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Steroidogenesis in the Fetal Testis and Its Susceptibility to Disruption by Exogenous Compounds

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Masculinization depends on adequate production of testosterone by the fetal testis within a specific "masculinization programming window." Disorders resulting from subtle deficiencies in this process are common in humans, and environmental exposures/lifestyle could contribute causally because common therapeutic and environmental compounds can affect steroidogenesis. This evidence derives mainly from rodent studies, but because there are major species differences in regulation of steroidogenesis in the fetal testis, this may not always be a guide to potential effects in the human. In addition to direct study of the effects of compounds on steroidogenesis, information also derives from study of masculinization disorders that result from mutations in genes in pathways regulating steroidogenesis. This review addresses this issue by critically reviewing the comparative timing of production and regulation of steroidogenesis in the fetal testis of humans and of rodents and its susceptibility to disruption; where there is limited information for the fetus, evidence from effects on steroidogenesis in the adult testis is considered. There are a number of fundamental regulatory differences between the human and rodent fetal testis, most notably in the importance of paracrine *vs.* endocrine drives during masculinization such that inactivating LH receptor mutations block masculinization in humans but not in rodents. Other large differences involve the steroidogenic response to estrogens and GnRH analogs and possibly phthalates, whereas for other compounds there may be differences in sensitivity to disruption (ketoconazole). This comparison identifies steroidogenic targets that are either vulnerable (mitochondrial cholesterol transport, CYP11A, CYP17) or not (cholesterol uptake) to chemical interference. **(*Endocrine Reviews* 30: 0000–0000, 2009)**

Abbreviations: AF, Amniotic fluid; AGD, anogenital distance; AR, androgen receptor; CAIS, complete androgen insensitivity syndrome; CG, chorionic gonadotropin; DBP, dibutyl phthalate; DEHP, diethyl hexyl phthalate; DES, diethylstilbestrol; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; e, embryonic day/gestational day; ER, estrogen receptor; GPR54, G protein-coupled receptor 54; hCG, human CG; HDL, high-density lipoprotein; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HSD, hydroxysteroid dehydrogenase; ITT, intratesticular testosterone; LDL, low-density lipoprotein; LHCGR, LH/CG receptor; MBP, monobutyl phthalate; MEHP, monoethyl hexyl phthalate; MEP, monoethyl phthalate; NADPH, nicotinamide adenine dinucleotide phosphate (reduced); POR, P450 oxidoreductase; PORD, POR disorder; PPAR, peroxisome proliferator-activated receptor; PVC, polyvinyl chloride; SF1, steroidogenic factor-1; SRB1, scavenger receptor class B, member 1 (also known as SCARB1); StAR, steroidogenic acute regulatory protein; TDS, testicular dysgenesis syndrome; TSPO, translocator protein (also known as PBR).

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I. Introduction and Background

Steroid hormones produced by the adrenals, gonads, and (in pregnancy) placenta play vital roles in day-to-day physiology and reproduction. Arguably, the most profound of all the numerous effects of steroid hormones is the role of androgens in "making a male" during fetal development, because this literally switches that individual from developing as a phenotypic female into developing as a phenotypic male. This is illustrated dramatically in the case of complete androgen insensitivity syndrome (CAIS), when the absence of a functional androgen receptor (AR) means that the fetus will develop into a phenotypic female irrespective of the otherwise normal formation and hormonal function of testes (1). CAIS is rare, as is partial androgen insensitivity syndrome, and both most commonly result from mutations in the AR (1). However, of much greater concern from a numbers perspective are disorders that may occur when the masculinization process fails in more subtle ways, such that the individual appears phenotypically male but may have a disorder, such as hypospadias or cryptorchidism. Such disorders can result from deficient androgen production or action (2), and they are among the commonest congenital disorders in children; the incidence of cryptorchidism and hypospadias at birth ranges from 2–9% (3, 4) and 0.2–0.7% (5) in various countries.

It has been hypothesized that cryptorchidism and hypospadias may represent part of a testicular dysgenesis syndrome (TDS), which also comprises testicular germ cell cancer and some cases of low sperm counts (6) and perhaps other adult-onset disorders such as low testosterone levels (7). This is based on the disorders being risk factors for each other and having shared risk factors, one of which is deficiencies in fetal androgen production or action (6, 7). The latter thus throws the spotlight onto fetal testicular steroidogenesis, especially because recent studies in the rat have established that inhibition of androgen production or action within a discrete fetal time frame will increase the risk of cryptorchidism, hypospadias, and smaller testis size (equates to reduced sperm production) and penis size at puberty or in adulthood (8–10).

TDS disorders may be increasing in incidence. This is certain for testicular germ cell cancer over the past approximately 60 yr (11), and there is some supporting evidence, although more contentious, for the other TDS disorders (6, 12). It is reasoned that any recent increase in TDS disorders is most likely to have environmental and/or lifestyle causes that are, by definition, preventable (7); however, TDS disorders may also arise due to genetic mutations or influences (6). In this context, it has become clear that humans are exposed to a number of widespread environmental chemicals that have the capacity to inhibit testosterone production by the fetal testis in some species (*e.g.*, certain phthalates, certain pesticides). Additionally, a range of widely used therapeutic drugs (*e.g.*, steroid hormones, statins, glitazones) have similar potential. Although these drugs are not usually prescribed to pregnant women, it is possible that a woman taking such drugs may become pregnant and, if unaware of the pregnancy or the risks, continue her medication. In these cases, fetal exposure to such compounds (via the mother) could theoretically inhibit testosterone production by the fetal testis and thus increase risk of downstream TDS disorders. A major obstacle to assessing the risk that such exposures may pose to the developing human male fetus is a fundamental lack of understanding about the regulatory mechanisms for fetal testicular steroidogenesis, their susceptibility to disruption, and their variation between species. The latter is an important issue because, for practical reasons, direct study of steroidogenesis in the human fetal testis is limited, and animal models will therefore be widely used.

These considerations prompted this review, which thus aimed to compare regulation and disruption of steroidogenesis in the human fetal testis with that in rodents (for which most information is available) to establish comparability and/or key differences. The main objective was to provide a guide as to when rodents may, or may not, be good models for the study of fetal testis dysfunction related to masculinization/reproductive development. This should also assist in studies directed at understanding the origins, and possibly the causes, of human TDS disorders.

