. These primordial follicles undergo several stages in development, and at each stage large numbers die. The processes governing the selection of oocytes and follicles are poorly understood, but in humans, the initial population of 5–7 million germ cells will produce an ovarian reserve at birth of about 700,000 follicles containing oocytes held in a state of mitotic arrest. This does not represent a static population, however, since follicles will leave this state of quiescence, grow and die during both prenatal and postnatal life. Many of the follicles that grow prenatally are abnormal, indicating that this process could at least in part be a way to eliminate damaged or suboptimal oocytes. From birth to puberty, the ovarian reserve diminishes from around 700,000 to 300,000, and post-pubertally numbers continue to decline, either through ovulation or atresia, until the ovarian reserve is depleted and menopause

occurs. During follicle development, theca cells surround the follicular granulosa cells and are analogous to the Leydig cells in the male. One of their primary functions is to secrete androgens and progestins under gonadotropin control. Luteinizing hormone (LH) stimulates production of androgens which are then converted into oestrogens by aromatase, which is produced by growing follicles in response to follicle-stimulating hormone (FSH). They differentiate from the stroma, appearing around follicles after the formation of two or more layers of granulosa cells [49, 50].

In the absence of androgens, the female genital phenotype manifests [51, 52]. There is no proportional increase in anogenital distance or fusion of the labioscrotal or urogenital folds. The labioscrotal folds instead become the labia majora, and the urogenital fold becomes the labia minora. Rather than extending forwards and growing into a phallus, the genital tubercle enlarges and folds inwards slightly to form the clitoris. The urogenital sinus enters a period of rapid growth in week 11, forming a series of evaginations known collectively as the sinovaginal bulbs. These fuse with the uterovaginal canal at 15 weeks to form the vaginal plate. This does not acquire a lumen until around week 20. Its caudal end slides down the urethra and acquires an independent perineal opening. The oviducts, uterus and the upper third of the vagina differentiate from the paramesonephric duct. The mesonephric duct degenerates in the absence of androgens and can leave three remnants. These are the epoöphoron and paroöphoron in the ovarian mesentery, and Gartner's cysts, a scattering of small inclusion cysts located near the vagina. Where the two paramesonephric ducts touch at the posterior wall of the pelvic urethra, they fuse to form a short tube called the uterovaginal or genital canal. This gives rise to the uterus and upper portion of the vagina, and the unfused portions become the oviducts. The differentiation of these three structures is largely controlled by cues from underlying mesenchymal cells which trigger the expression of different HOX protein-controlled signalling pathways [53]. Gubernacula similar to those found in males form. The upper set attached to the ovaries and the lower extend down through rudimentary inguinal canals on either side of the vagina and attach to the labioscrotal folds. Unlike the male versions, these gubernacula do not shorten or thicken but attach to the paramesonephric ducts during the seventh week of gestation. As these are drawn together to form the uterus, the gubernacula pull the ovaries into their final position in the broad ligament of the uterus, a fold in the peritoneum. The gubernacula persist throughout development and adulthood, the upper pair becoming the round ligament of the ovary and the lower becoming the round ligament of the uterus [26].

EDC Effects on Reproductive Development

The reproductive tract, particularly that of the male, is the most studied developmental target of endocrine-disrupting chemicals (EDCs). Exposure to exogenous oestrogens, xeno-oestrogens or anti-androgens has been consistently shown to hinder testicular development via multiple pathways [54–57]. Similarly, development of the ovary is adversely affected by anti-oestrogens, xeno-oestrogens, over- or

underexposure to endogenous oestrogens and exposure to androgens [58–61]. EDCs can affect gonadal development in three ways: (1) direct alteration of gene expression by mimicking the action of an endogenous hormone, or interfering with its synthesis breakdown or transport; (2) changing the neuroendocrine mediation of the later stages of gonadal development; and (3) altering the epigenetic regulation of gene transcription [62]. Many EDCs interfere with endocrine-mediated signalling pathways other than those controlled by androgens and oestrogens, but little research on other types in the context of reproductive development has been carried out to date [18]. For this reason, this review is mostly restricted to compounds affecting the oestrogen receptors (ERs), androgen receptor (AR) or other components of the HPG system. Elucidating the effects of individual endocrine-disrupting chemicals on this process can be difficult, since they tend to be present in the environment together with many other EDCs, and many form numerous different metabolites which can have very different effects to their parent compounds. Methoxychlor, for example, is an organochlorine insecticide with a weak affinity for ER α and a slightly greater affinity for ER β [63]. Its major metabolites, however, include a compound which is an ER α agonist and an ER β and AR antagonist and shows oestrogenic activity which is approximately 100 times greater than that of the parent methoxychlor compound [64].

The effects of certain xeno-oestrogens on folliculogenesis are a good example of the consequences of the potential for direct actions of endocrine disruptors. In the normally developing ovary, inhibin and activin act as mutually antagonistic paracrine factors which regulate follicular development [65]. In mice, rats and baboons, oocytes and pre-granulosa cells express activin receptors abundantly whilst inhibin receptor expression is minimal. This is regulated by intraovarian oestrogen, and if insufficient oestrogen is present, activin receptor expression decreases whilst inhibin receptor expression increases and folliculogenesis is disrupted [66, 67]. An increased number of multi-oocyte follicles is an indication that this pathway has been perturbed [68]. Overexposure to oestrogens or exposure to synthetic oestrogens has been shown in a mouse model to impede follicular formation by disrupting this balance, inducing the production of multi-oocytic follicles, decreasing the number of small antral follicles present and decreasing activin synthesis [69]. These phenomena and reductions in follicular reserve have been found in rats injected with high doses (20 mg/kg/day) of bisphenol A during follicle formation [70] and in the ovaries of alligators from lakes heavily contaminated with EDCs [71], although there is no evidence that humans are similarly affected.

The chromophils-producing FSH and LH appear in the foetal adenohypophysis at around 22–28 weeks of gestation [72], enabling the development of the hypothalamus-pituitary-gonadal neuroendocrine axis. A functional foetal hypothalamus-pituitary-gonadal axis during the third trimester of gestation appears to be essential for development of the normally formed gonads. The gonads of male anencephalic foetuses are generally reported to be small, as are their penises, although both are normally formed [73]. Such females have normal numbers of oocytes but lack the small and growing antral follicles seen in healthy foetuses, and both genders have reduced populations of gonadal interstitial cells [73]. In order to correctly

regulate reproductive function and development, the HPG axis must adopt either a male or female phenotype post-pubertally. In rodent models, sexually dimorphic areas include the anteroventral periventricular nucleus (AVPV), the sexually dimorphic nucleus of the pre-optic area (SDN-POA), areas which are rich in the oestrogen receptors (ER α , ER β), androgen receptor (AR) and progesterone receptor (PGR) and are central to the regulation of the HPG axis. They are subject to extensive steroid-mediated sexually dimorphic prenatal programming which is exquisitely vulnerable to the actions of certain EDCs [74]. In the absence of testosterone, the HPG adopts a female phenotype. In adulthood, the pulsatile release of gonadotropin-releasing hormone (GnRH) varies over the course of the ovulatory cycle, stimulating the production of follicle-stimulating hormone (FSH) to drive follicle maturation and oestrogen production, and a surge of luteinizing hormone (LH), triggering ovulation. If testosterone is present, then a male phenotype is adopted and the GnRH pulses are released at a constant rate, stimulating FSH, LH and testosterone production and enabling constant spermatogenesis [75].

Sexual differentiation of the rodent brain, including the hypothalamic-pituitary unit, is determined around the time of birth. The male brain is masculinized primarily by the actions of oestrogen, although androgens also play a role, and exposure to both natural and man-made oestrogenic compounds can masculinize the female brain if they are present in sufficient quantities. In humans, however, masculinization is solely androgen mediated [76]. Therefore, only EDCs with anti-androgenic properties are expected to possibly impair human brain masculinization.

To date, most research in this arena has been done in rodents. In experimental studies, different classes of EDC affect sexually dimorphic brain areas such as the rodent AVPV and SDN-POA in different ways causing different effects. Some phyto-oestrogens and their metabolites, for example, act as weak oestrogen receptor agonists or antagonists [77], only targeting the oestrogen receptors, whilst the effects of PCBs are less predictable. Their modes of action are poorly understood, involving the perturbation of multiple hormonal targets including androgens and the thyroid hormones, and affecting the dopamine, serotonin and acetylcholine neurotransmitter systems as well as binding to oestrogen receptors [78]. Prenatal exposure to phyto-oestrogens and other oestrogenic and anti-androgenic EDCs such as bisphenol A and methoxychlor have been shown to decrease GnRH activation in adulthood [79, 80]. In rabbits, the anti-androgenic fungicide vinclozolin decreases the number of GnRH neurons in the mediobasal hypothalamus of both males and females but increases GnRH neuron number in the terminals in the region of the median eminence [81]. Since GnRH neurons do not express androgen receptors themselves, the mechanism by which vinclozolin causes this decline and the contribution, if any, that this makes to vinclozolin's effects on reproduction are still to be determined. Foetal vinclozolin exposure causes reproductive malformations identical to the androgen antagonist flutamide in rats [82]. What effects these chemicals may have, if any, on the sexual differentiation of the human brain remains to be elucidated.

Experimental models of the effects of foetal EDC exposure usually use high doses of single compounds rather than mixtures of low concentrations of these compounds, their metabolites and natural EDCs as usually occurs outside the laboratory.

Compounds have frequently been found to act together additively, even when compounds have been present at levels below those conventionally thought as toxic [83]. For example, foetal sheep from ewes grazed on pastures treated with sewage sludge, which contains a variety of EDCs, have been found to have significant disruption of the hypothalamic pituitary axis even although none of the EDCs present reach the animals in concentrations that are deemed hazardous by the ‘no observed effect levels’ reported by studies assessing their toxicity and endocrine-disrupting potential individually. Effects include reduced hypothalamic GnRH mRNA expression and reduced hypothalamic and pituitary GnRH receptor, galanin receptor and KISS1 expression [84]. Ovarian and testicular development [85, 86], maternal bone density [87] and the behaviour of adult offspring [88] have also been shown to be adversely affected by foetal exposure using this model.

As they migrate down the genital ridge and into the developing gonad, primordial germ cells undergo a unique process. Their genes are completely demethylated, erasing previous epigenetic programming [89]. During sexual differentiation, the genes are remethylated, receiving sex-specific programming appropriate for the type of gamete they will later become [90]. Altered DNA methylation within the somatic cells resulting from EDC exposures in previous generations is postulated as the means by which epigenetic changes are passed down the germ cell line [91]. Although true transgenerational effects resulting from exposure to EDCs have been documented in animal models, they have yet to be demonstrated in humans. This is unsurprising given the limited number of studies available, the length of human lifespan and reproductive cycles, their genetic and epigenetic heterogeneity, and the diverse chemical exposures to which they are subject.