II. Scope of This Review

To provide appropriate perspective, the basic cascade of steps in steroidogenesis and their regulation are detailed first, and these form a backdrop to the remainder of the review that considers their susceptibility to perturbation. For the latter, we have chosen therapeutic and environmental compounds to which humans are likely to be exposed and/or for which there is evidence from animal studies that they can disrupt steroidogenesis. In many instances, there is a lack of detailed information available for the fetal testis, and in such instances, use is made of any information that is available for effects on the adult testis; the latter is also described where there is evidence for con-

trasting effects on, or regulation of, testicular steroidogenesis between fetal life and adulthood. Considerable space is devoted to comparison of differences in the basic drive to the fetal testis to stimulate testosterone production because there are fundamental species differences that may have implications regarding relative susceptibility to disruption; comparative analysis of humans and mice deficient in component parts of the hypothalamic-pituitary-testis axis is particularly informative in this regard. Finally, because direct information on modulation of testosterone production in the human fetal testis is extremely limited, wide use is made of fetal masculinization disorders as an index of altered testicular steroidogenesis. This has necessitated provision of background information on how and when such disorders can arise and, in particular, how they may reflect altered androgen action at different fetal ages.

III. The Steroidogenic Cascade and Its Regulation

A. Preferred sources and mobilization of cholesterol

Cholesterol is the precursor of the steroid hormones (13), providing the backbone of the steroid molecule. The biosynthesis of testosterone directly from cholesterol can only occur in the Leydig cells (14) because the adrenal glands are usually only capable of synthesizing the testosterone precursors, dehydroepiandrosterone (DHEA) (sulfate) and androstenedione. Estrogen synthesis in the human fetal placental unit requires fetal adrenal-derived DHEA (sulfate) to be converted to androstenedione [placental steroid sulfatase and type 3β-hydroxysteroid dehydrogenase (3β-HSD) actions], followed by placental aromatase action to produce estrone. Estradiol dehydrogenase [type 1 17β-hydroxysteroid dehydrogenase (HSD17B1)] is the principal reductive 17β-HSD in the placenta and is most efficient at converting estrone to estradiol. Only very limited amounts of testosterone must escape further metabolism (15). Cholesterol can be obtained from within the cell membrane, synthesized *de novo* from acetate, or imported from the circulation in the form of high-density lipoprotein (HDL) or low-density lipoprotein (LDL). HDL, the principal form of circulating cholesterol in rodents, is imported via scavenger receptor class B type 1 (SRB1), which is a cell surface HDL receptor that is expressed in the fetal mouse testis throughout the key period of testosterone production (16, 17). In the human, LDL cholesterol is considered the principal circulating form of cholesterol used by steroidogenic tissues being taken up into these cells via the LDL receptor. The human fetal testis certainly expresses functional LDL receptors in samples obtained from gestational wk 10 onward, similar to the human fetal adrenal (18). Cholesterol can also be obtained from the conversion of intracellular C2-acetyl units via the activities of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase and HMG-CoA reductase (19). Testes of 10- to 20-wk fetuses also have the ability to utilize *de novo* synthesized cholesterol for testosterone formation (18); however, similar information on testes before 10 wk is lacking. It is notable that the transcription factor, steroidogenic factor-1 (SF1, NR5A1), a member of the orphan nuclear receptor superfamily that regulates expression of downstream steroidogenic enzymes (see *Section III. E*), also regulates expression of SRB1 (16) and HMG-CoA synthase and HMG-CoA reductase (20).

Because the fetal Leydig cell has various possible routes for obtaining cholesterol, it is perhaps not surprising that blockade/inactivation of any one pathway tends to be without major effect. For example, knockout of SRB1 in mice has no apparent effect on masculinization or male fertility (21). This probably also explains in part why lowering of circulating cholesterol via the therapeutic use of statins has no apparent effect on masculinization (see below). Normal Leydig cells from different species contain vastly different numbers of lipid droplets, with rat Leydig cells containing the fewest. However, because all species are equally efficient at secreting testosterone, this is suggestive that Leydig cells must utilize a number of mechanisms to meet the demand for cholesterol, for example, *de novo* synthesis and possibly immediate utilization of circulating lipoprotein-borne cholesterol (22).

B. Cholesterol transport within the Leydig cells

The first step of steroidogenesis requires the transport of free cholesterol from the outer to the inner mitochondrial membrane (23). This is where the first "steroidogenic" enzyme reaction occurs, catalyzed by the CYP11A enzyme (Fig. 1), which is located on the matrix side of the inner mitochondrial membrane (24). Although the outer mitochondrial membrane itself is relatively cholesterol-rich and does not provide a barrier to cholesterol, the space between the outer and inner mitochondrial membranes is filled with an aqueous fluid that only permits the free passage of water-soluble molecules, therefore preventing the passage of lipophilic cholesterol (25). Consequently, the necessary translocation of cholesterol to the inner mitochondrial membrane is facilitated by steroidogenic acute regulatory protein (StAR).

StAR is a short-lived molecule, rapidly synthesized in response to tropic hormones, that actively transports cholesterol from the outer to the inner mitochondrial membrane (26) and allows CYP11A, which is located in the inner membrane, access to cholesterol and as such regu-