Effects of Prenatal Endocrine-Disrupting Chemical Exposure on Puberty

In Western Europe and North America, puberty typically occurs between the ages of 12–13 and 17–18 in boys and 10–11 and 16–17 in girls. To give a means of objectively measuring the onset and progress of puberty, the external features of children and adolescents can be classified according to five stages defined by James Tanner in 1969 and henceforth known as Tanner stages [92]. In girls (Fig. 2.3) and boys (Fig. 2.4), stage 1 represents the prepubescent child. Individuals lack pubic hair; the breasts of females lack glandular tissue and have areolae that lie flush with the surrounding skin. Boys have testis with volumes of less than 1.5 ml and a penis of 3 cm or less. As puberty progresses in females, the breast buds and associated glandular tissue begin to form; the areolae enlarge slightly and a small amount of long, slightly pigmented but fine hair appears along the labia majora during stage 2. In males, the scrotum thins and reddens, the testicles begin to enlarge, reaching 1.6–6 ml and downy pubic hair begins to emerge around the base of the penis and scrotum. Stage 3 is defined by the beginning of penile growth in boys, as the penis elongates to around 6 cm. The scrotum enlarges as the testicles reach between 6 and 12 ml in

Fig. 2.3 Development of the breasts and pubic hair at the five Tanner stages

Stage 1



Stage 2



Stage 3



Stage 4



Stage 5



volume, and the breasts of girls begin to become elevated compared to the surrounding skin and begin to extend beyond the areola, which continue to enlarge. In both sexes, the pubic hair extends laterally and begins to darken, coarsen and curl. In stage 4, the pubic hair becomes fully adult in texture, covering the pubis but not the inner thigh. The quantity of pubic hair present and the rate at which it is acquired can vary between individuals. Testicular volume at this stage should lie between 12 and 20 ml, and the penis should be around 10 cm in length. In girls, the breasts continue to grow, and the areolae and papillae begin to stand proud of the breast, forming a secondary mound. By stage 5, this secondary mound disappears, and the areolae once again lie flush with the surrounding breast. The breasts reach their adult size. In boys, the penis reaches a flaccid length of around 15 cm, although this

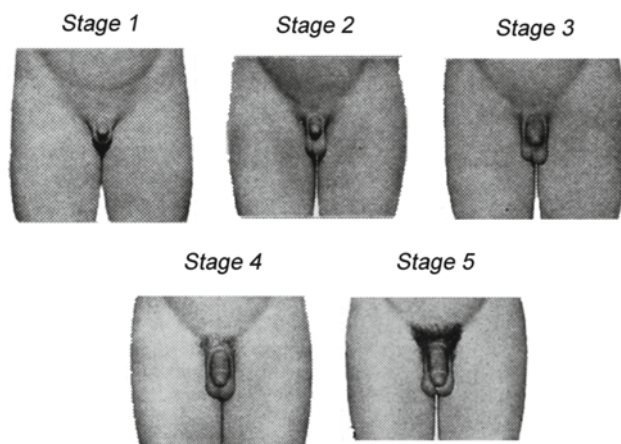


Fig. 2.4 Development of the penis, scrotum and pubic hair at the five Tanner stages

can differ widely between individuals and the testicular volume exceeds 20 ml. The pubic hair of both sexes attains its adult distribution, extending to the medial surface of the thighs and up the central line of the abdomen on many individuals.

Known effects of EDCs on the prenatal programming of the central neuroendocrine systems controlling puberty have recently been reviewed [93, 94]. Many of these effects involve changes in the central regulation of the HPG and hypothalamic-pituitary-thyroid (HPT) axes. Although the hypothalamic-pituitary-adrenal (HPA) axis is likely to be similarly vulnerable, it remains relatively unstudied [95], probably because its role and importance is radically different in primates and evolutionarily distinct animals such as rodents. Effects on puberty are gender specific, which at least partly reflects sex differences in the development of the brain regions involved in central control of the neuroendocrine axes, namely the hypothalamus and limbic systems. In crucial sexually dimorphic areas of the rodent brain such as the sexually dimorphic nucleus of the pre-optic area of the hypothalamus (SDN-POA), the mediobasal hypothalamus and the anteroventral periventricular nucleus (AVPV), exposure to EDCs can change both the number and phenotype of cells. Prenatal exposure to high doses of bisphenol A has been shown to benignly advance pubertal maturation of spermatogenesis slightly in male rats, probably by increasing FSH levels [96]. Similar exposure to the phyto-oestrogen genistein changes pubertal timing in female rats, with some studies showing an acceleration and others showing a delay [97, 98]. At puberty, male rats receiving the same compound have lower body weights, smaller testis and are less likely to achieve preputial separation than unexposed males. They also have lower circulating testosterone levels and impaired masculine sexual behaviour later in life [99, 100].

The peripheral control of puberty by endocrine organs and tissues can also be affected by exposure to EDCs in utero. The gonads are possibly the best-researched peripheral target of EDC action. As discussed above, exposure of the developing testes or ovaries to exogenous oestrogens and xeno-oestrogens, or the incorrect

levels of endogenous sex steroids, and exposure of the developing ovaries to anti-oestrogens has been consistently shown to hinder their development via multiple pathways [54–61]. Every step of thyroid hormone production and metabolism is sensitive to disruption by pollutants, and many have been shown to disrupt foetal thyroid development [101]. The mammary gland is also affected (see Chap. 7). Prenatal and perinatal exposure to environmentally relevant concentrations of bisphenol A disrupts mammary gland development and sensitizes it to the action of oestrogen later in life. Overall development in utero is hastened. Absolute ductal area and ductal extension are increased, as is epithelial proliferation and penetration into the fat pad [102, 103]. The sensitivity of the tissue to estradiol and its expression of progesterone receptors at puberty are increased dramatically. Fewer of the terminal end bud cells undergo apoptosis, which, together with the increased progesterone expression, may explain why bisphenol A-treated animals tend to have mammary glands that have more branched ducts and are more densely packed with alveolar buds later in life [104]. Other EDCs have been shown to hasten or delay mammary development in animal models [105–107], and there is concern that such prenatal changes may contribute towards early or late thelarche in humans [108–110], although there is little direct evidence for this.

Finally, since the initiation of puberty depends to a large extent on energy availability, its disruption by EDCs may at least partly be due to the alteration of neuroendocrine set points regulating homeostasis or by direct effects on adipocytes [97] (see Chap. 10). Adipogenesis and lipid accumulation have been demonstrated in vitro in response to EDCs such as organotins, 4-nonylphenol and bisphenol A [111, 112]. At a neuroendocrine level, EDCs can change the sensitivity of the brain to peripheral signalling agents such as leptin, adiponectin and IGF-1, changing the expression of hypothalamic factors involved in homeostasis such as Agouti-related protein (AgRP) and GnRH [18, 97, 113] (See Chap 10).

Tobacco and Prenatal Development

General Effects

Over 4,800 chemicals are found in tobacco smoke, including a wide range of reproductive toxicants such as carbon monoxide, nicotine, arsenic, numerous heavy metals and polycyclic aromatic hydrocarbons (PAHs), and many of the adverse affects of exposure to it are well documented [114–116]. One of the most marked differences between babies of smokers and non-smokers is size. The babies of smokers suffer restricted growth in the womb [117, 118], and this effect is more pronounced in males than in female fetuses [119]. Like other causes of intrauterine growth restriction, this causes changes in the neuroendocrine programming affecting growth and homeostasis, which although intended to be of benefit in a hostile, hungry environment, can lead to the development of illnesses later in life if the postnatal

environment does not match that anticipated by the prenatal conditions [120, 121]. Smoking-exposed babies tend to be smaller and thinner than average at birth, but display accelerated ‘catch up’ growth and greater adiposity during childhood, and have a higher incidence of obesity, cardiovascular disease and type 2 diabetes later in life [122–124], changes that are common to many babies whose foetal growth was subnormal irrespective of the primary cause. As will be explored in the remainder of this section, maternal smoking also has adverse effects on the development of the foetal reproductive tract and later fertility and fecundity, the neuroendocrine axes regulating growth and reproduction, and is thought to influence pubertal timing. In addition, many of the agents in tobacco smoke, including its principle addictive component nicotine, target the nervous and respiratory systems. Associations between maternal smoking and neurodevelopmental defects such as attention deficit disorder, impaired visual motor coordination, reduced IQ and autism are becoming well established [13, 125, 126]. Disorders of the respiratory tract and the regulation of breathing are more common in the infants and children of smokers. Maternal smoking and an infant age of <9 months are the only two consistent background characteristics of infants who succumb to sudden infant death syndrome (SIDS). Prenatal exposure impairs lung development, and the response of the respiratory system to hypoxia causes neurotransmitter abnormalities in multiple areas in the infant brainstem, midbrain and forebrain and can increase pro-inflammatory responses to later tobacco smoke in some infants, factors which are thought to contribute to the fourfold increase in the risk of SIDS in prenatally exposed babies [127]. It is interesting to note that lung development, just like Sertoli cell proliferation, is modulated by locally produced androgens and therefore could be vulnerable to the effects of anti-androgens [128]. Wheezing and asthma are also more prevalent in the children of smokers [129–131]. A fuller review of the medical conditions prenatal tobacco exposure contributes to is available in the recent literature [114].

The Reproductive Tract

Adverse effects of maternal smoking on gonadogenesis and later fertility and fecundity have been observed in both genders. In females, reductions are seen in ovarian follicle numbers [132] and the uteri of exposed girls tend to be smaller [133]. Fertility and fecundity in adulthood is reduced, particularly if the women exposed in utero themselves smoke [16, 134–136]. There is also evidence that they are less likely to carry to term babies conceived using assisted reproductive technologies [137] and that they go through the menopause earlier than the daughters of non-smokers [138]. A large part of the adverse effects on female fertility and fecundity are thought to arise from depletion of the foetal ovarian reserve. A decrease of 41% in germ cell numbers and a 29% reduction in somatic cell numbers in females exposed during the first trimester versus controls was found in a recent study of human foetuses [139]. By suppressing the action of aromatase, nicotine and cotinine inhibit oestrogen-mediated oocyte development, reducing the number of