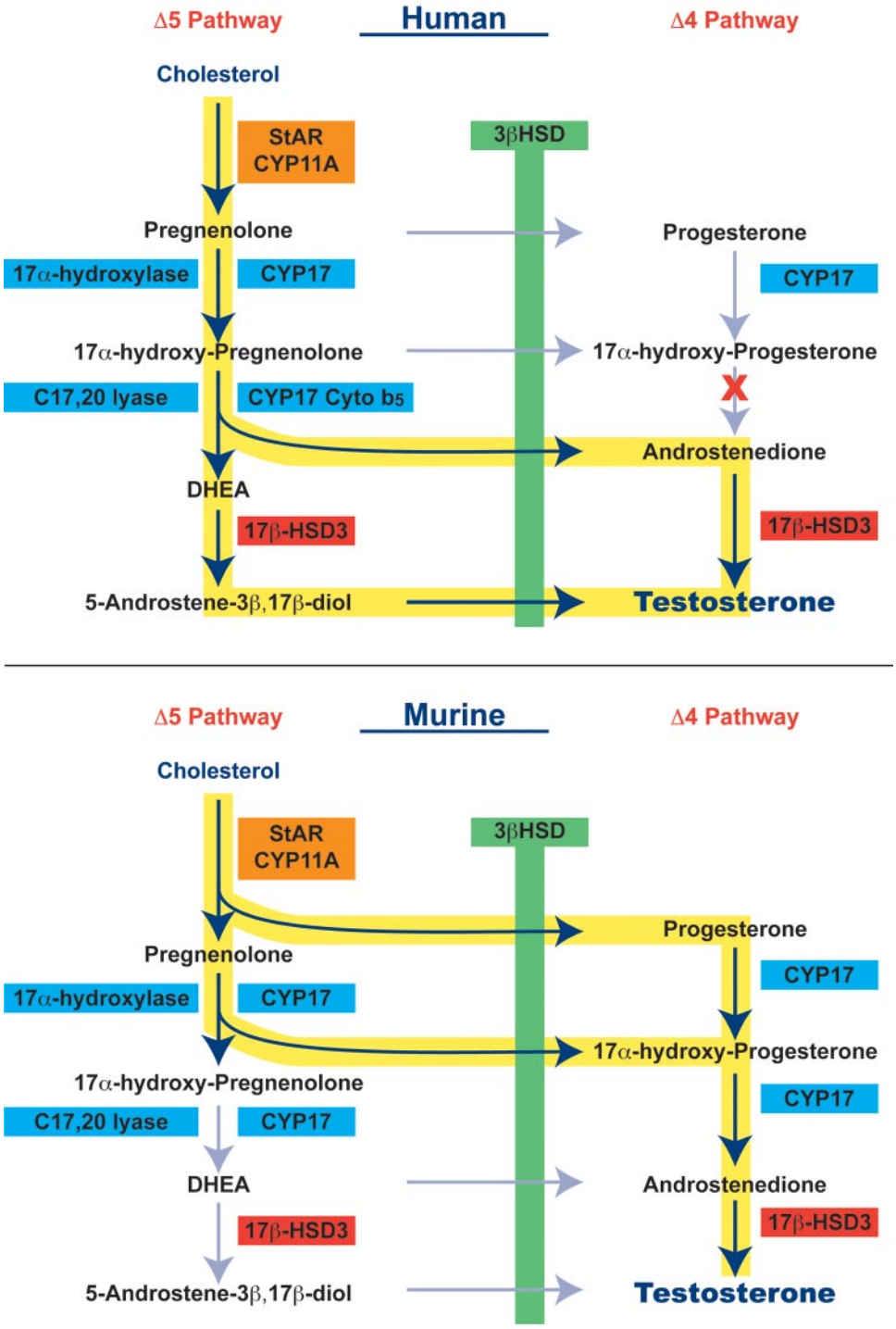

FIG. 1. Main components of the steroidogenic pathway in the human (*top*) and murine (*bottom*) fetal Leydig cell. The *yellow background coloring* shows the preferred pathways of steroidogenesis, which differ between human and murine; note that there is still a degree of flexibility in the pathways that can be used. The *red X* in the *top diagram* indicates that this reaction does not occur in the human.

lating steroid flux through the pathway (Fig. 1). It has been reported that SF1 and fellow subfamily member liver receptor homolog-1 can regulate StAR by activating the promoters of human StAR, enhancing its expression (27). StAR expression is mainly regulated by LH-mediated activation of cAMP-dependent pathways, which ultimately lead to transcriptional activation (28). StAR protein has been shown to be a substrate of ERK1/2 (29). Appropriate activation of ERK1/2 in the fetal testis could well be a trigger for fetal testosterone production. Because steroidogenesis is completely dependent on the movement of cholesterol across the intramitochondrial space, it is not surprising that mutations in StAR have dramatic effects on masculinization due to the absence/reduction in testoster-

one production during the masculinization programming window. Thus, absence of StAR protein in 46XY genetic males results in wholly female genitalia as well as congenital lipoid adrenal hyperplasia (30).

The translocator protein TSPO, previously known as peripheral-type benzodiazepine receptor, is a mitochondrial protein also involved in the regulation of cholesterol transport from the outer to the inner mitochondrial membrane. *In situ* and *in vitro* studies have demonstrated that TSPO mediates the StAR-induced cholesterol import into mitochondria. It has also been shown that cell-specific TSPO expression in steroidogenic cells is due, at least in part, to the expression of Sp1/Sp3 transcription factors (31). The role of TSPO in fetal Leydig cell steroidogenesis in early human or rodent gestation remains to be evaluated.

C. Steroid biosynthetic pathways in Leydig cells

The synthesis of testosterone from cholesterol requires the action of several enzymes (Fig. 1), and these fall into two categories: the cytochrome P450 enzymes, CYP11A and CYP17; and the HSD enzymes, 3β-HSD (HSD3B2 in humans but HSD3B1 in all other species to date) and 17β-HSD [HSD17B3 in the principal sites of testosterone formation, fetal and adult testis, and adult brain, but possibly HSD17B5 (ARK1C3) in other tissues of adults] (32). The P450 enzymes catalyze the hydroxylation and cleavage of the steroid substrate utilizing molecular oxygen and nicotinamide adenine dinucleotide phosphate (reduced) (NADPH) as the source of reductive potential (32). The HSD enzymes catalyze the oxidation and/or reduction of steroid hormones and respectively require nicotinamide adenine dinucleotide (oxidized) and/or NADPH as electron acceptor/donor (32). Whereas each P450 enzyme is the product of a single gene, the HSD enzymes have several isoforms, each the product of a distinct gene (32).

Once cholesterol has been transported to the inner mitochondrial membrane, the first of several enzymatic reactions take place. The initial steroidogenic enzyme, CYP11A, cleaves the side chain of cholesterol (a C27-sterol) generating the C21 steroid, pregnenolone (33) (Fig. 1). This is the first step and the rate-limiting enzymatic step in testosterone biosynthesis (32, 34). Pregnenolone then passes from the mitochondria to the smooth endoplasmic reticulum, where the remaining enzymatic reactions occur (35). There is little, if any, evidence that a specific carrier protein is required for pregnenolone exit from the mitochondria; for example, monkey transformed kidney (non-steroidogenic) COS-1 cells transfected with CYP11A readily allow egress of pregnenolone into the medium (36). It is thought that CYP11A deficiency is incompatible with human pregnancy and consequently 46XY genetic males with haploinsufficiency and/or partial inactivation of CYP11A are the only cases to have been reported (37, 38), although this can include major deficiencies in masculinization (37). CYP11A deficiency has been observed in rabbits, giving rise to congenital lipoid adrenal hyperplasia (39); however the newborn −/− homozygotes do not survive.

D. Conversion of pregnenolone to testosterone

The combined enzymatic actions of 3β-HSD and CYP17 catalyze the overall conversion of pregnenolone to androstenedione, the precursor of testosterone. This conversion can occur down one of two main pathways, either via Δ^5 steroid pregnenolone and its intermediates, 17α-hydroxypregnenolone and DHEA, or via Δ^4 steroid progesterone and its intermediate, 17α-hydroxyprogesterone (40) (Fig. 1). It is also possible to converge from the Δ^5 to the Δ^4 pathway (Fig. 1). Although the steroid biosynthetic pathway in the Leydig cell is the same in humans and rodents, there is a preference for either the Δ^4 or Δ^5 pathways, and this may be both species- and age-dependent (41). Species differences in preferred pathway (Δ^4 or Δ^5) are likely to depend upon relative substrate affinity of the CYP17 enzyme (41). In the human and higher primates, the Δ^5 pathway predominates in the adult (42–45) and fetal testis (46) because the human CYP17 enzyme readily converts 17α-hydroxypregnenolone to DHEA but has little 17,20-lyase activity when 17α-hydroxyprogesterone is the substrate (Fig. 1) (47). In the rat, CYP17 readily cleaves both the Δ^4 and Δ^5 C_{21} steroids (48), but in contrast to the human, has a preference for the Δ^4 pathway (Fig. 1) (49–51). All routes of conversion require 3β-HSD and CYP17, and because CYP17 sequentially catalyzes both 17α-hydroxylase and 17,20 lyase activities (Fig. 1), it is regarded as the qualitative regulator of steroidogenesis (1).