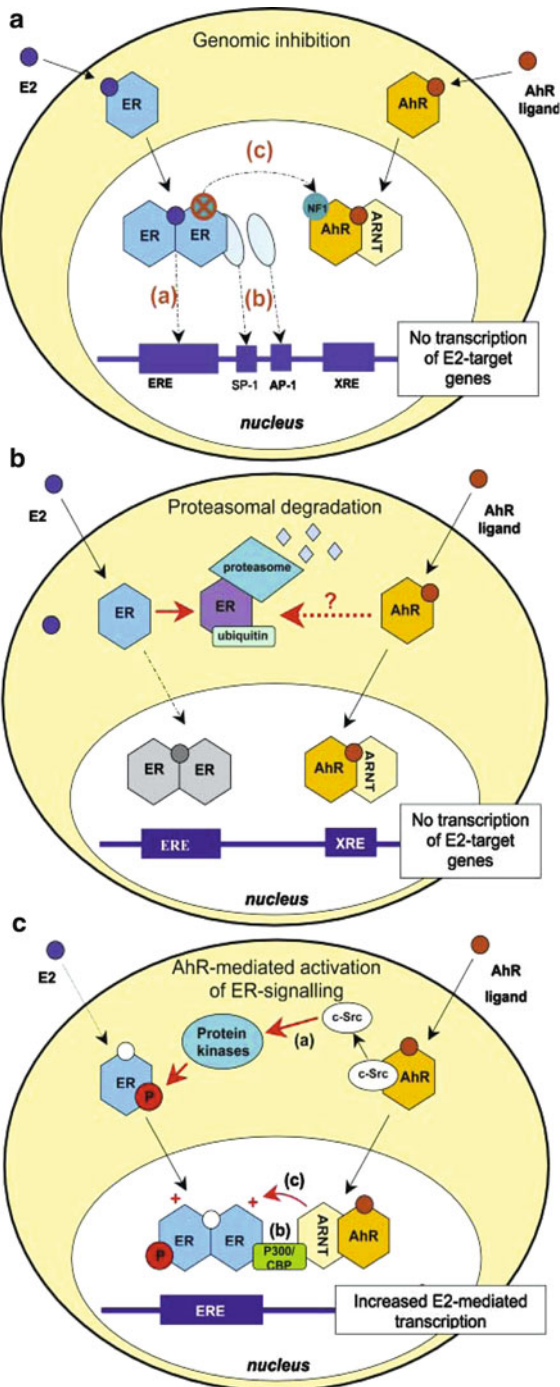


Fig. 2.5 (a) Mechanisms of genomic inhibition of steroid-mediated transcription by ligand-activated AhR. Transcriptional inhibition depends on iXREs within promoter regions of oestrogen-regulated

oocytes reaching metaphase II, and may reduce oocyte fertilization rates in adulthood [140]. The aryl hydrocarbon receptor (AhR) plays a key role in the death of oocytes during ovarian development [141]. The interaction of polycyclic aromatic hydrocarbons (PAHs) in tobacco smoke with the AhR is thought to be at least partly responsible for the reduced oocyte numbers and viability observed in the daughters of smokers, as emphasized by a recent study of differentiated human embryonic stem cells as an analogue of germ cells [142]. By increasing oocyte expression of Bax, a pro-apoptotic cell death regulator from the Bcl-2 family, PAHs can induce oocyte apoptosis by inducing expression of the Bcl-2 protein Harakiri [143]. In addition, nicotine, but not cotinine, has a direct toxic effect on oocytes [144] and inhibits meiotic maturation in foetal oocytes [145, 146].

In addition to depleting the reserve of oocytes formed prenatally, maternal smoking may change hormone production and responsiveness later in life, increasing the incidences of certain reproductive disorders. Prenatal nicotine exposure increases plasma testosterone in female adolescent mice and sheep [147]. In humans, increased serum testosterone levels have been reported in women who smoke during pregnancy and then later in their adult daughters [148]. Elevated serum testosterone levels are associated with polycystic ovary syndrome (PCOS), a reproductive and metabolic disorder thought to arise due to disturbances in GnRH secretion and that may be caused at least in part by prenatal exposure to androgens [149–152]. Any link existing between maternal smoking and PCOS, however, remains to be confirmed.

Steroid hormone receptors are highly vulnerable to disruption by PAHs since, by binding to the AhR receptor, PAHs can inhibit or increase oestrogen receptor-mediated transcription, as shown in Fig. 2.5. In addition to the effects on steroid hormone synthesis, the action of PAHs on the AhR in reproductive organs other than the ovary may be responsible for some of the abnormalities affecting the foetuses of smokers. The AhR is expressed abundantly throughout the oviduct, uterus and vagina. Studies of AhR-knockout (AhRKO) mice and the effects of the toxic PAH TCDD have demonstrated that the AhR plays numerous roles contributing to the successful initiation and maintenance of pregnancy and that these can be compromised by exogenous ligands and can be found reviewed in depth by [153]. Mice exposed in utero to the PAHs in tobacco smoke have severely reduced ovarian

Fig. 2.5 (continued) genes which prevent ER transcriptional activity by (a) being located close by to neighbouring EREs, (b) disrupting the formation of ER–SP-1 or ER–AP-1 DNA complexes and (c) competing for limited cofactors (e.g. NF-1). These mechanisms apply to interactions of the AhR with all sex steroid hormone receptors; interactions are shown here for the oestrogen receptor (ER). **(b)** Activation of AhR by exogenous ligands can rapidly reduce the levels of ER protein by proteasomal degradation. The exact mechanism by which liganded AhR initiates that degradation is presently unknown. **(c)** AhR-mediated mechanisms leading to the activation of sex steroid hormone signalling in the absence of oestrogen. (a) Upon binding of the ligand, a conformational change causes the release of active c-Src from the cytoplasmic AhR–protein complex. c-Src activates several protein kinases leading to phosphorylation and activation of ER. (b) A direct association of AhR–ARNT complexes with nuclear ER and the cofactor p300/CBP in the absence of E2. (c) Activation of steroid hormone receptor by direct protein interaction with the AhR–ARNT complex in the absence of hormone has so far only been observed for the ER [160] ©Society for Reproduction and Fertility 2005, reproduced by permission

reserves due to the activation of the AhR-mediated initiation of the transcription of *Harakiri*, one of the genes which regulate apoptosis [154].

Heavy metal exposure via maternal smoking is also likely to affect gonadogenesis in both genders. In an experimental mouse model, cadmium reduces primordial germ cell numbers and motility, leading to a smaller genital ridge size. Primordial germ cell degeneration is increased, and gonad size is reduced. In adulthood, males but not females exposed in utero showed impaired fertility, and their sperm had less capacity to fertilize eggs in vitro [155]. Arsenic and lead suppress estradiol and progesterone production in adult mice exposed in utero [156], and irregularities in the oestrus cycle and corpus luteum failure are both seen in the lead-exposed animals [157, 158]. In males, lead and arsenic exposure in adulthood damage spermatocyte DNA, reducing sperm viability [159].

The male reproductive tract is very sensitive to the effects of maternal smoking. A recent study of human foetuses exposed during the first trimester showed that germ cell numbers are reduced by 55% [138]. Men exposed in utero have smaller testes, their sperm count is reduced by up to ~40% and they are less likely to father children [134, 160, 161]. As babies, they have increased incidences of the reproductive tract defects associated with testicular dysgenesis syndrome (TDS) such as undescended testicles, cryptorchidism [141, 162, 163] and hypospadias [164]. The risk of cryptorchidism may also be increased by the use of nicotine patches as a tobacco replacement [165] although not all data are supportive [166, 167].

Foetal development of Sertoli and Leydig cells is a key process which is both vulnerable to disruption by EDCs and strongly associated with TDS [40, 48]. Decreased Leydig cell function has been reported in adult smokers and animal models [168, 169]. Whether the number of Sertoli or Leydig cells present is reduced by maternal smoking is unknown, but a 37% decrease in the number of somatic cells present in the testis of human fetuses exposed in utero was found in a recent study [139], and the fall in sperm counts in adult men whose mothers smoke is thought to be explained by reduced Sertoli cell number [170]. There is also compelling evidence of developmental impairment at the molecular level. The morphogen desert hedgehog (*Dhh*) is expressed specifically in the foetal Sertoli cells [171, 172] and is essential for Leydig cell development and subsequent normal development of the male reproductive tract [173]. Maternal smoking halves *Dhh* expression during the second trimester, which might be expected to affect foetal Leydig cell development [174].

The Neuroendocrine Axes

Cigarette smoking affects the neuroendocrine control of peripheral endocrine tissues in both the mother and foetus and alters the programming of set points in the foetal neuroendocrine axis that guide later reproductive development and function. The hypothalamic-pituitary-adrenal axis affects reproductive development because its activation suppresses that of the hypothalamic-pituitary-gonadal axis [175, 176]. Nicotine strongly activates the HPA axis [177] and has been shown to inhibit normal

testosterone synthesis in foetal and neonatal rats, an effect which can be reversed by treatment with the corticosterone-blocking drug metyrapone [178]. Intrauterine hypoxia has also been shown in animal models to activate the HPA axis and make it hyperactive later in life [179]. In the foetuses of smokers, carbon monoxide levels are higher than in the mother [180]. Carboxyhaemoglobin, which is formed by the binding of carbon monoxide to haemoglobin, is removed slightly less efficiently from the foetal blood stream than from the adult, [181] reducing the foetus's ability to transport oxygen more than the mothers. Chronic exposure to nicotine contributes further to the hypoxic intrauterine environment by causing the arteries supplying blood to the uterus to contract [182, 183], both via its direct action on the arterial walls and by activating the maternal HPA axis. In exposed newborn human infants, adrenocorticotrophic hormone (ACTH) levels have been found to be significantly elevated without a concurrent rise in cortisol, indicating that sensitivity to or production of ACTH is changed [184].

In rodent models, maternal smoking affects the HPG axis in a sex-dependent manner, demasculinizing the sexually dimorphic areas of the brain involved in its control in males and masculinizing them in females. Nicotine and cotinine exert a demasculinizing effect on these areas of the male brain without impacting upon the sexual dimorphism of the female brain by inhibiting aromatase activity, with cotinine being approximately twice as potent as nicotine [182, 185]. In females, testosterone synthesis is increased both pre- and postnatally [147, 185], which has the potential in rodents to masculinize their GnRH production and SPN-POA and AVPV volumes [60, 186]. In female animals experimentally exposed to androgens in utero, oocyte numbers are reduced, and oestrus cycles are impaired in adulthood. This occurs via increased GnRH neuron activity, inhibiting the sensitivity of the HPG axis to the progesterone-mediated negative feedback necessary to promote follicle-stimulating hormone (FSH) synthesis and ovulation [187, 188].

AhR ligands can have a lasting effect on the sexually dimorphic neuroendocrine programming of the HPG axis. The distribution of AhR gene expression in the brain follows the expression of the genes encoding for glutamic acid decarboxylase 65 and 67 [189, 190], which are essential for GABA synthesis. GABAergic neurons in the AVPV and mediobasal hypothalamus and the AhR and ER expressing GABA/glutamatergic neurons unique to the AVPV [191] are essential for transmission, amongst other things, of the estradiol-mediated signals needed to trigger the GnRH-stimulated LH surge in the female [192]. The gender-specific apoptosis of these neurons in the AVPV, mediobasal hypothalamus and SDN-POA, which allows their sexual differentiation, is mediated by the AhR via Bax. Exposure to AhR ligands, such as 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), during periods of gender-specific brain differentiation in rodents produces lifelong changes in sex-specific behaviour and reproductive competence [193]. Male rats exposed to TCDD have demasculinized patterns of GnRH release and sexual behaviour [193, 194]. In females, the SDN-POA is masculinized and masculine behaviour results [195].