The final step in testosterone synthesis is catalyzed by a reductive 17β-HSD, to reduce androstenedione to testosterone (Fig. 1). There are multiple reductive 17β-HSDs, but the type 3 is vitally involved in adult testis function (52). It also seems likely that this same enzyme has a role in the fetal Leydig cell, although a possible role of the type 5 17β-HSD (AKR1C3) in the fetal Leydig cell cannot be dismissed.

E. Regulation of steroidogenic enzymes

The majority of what is known about the regulation of steroidogenic enzymes in both the human and rodent derives from studies of postnatal or adult testes. There is little information on the regulation of gonadal steroidogenic enzyme expression during the first trimester of human development (53) or in fetal rodents. It is important to emphasize that fetal and adult Leydig cells arise from distinct lineages, and there are several morphological and func-

tional differences between the two cell types (34). The fetal Leydig cells are responsible for fetal and neonatal masculinization, after which they regress. The adult Leydig cells, which emerge around postnatal d 10 in the rat (54, 55) and at the beginning of puberty in the human (56), are required for pubertal masculinization (34).

Cell-specific expression of the P450 enzymes is dependent on expression of SF1 (57, 58), which binds to the proximal promoter region of all P450 enzymes (59). SF1 is essential for CYP17 expression in gonadal cells (60), but other factors may then determine maximal and cell-specific expression of the P450 enzymes (32). For example, chronic but not acute LH stimulation leads to activation of adenylate cyclase and consequent increase in cAMP, which initiates the increased synthesis of steroidogenic P450 enzymes (61). Expression of CYP17 is unique in that it depends solely on cAMP stimulation in adult Leydig cells (62, 63), and although this might apply to fetal Leydig cells in the later stages of gestation in rodents, it is obvious that other factors must be involved before the ontogeny of LH action (see below).

Species differences in the regulation of CYP11A activity are apparent between postnatal rats and mice based on studies using cultured Leydig cells. In mouse Leydig cells, CYP11A activity is at maximal capacity during short-term LH stimulation but can be inhibited after chronic stimulation. Immature rat Leydig cells, however, are running at only a fraction of their potential CYP11A activity during short-term LH stimulation, and this is unaffected by long-term LH action (64). This could suggest that mouse CYP11A activity is more susceptible to perturbation because it is already running at full capacity. Because CYP11A activity is the rate-limiting step of testosterone biosynthesis (32), any interference at this step could compromise the whole pathway. In neonatal rat Leydig cells, high doses of human chorionic gonadotropin (hCG) stimulate CYP17 mRNA levels, but in the adult Leydig cell, CYP17 mRNA levels are reduced in response to high hCG doses (65). This LH/hCG-induced down-regulation is an established feature difference between adult and fetal type Leydig cells in rodents and in humans (66), and it is thought to be one way in which adequate fetal testosterone synthesis is ensured for masculinization (67).

In addition to the P450 enzymes, Leydig cell expression of 3β-HSD also appears to be dependent upon SF1 (32). There is an SF1 response element in the proximal promoter region of the human type 2 (gonadal/adrenal) 3β-HSD gene (68), and the mouse type 1 gonadal/adrenal 3β-HSD promoter has three potential SF1 consensus binding sites (69). Differences have been shown in the way that cultured rat and mouse Leydig cells respond to cAMP. In mouse Leydig cells, cAMP stimulates testosterone production, which then suppresses 3β-HSD mRNA (70), whereas addition of LH or cAMP to cultured rat Leydig cells increases 3β-HSD mRNA, protein, and activity after 24–72 h (71).

It is currently unknown whether gonadal 17β-HSD(s) is/are regulated by SF1. Studies using hpg and testicular feminized (*tfm*) mice have shown that in early postnatal development, gonadal 17β-HSD expression is independent of gonadotropin stimulation, but after puberty it is dependent upon gonadotropins and androgen action (72). Consequently, it has been suggested that androgens are required transiently to ensure optimal 17β-HSD expression during adult Leydig cell development (41). In the fetal mouse, type 3 17β-HSD is expressed in the Sertoli cells and not in the fetal Leydig cells, whereas at puberty 17β-HSD expression relocates to the adult-type Leydig cells (73). It is unclear whether this cellular switch in 17β-HSD expression is peculiar to the mouse or has counterparts in other species.

In view of the critical role that SF1 plays in ensuring expression not only of key genes in the steroidogenic pathway, as discussed above, but also in the development of the adrenal gland and testes in both mice and humans (74–76), it is not surprising that 46XY individuals with mutated SF1 have XY sex reversal (77, 78) or ambiguous genitalia (79), indicative of disrupted fetal testosterone biosynthesis and masculinization.

IV. Steroidogenesis by the Fetal Testis and Species Differences

A. Role in masculinization, regulation, and timing

The timing and evolution of steroidogenesis by the fetal testis are well described for humans and rodents (see below), but the finer details as to what initiates the onset of steroidogenesis and regulates it in early fetal life are still not definitive, although enough is known to recognize some fundamental differences between the human and rodents. These differences are best appreciated by considering the normal endocrinology of the developing male fetus and how testosterone levels change in relation to potential stimulatory hormones [LH, chorionic gonadotropin (CG)]. In evaluating this information, it should be recognized that absolute and relative hormone levels at different gestational ages depend to a large extent on the compartment in which they are measured, and this is particularly the case for testosterone in the human. For example, it is clear that measurement of testosterone levels in maternal blood in pregnancy is not an accurate guide to fetal levels (80), and although measurements in umbilical cord blood are more accurate, most such measurements are made at birth, at which time testosterone secretion by the human male fetus is relatively low and does not reflect levels ex-

perienced earlier in gestation during the period of masculinization (Fig. 2) (81–83). Amniotic fluid (AF) is presumed to provide a more accurate measure of fetal hormone levels, and, in general, relative changes in testosterone or of LH and hCG in this compartment appear comparable to those in fetal blood (82, 84, 85). Finally, although the testosterone that circulates in blood is of primary importance for masculinization of the reproductive system in males, measurement of levels within the testis should also capture this on the presumption that the testis is the main source of circulating testosterone. Intratesticular testosterone (ITT) is commonly reported in the