The effect of nicotine on maternal sex hormone production and metabolism may have a significant effect on the reproductive development of both genders. In addition to suppressing oestrogen production at all levels via the activation of the HPA

axis, nicotine and cotinine actively inhibit their production by ovarian granulosa cells and have a synergistic effect when present together [196]. In female foetuses, reduced levels of the maternal sex steroids cause premature nest breakdown and a reduction in oocyte number, although follicle maturation can still occur [197, 198]. In adult female rats prenatally exposed to nicotine, steroidogenesis is altered (decreased oestrogen, increased progesterone), and the time to pregnancy is increased [199]. In the human, maternal chorionic gonadotropin (hCG) production is lowered, which, in turn, may affect LH and subsequent testosterone production in male foetuses. The ratio of the active isoform of the LH receptor is increased relative to the inactive form in exposed foetuses, which may be part of an adaptive strategy [33].

Exposure to the heavy metals present in tobacco, such as cadmium and lead, can alter maternal HPG function. Gonadal and placental oestrogen and progesterone secretion are suppressed by cadmium [200–203]. Hypothalamic serotonin, dopamine and noradrenaline are reduced by both cadmium and lead, diminishing gonadotropin secretion [204].

Some of the adverse effects of maternal smoking on reproductive development may be mediated by the hypothalamic-pituitary-thyroid (HPT) axis. Its function and the conversion of T_4 to T_3 in peripheral tissues are suppressed by activation of the HPA axis [205–207]. As with the HPA and HPG axes, the set points controlling the sensitivity of the HPT axis are organized before birth and are sensitive to change by exogenous pollutants [208]. During adolescence, HPT underactivity is usually associated with delayed puberty [209] and overactivity with precocious puberty [210]. Cigarette smoke contains numerous chemicals capable of interfering with thyroid activity, although the effects of some may be moderated by iodine intake and existing thyroid status [211]. Smoke may exhibit a weakly pro-thyroid action in subjects with normally functioning thyroid glands [212] and an anti-thyroid action in those with existing hypothyroidism [213]. Thiocyanate was one of the first thyroid-disrupting components of tobacco smoke identified. It competitively inhibits iodine uptake [214], inhibits its organification and reduces the synthesis of thyroid hormones [215]. Benzopyrene and other PAHs in tobacco have been found to affect the action and excretion of thyroid hormones in a number of different ways. Some interact directly with T_3 receptors, acting as agonists [216], others can act as antagonist by dissociating the thyroid hormone response element from the thyroid receptor [217]. T_4 levels can be reduced by the induction of glucuronyl transferase activity, increasing the formation and subsequent excretion of T_4 glucuronides [218]. The structure of the thyroid-stimulating hormone receptor may be altered by maternal smoking in such a way that it makes the thyroid more immunogenic and therefore increases susceptibility to autoimmune thyroid diseases [219]. In addition, maternal smoking reduces TSH levels, increases thyroglobulin levels and decreases the effect of thyroid hormones on peripheral tissues [219]. Thyroid volume is also increased in pregnant smokers [220] even though TSH production is reduced [221], while neonates have elevated thyroglobulin levels which persist into infancy [222, 223].

Puberty

There are few studies of the effects of maternal smoking on the timing and progression of human puberty, and the results are scant and conflicting. Two small early studies in Poland found that the daughters of smokers tended to have earlier menarche than those from non-smoking families, that maternal smoking was a greater factor than paternal smoking and that girls from smoking families were also at greater risk of precocious puberty [224]. One later large study linked heavy maternal smoking during pregnancy with a higher ponderal index and earlier voice change and initiation of shaving in adolescent boys after adjustment for other factors. The ponderal index is calculated by dividing an individual's body weight, in kilograms, by their height in metres cubed. It gives a measure of body composition, with larger values indicating greater fatness. No association was found between smoking and the onset of menarche or self-reported breast development in girls, but the likelihood of late menarche was reduced [225]. Two further large longitudinal studies have found conflicting associations between the onset of menarche and maternal smoking or smoke exposure. The first involved 994 female adolescents and amongst those whose mothers were exposed to high levels of ETS during pregnancy, the adjusted mean age was 4 months earlier than in unexposed girls. Those whose mothers smoked over 20 cigarettes a day and who were also heavily exposed to ETS were half as likely to start menarche late but showed no reduction in age of menarche after adjustment for other factors known to affect menarcheal age such as body weight [226]. The second enrolled a cohort of 1556 girls and found a mean delay of 4 months amongst the daughters of heavy smokers after adjustment [227]. Results of a smaller longitudinal study of 262 daughters from a US cohort were published recently. It was found that girls exposed to tobacco prenatally, to environmental tobacco smoke (ETS) as children or both were more likely to start menarche later than the median age of 12 [228].

Puberty in humans can be hard to measure experimentally, especially in males since markers that can be used to gauge the onset of puberty in females, such as breast growth and menarche, are more visible than testicular growth. Therefore, more data are available for girls than boys [94]. Different events during puberty are controlled by different processes – development of the reproductive tract, for example, is regulated by the HPG axis in females, whilst pubic hair development is stimulated by adrenal androgens [229, 230]. Markers used to measure pubertal processes must therefore be chosen carefully since, for instance, the appearance of pubic hair reveals little about the maturation or functional status of the ovaries. Environmental insults affecting puberty, particularly those, such as maternal smoking, which result in exposure to many different compounds with different modes of action, are therefore likely to have multiple measurable end points. Studies using markers that cover a very limited range of end points, such as age of menarche, or markers which result from numerous physiological processes, such as the age of voice change in boys, could easily miss effects on the process of puberty as a whole. The combinations of varying environmental chemical exposures and the genetic variations between

individuals in a population make it difficult to distinguish the effects of individual chemicals [231] especially since phenotypic variation often increases with exposure to chemicals and other environmental insults [232].