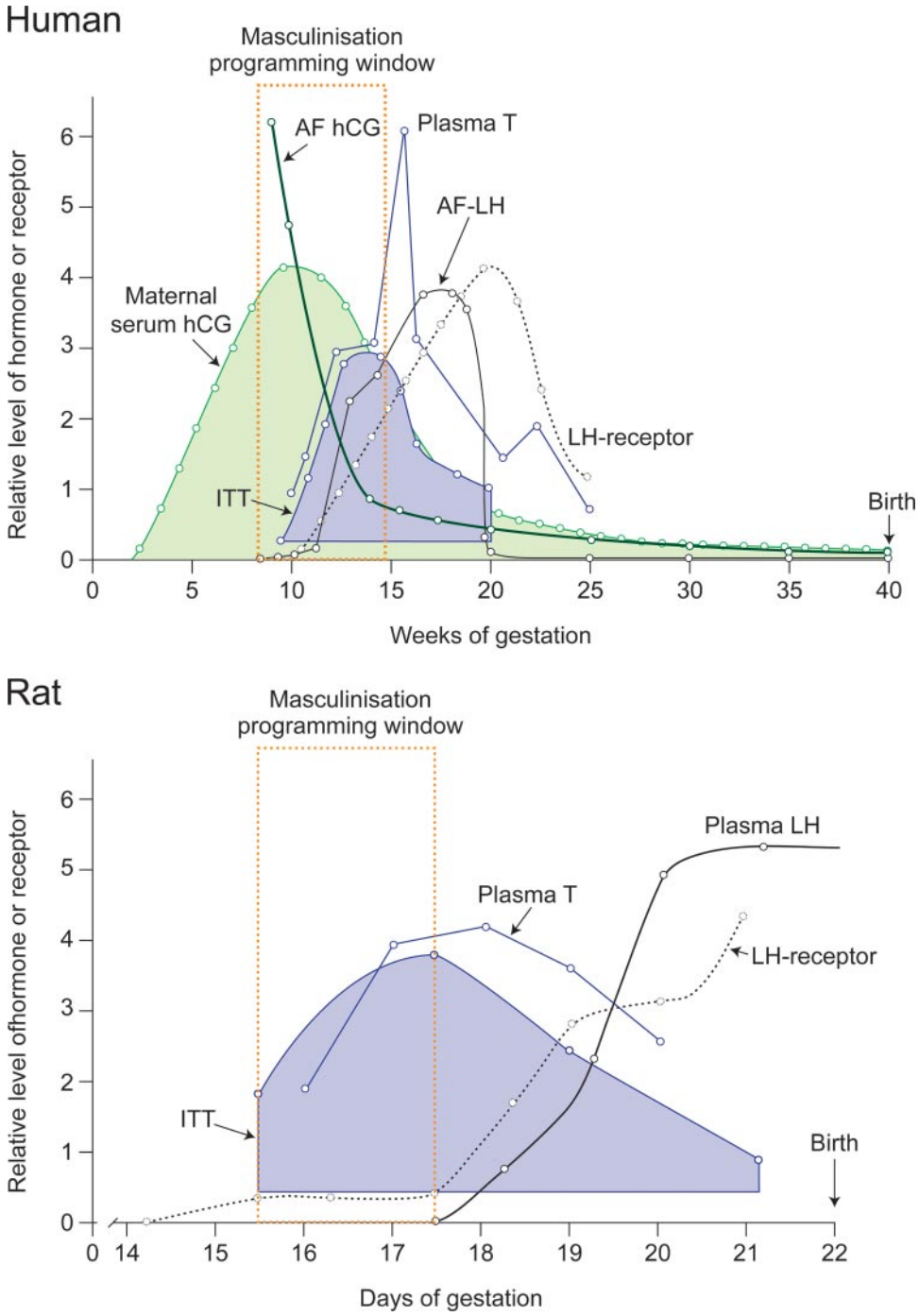

FIG. 2. Onset of testosterone production by the fetal testis in the human (*top*) and rat (*bottom*) in relation to the ontogeny of LH secretion, LH receptor appearance in the testis, production of hCG (in the human), and the time window in which masculinization of the reproductive tract by androgens occurs (masculinization programming window) (9). Note that both ITT and plasma testosterone levels are illustrated. In the human, hCG levels are shown in both maternal serum and AF to illustrate that hCG passes readily into AF (indicating fetal exposure). Note that in the rat, but not the human, coavailability of LH receptor and a suitable ligand (CG or LH) does not occur during the masculinization programming window, indicative that testosterone production during this time window is LH receptor-independent. For each parameter, each point indicates a data point or average value derived from one or more studies in the literature (8, 9, 80, 82–84, 88, 94, 102–104).

rat in terms of total content per testis (86), whereas the majority of data available for the human has expressed it per unit weight of testis (83). The latter is arguably a better measure anyway because the testis grows more than 8-fold during the period of masculinization in both the rat (H. M. Scott, unpublished data) and human (83), and so the content of testosterone per testis will increase because of this, which could be misleading. Therefore, in the comparison of human and rat in this review, ITT per unit weight of testis has been used as the comparator (Fig. 2), although it is recognized that variation in Leydig cell number/concentration according to species and age will affect this parameter.

B. Ontogeny of testosterone secretion by the human fetal testis

In the human male, the fetal Leydig cells begin to produce testosterone at around 8 wk gestation (87), and production peaks at around 11 or 12 to 14 wk gestation as determined by measurements in the testis (83, 88) and fetal blood (Fig. 2) (82). Between 12 and 20 wk, serum testosterone levels in the male fetus are between 3- and 8-fold higher than in the female (80, 89). However, at the individual level, there is not always a clear separation between testosterone levels in male and female fetuses, especially when measurements are made in AF (80, 90, 91). One study has shown that blood testosterone levels begin to decrease at 17 wk (92) (Fig. 2), whereas another demonstrated that testosterone levels gradually decrease at around 20 wk (89). Both studies concluded that by term, there are no differences in blood testosterone levels between the male and female fetus (89, 92), although not all studies agree (93).

Production of hCG in human pregnancy peaks between 8 and 12 wk gestation (94, 95), in a similar pattern and concentration in both maternal serum and AF (Fig. 2), indicative of direct hCG diffusion from the placenta (95). Direct measurement of hCG in fetal blood confirms that levels are high (and independent of fetal sex), although they are approximately 3-fold (<12 wk gestation) and approximately 9-fold (12–29 wk) lower than corresponding levels in AF (84). A similar temporal pattern of change in CG has been shown in the chimpanzee during pregnancy (83). The LH receptor is first reported in the human fetal testis at around wk 10, and maximal LH receptor binding capacity is attained between 15 and 20 wk (Fig. 2) (96). At 12 wk, when hCG levels begin to decline, LH secretion is first detected and begins to rise, such that levels in blood and AF (84) peak at around 16 wk (Fig. 2). However, it is important to note that although hCG levels are declining at a time when LH levels are increasing, data from radio-receptor assays (97) and *in vitro* bioassays (98) have shown that CG is two to six times more potent than LH on a weight basis, so hCG may still be a more important ligand than LH for stimulating steroidogenesis at 15–20 wk.

As will be clear from data discussed below, it is evident that LH receptor-mediated drive is essential for testosterone production by the normal human fetal testis, although it remains unclear whether testosterone production at very early fetal ages (7–10 wk) is partially or completely LH/CG-independent (Fig. 3). Although there are no direct reports of LH receptor protein expression before the time of onset of testosterone production (7–10 wk; Fig. 2), *in vitro* studies with human fetal testis explants have shown LH/hCG-responsiveness of steroidogenesis at 7–12 wk, suggesting that functional LH receptors are already present (99). However, the same authors have also shown that testis explants from fetuses at approximately 7 wk gestation do not require LH/hCG stimulation to maintain *in vitro* testosterone production, although addition of retinoic acid will increase secretion at this age but not at later ages (100). Therefore, from the available data, it is evident that steroidogenesis by the human fetal testis could be regulated by hCG, LH, or paracrine factors, retinoic acid being one such possibility for the latter.

C. Ontogeny of testosterone secretion by the rat fetal testis

The set-up in the male rat fetus is markedly different from that in the human, primarily because the rat does not produce CG (101). Testicular testosterone production starts at embryonic day (e) 14.5–e15.5 (102, 103), at around the same time that detectable LH receptors first appear (86, 104), although levels of LH receptor at this time may be markedly lower than between e18.5 and e21.5 (Fig. 2). Testosterone concentrations in the testis (Fig. 2) (9) and in blood (105, 106) show a modest peak at around e16.5–e17.5, although in most studies ITT levels have been expressed as content per testis, and this shows a more pronounced peak at a later age (e18.5–e19.5) (9, 102, 107). In studies in which whole body testosterone levels were measured, the fetal testosterone peak also occurred at e18.5–19.5 (108). It was also reported that whole body testosterone levels were significantly higher in males compared with females, on these 2 d alone, and on e16, e17, e20, and e21, they were described as being the same (108). Despite the earlier presence of LH receptors, LH secretion does not start until e17.5 and reaches its peak at e20.5–e21.5 (Fig. 2) (109). In fact, evidence suggests that hypothalamic control of gonadotropic function is not operative until d 19 of fetal life (110, 111). Consequently, it is clear that neither CG (because it is not present) nor LH can be responsible for initiating the onset or regulating the early phase of testicular steroidogenesis. It is therefore pre-

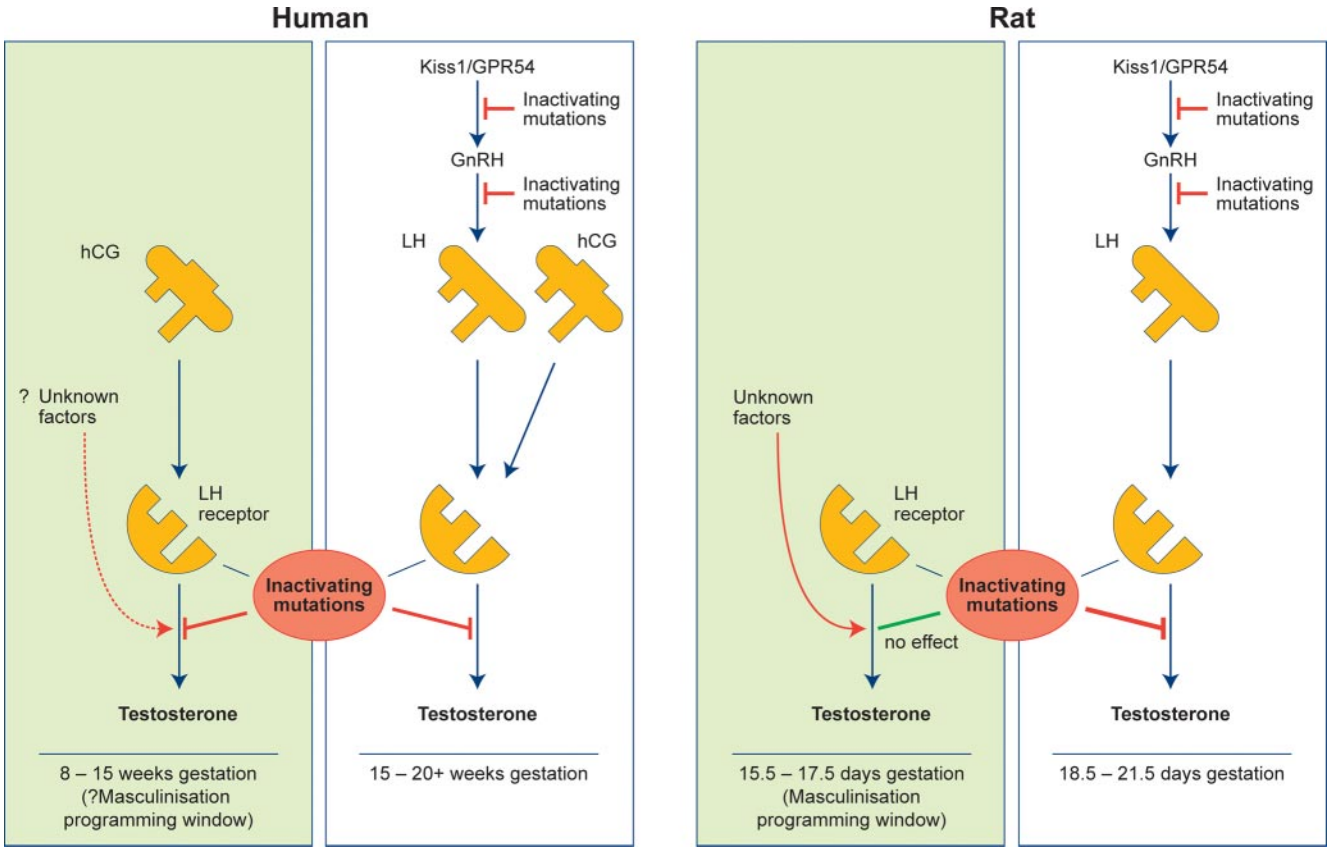

FIG. 3. Diagrammatic representation of the regulation of testicular steroidogenesis during the masculinization programming window (in early gestation) and in the period following this (in later gestation) in the human and rat. The impact of inactivating mutations in genes encoding key component parts of the hypothalamic-pituitary-testicular axis and whether this is able to block (*red bars*) steps in the pathway of regulation is also illustrated. Details are provided in the text. Kiss1, Kisspeptin 1.

sumed that regulation of steroidogenesis by the fetal rat testis is fundamentally different from that in the human and is regulated either autonomously or by paracrine factors (112) before the onset of LH secretion (Fig. 3). This conclusion is reinforced by experimental studies involving absence of LH or LH receptors (see below). Nevertheless, it is established that the LH receptors that are expressed before appearance of LH are functionally coupled because stimulation of fetal rat testis explants at e14.5 with LH/hCG is able to stimulate testosterone secretion (113).