Maternal Smoking and Reproductive Development: Lessons

It is hard to imagine a more noxious combination of prenatal chemical insults than those delivered by maternal cigarette smoking. In addition to the direct toxicity of nicotine, heavy metals and tobacco combustion products that cross the placenta, and the perturbation of vital maternal endocrine signals, the foetus is subject to an environment that is oxygen deprived. It is no surprise that women who smoke suffer more adverse pregnancy outcomes and reduced fecundity compared to those who do not and that their children are at greater risk of numerous developmental effects [114]. The adverse effects resulting from prenatal exposure to environmental tobacco smoke demonstrate that the chemical mixture plays a significant role in this, independently of hypoxia [233]. Unlike studies involving laboratory animals or wildlife-based observations, human studies of the effects of prenatal tobacco exposure give indications of how a mixture of EDCs and other reproductive toxicants affects human physiology. It affects many of the same targets, such as the AhR, as do other environmental EDCs and EDC mixtures. Measuring the effects of EDCs in a human population, however, can be very challenging. As with any outbred animal population, there is considerable genetic variation amongst humans that can affect how they respond to these toxins. Many of the processes affected, such as puberty, are very complex involving multiple tissues and signalling pathways that are vulnerable to perturbation by EDCs and other toxicants. Identifying measurable end points that are reliable indicators of the effects of prenatal tobacco exposure on these processes is essential, but can be very difficult, especially in human studies. Multiple processes can affect individual end points, and the use of some end points is limited by expense, time and ethical considerations. This is especially true for those requiring repeated measurements, such as testicular growth, or which can only be measured using invasive procedures and/or tissue samples, such as amniocentesis or the collection of human foetal tissues from aborted or miscarried foetuses. Further longitudinal studies of environmental factors affecting prenatal, postnatal infant, child and adolescent sexual development that measure multiple developmental end points are certainly required.

Many of the reproductive end points identified in studies of prenatal tobacco exposure have been observed to result from prenatal exposure to other EDCs, some of which have been studied extensively. These include oocyte and male gonocyte loss, reproductive tract malformations and reduced fertility and fecundity in adulthood. For these disorders, a number of common aetiologies have been identified. Loss of germ cells, for example, can occur due to direct toxicity to the developing cells, the activation of AhR-mediated apoptosis or the alteration of foetal or maternal hormone levels. The activation of the maternal and foetal HPA axis and subsequent

HPG and HPT axis suppression, however, has not been studied to any great extent in any EDC exposure models. Maternal and foetal HPA axis activation and the subsequent programming of the foetal HPA are implicated extensively as a factor in adverse birth outcomes such as intrauterine growth retardation (IUGR), and developmental and neurodevelopmental disorders such as diabetes and mental illnesses which manifest later in life [234–236]. The HPA axis is susceptible to many toxins, including EDCs, but little is known about the extent to which these affect humans [18, 236]. At least two components present in tobacco smoke, carbon monoxide and nicotine, strongly activate the HPA axis and other toxins present such as cadmium are highly toxic to the adrenal gland [237]. One key lesson which must be learned from the effects of prenatal tobacco exposure is that perturbations of the foetal and maternal HPA axis function and foetal HPA axis development can occur as the result of exposure to environmental toxins and play an important role in the effects of the exposure on development. Further research into the effects of EDCs on adrenal gland and HPA axis is therefore vital.

Quantification of the roles epigenetic changes play in the effects of maternal smoking is still in its early stages. Two studies of children have found changes both in global and gene-specific methylation induced by maternal smoking. The first found an overall increase in DNA methylation in mononuclear cells isolated from blood [238]. The second, which used buccal cells and a more sensitive technique, found a global decrease which was linked to the maternal genotype of GSTM1, a gene involved in the metabolism of tobacco smoke [239]. What these changes mean in terms of reproductive development is yet to be elucidated, but a recent study found changes in the methylation of brain-derived neurotrophic factor-6, which plays a role in neuron and axon survival and is perturbed in neurodegenerative disease [240]. So far, transgenerational effects on human reproduction arising as a consequence of grandparental smoking have not been documented. However, there is evidence that other adverse effects are transmissible for more than one generation. Grandmaternal smoking is associated with asthma in humans [241], and elevated insulin levels have been found in the F2 generation of offspring from nicotine-exposed rats [242]. Transgenerational reproductive effects should be considered as a possible outcome in future studies of maternal smoking and environmental tobacco smoke exposure and could have much to teach us.

Like virtually all exposures to EDCs and other toxins that occur outside laboratory studies, prenatal tobacco exposure via maternal smoking involves a complex mixture of compounds, many of which have not yet had their toxicities or effects on human reproduction quantified. Some of these compounds or compound classes, such as benzopyrene and other PAHs, can reach humans via a variety of other sources, and the study of the effects of maternal smoking can be used to inform investigations of the consequences of exposure from them, and vice versa. Others such as nicotine are unlikely to reach humans via other pathways, but knowledge of their effects on development at the molecular level can still give insight into the effects of other chemicals which target the same processes as nicotine within the body. Studies of the interaction between maternal smoking, genetic factors and

developmental outcomes in their children can pinpoint genetic polymorphisms vulnerable to environmental perturbations. So far, little work has been done looking at how these affect foetal reproductive development, but gene polymorphisms have been identified which are associated with reduced infant birth weights, orofacial clefts and neurodevelopmental abnormalities in the children of smoking mothers [243–245]. Being exposed to tobacco combustion products in utero, the children of smokers are subject to a genetic roulette wheel of developmental risk. The consequences may not be apparent immediately at birth but can manifest later in life, and future generations may be at risk. It is therefore imperative that anti-smoking measures continue to be implemented, that prospective parents be strongly encouraged to give up smoking and supported in their efforts to do so.

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