The identity of the putative paracrine stimulators of testosterone production by fetal rodent Leydig cells is unclear, but several factors have been identified that can stimulate testosterone production by the fetal rat testis *in vitro* (Table 1). The role and importance of these factors *in vivo* is not known, and it is possible that a "fail-safe" mechanism operates such that any one of a number of factors can stimulate steroidogenesis so as to ensure masculinization. Whether these factors play a role in the human fetal testis is unknown, but retinoic acid, which stimulates testosterone production by fetal human testes at a comparably early age (100), has only inhibitory effects on steroidogenesis by rat fetal testes (114).

D. Relationship between testosterone production by the fetal testis and masculinization

Sexual differentiation and masculinization are terms that are sometimes confused (especially in the older literature) and can lead to ambiguous meaning or interpretation. Formation of a phenotypic normal male involves a cascade of changes initiated genetically by activation of the SRY gene. This leads to testis formation, and subse-

TABLE 1. Factors shown to stimulate steroidogenesis by the fetal rat testis *in vitro* and which are therefore potential candidates for paracrine regulation of steroidogenesis during the masculinization programming window (e15.5–e17.5)

Factor	Fetal age	Refs.
IGF-I[a]	e16.5	439
PACAP-27[b]	e18.5	440, 441
VIP[b]	e18.5	442
ANP[b]	e18.5	443
BNP[b]	e18.5	443
CNP[b]	e18.5	443

[a] IGF-I production detectable within the fetal rat testis from e16.5; IGF-I also increased the numbers of fetal Leydig cells.

[b] Receptors for these compounds also shown to be present from e15.5.

quent testosterone production by the latter then leads to bodywide masculinization. The term sexual differentiation is generally used to describe the moment when a recognizable testis is first formed because this is the very first point at which a fetus can be morphologically distinguished as being a male (115, 116). Masculinization refers to the process via which the sexually indifferent fetus with a testis is then transformed into a phenotypic male with internal and external male genitalia, which cannot occur until testes have differentiated and begun to secrete hormones (117). Therefore, masculinization follows sexual differentiation but is separate from it. Thus, a genotypic male (XY) fetus can undergo sexual differentiation and form a testis but fail to completely masculinize, as occurs in CAIS, or a genotypic female (XX) fetus without a testis can masculinize if exposed to sufficient androgens (1). Because much of the masculinization process involves androgen action and the main source of androgens is the fetal testis, disorders of androgen-dependent masculinization can provide a "read-out" of fetal testicular steroidogenic function. Regression of the Müllerian duct in the male fetus under the influence of anti-Müllerian hormone will not be considered in this review because this does not involve steroidogenic function of the fetal testis. It should also be noted that masculinization of the fetus is only the first, although most important, step toward formation of a phenotypic male because androgen production/action postnatally, and especially at puberty, is also essential for this to happen.

Masculinization of the reproductive tract involves the differentiation of the internal (epididymis, vas deferens, seminal vesicles, and prostate) and external (penis, scrotum, and perineum) genitalia (118), but these events do not occur synchronously. The prostate forms from gestational wk 10–13 in the human (83) and e18.5–19.5 in the rat (119). The penis forms through wk 11–13 in the human (83) and beyond e17.5 in the rat (120). The seminal vesicles develop over the period of 14–16 wk in the human (121) and at e19.5 in the rat (120). The androgen-dependent phase of testis descent into the scrotum occurs much later in gestation, during wk 27–35 in the human (122, 123) and during postnatal wk 3 in the rat (124). Once the reproductive tract structures have differentiated as the result of androgen action, their continued growth may also be driven by testicular androgens, as is the case for example for elongation of the penis postnatally (Fig. 4) (10, 125–127). This is an important point because some studies in humans that may involve androgen deficiency at various gestational ages may not have distinguished masculinization of the phallus (*i.e.*, formation of a penis) from its subsequent growth (*e.g.*, micropenis), and this could lead to misleading interpretations. Masculinization of the brain, which will not be discussed in this review, occurs late in gestation in primates (128) and perinatally in rodents (129, 130).

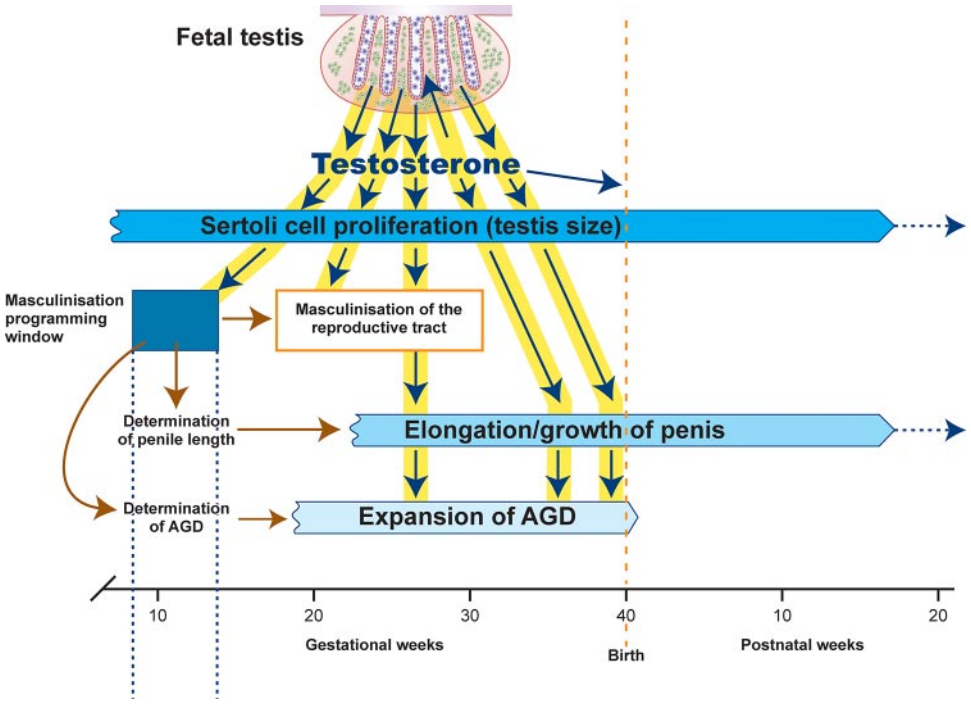

FIG. 4. Illustration of the main effects of fetal testicular testosterone (or other androgen metabolites) in relation to the stage of gestation and the masculinization programming window. Note that androgen action within the masculinization programming window is what sets up normal development of the penis and AGD in males (*i.e.*, determines what size they will grow to) but that it is androgen action after this time window, and extending into the postnatal period, that is responsible for realizing this growth (9, 10). In contrast, testosterone-driven proliferation of Sertoli cells is independent of the masculinization programming window and extends also into the postnatal period (8).

It has been widely assumed that androgens induce masculinization of the reproductive tract at around the time that these structures morphologically differentiate and thus appear different from those in the female. One consequence of this assumption was that researchers investigating the mechanisms of androgen-dependent masculinization in rodents have concentrated on the later period of gestation (*e.g.*, e18.5–e21.5 in the rat). However, recent studies in the rat have shown that the critical window for androgen-dependent masculinization (e15.5–e17.5) actually precedes the morphological differentiation of the structures (Fig. 4), and this has been termed the "masculinization programming window" (9). This period therefore coincides with the very start of testosterone production by the fetal rat testis, when LH is not present and the stimulus for testosterone production is presumed to be paracrine (Figs. 2 and 3). It is thought that a similar masculinization programming window occurs in the human and is predicted to be approximately 8–12 wk gestation (9) (Figs. 2–4). It has been shown that if there is insufficient testosterone production/action during the masculinization programming window in the fetal rat, it can result in disorders of masculinization such as lack of formation of the penis or its malformation (hypospadias), cryptorchidism, an underdeveloped prostate and reduced anogenital distance (AGD) and penis length (9, 10). In contrast, blockade of androgen action after the masculinization programming window in the rat, when both LH and the LH receptor are expressed (Fig. 2), does not affect the masculinization process, but may affect elongation of the penis (9, 10) or testis size (due to reduced Sertoli cell number) (Fig. 4) (8, 107). It has therefore become evident that the mechanisms that regulate testicular testosterone production, before regulation by LH, are of paramount importance in the rat (Fig. 2) because it is likely that it is these mechanisms that are disrupted when testosterone production is reduced, resulting in disorders of reproductive tract masculinization. Arguably, AGD provides the simplest and least invasive measure of reduced androgen exposure during the masculinization programming window (9, 10).

It is also important to note that the difference between external genitalia being undermasculinized (*i.e.*, penis formation is abnormal, as in hypospadias) and underdeveloped (*i.e.*, of subnormal length as in micropenis) is likely to be the result of androgen deficiency in two different periods (10, 131): the former, during the masculinization programming window, and the latter, after this window, including after birth (Fig. 4). Therefore, the type of genital abnormality that is present may provide information as to when the deficiency in androgen action occurred. This has relevance when considering evidence from natural mutations, in humans and animals and in transgenic animal models, that affect LH or the LH receptor, although it should also be remembered that hypospadias may occur for reasons other than deficient androgen production/action (132). Similarly, although cryptorchidism is usually taken to indicate deficient hormone action, this may involve insulin-like factor 3 as well as androgens (133). Because the diagnosis and reporting of cryptorchidism is also problematical (133), it will not be considered in this review as a potential guide to fetal androgen production/action.

V. Inferences Regarding Regulation of Fetal Steroidogenesis from Masculinization Defects in Humans and Animals

A. Effects of anencephaly (LH deficiency)

Anencephaly is the consequence of a neural tube defect that results in the absence of a major portion of the brain. As such, it has been equated with the absence of the hypothalamus and pituitary gland and consequent absence of LH. Although there have been suggestions that this is an overinterpretation and that varying amounts of pituitary tissue may be present in anencephalic fetuses (134), it is generally agreed that they are LH deficient (135). Despite having absent or low levels of pituitary LH, human anencephalic male fetuses are still able to undergo normal masculinization of the reproductive tract and external genitalia, indicative of normal testicular function and androgen production, at least during early gestation and encompassing the masculinization programming window (Fig. 3). However, although the penis and scrotum are normally differentiated, they are often small and the testes are often undersized (136–139). This implies that although testosterone synthesis in early gestation is normal (probably driven by hCG; see below), once hCG secretion starts to diminish at 12–15 wk gestation (Fig. 2), levels are no longer sufficient to compensate for the absence of LH, so androgen-dependent growth of the external genitalia is compromised (134). This is consistent with evidence from the rat that penis formation is regulated exclusively by androgen action during the (early fetal) masculinization programming window, whereas its subsequent elongation/growth in size is regulated by androgen action in late gestation as well as after birth (10, 131), and the same appears to apply to the human (118, 131) and nonhuman primate (125) insofar as data are available (Fig. 4). Nevertheless, because anencephalics will also lack pituitary hormones other than LH, it is not possible to draw definitive conclusions based on such examples.

These findings imply that the human fetal testis does not come under control of its hypothalamic-pituitary system until later in gestation (Fig. 3), by which time masculinization has occurred and the high levels of hCG have

declined (134). Some anencephalics are reported to have normal-sized genitalia, but it is likely either that these individuals have a greater portion of undamaged pituitary or that their mothers have higher levels of hCG that are able to compensate for low LH levels later in gestation (140).

The anencephalic state in the human has been recapitulated experimentally in animal models using *in utero* hypophysectomy to investigate the role of gonadotropic hormones in the regulation of fetal testicular testosterone synthesis. In fetal mice, hypophysectomy using x-ray irradiation before reproductive tract differentiation was followed by normal masculinization at birth (141). Additionally, transgenic mice, which are null for the thyroid-specific enhancer-binding protein (*T/ebp* or *Nkx2.1*) gene and which do not develop a pituitary gland, are normally masculinized at birth (142). There have been several studies in which fetal rats have been hypophysectomized *in utero* by decapitation. Rats that were decapitated in early sexual differentiation were described as having slight underdevelopment of the reproductive tract at birth (141). Although this phenotypic description does not elucidate whether the rats were normally masculinized but underdeveloped, other more recent studies have revealed that the fetal decapitation of rats from e16.5 onward does not affect normal masculinization (Wolffian ducts develop normally and AGD is comparable to controls) and that testosterone production is only reduced in animals decapitated after e19.5 (143, 144); this suggests that testosterone synthesis is independent of LH stimulation before e19.5 (Fig. 3). A study of the pituitary-gonadal axis by measuring plasma LH and testosterone in normal fetal rats and those that had been castrated and then exposed to either flutamide or ethylene dimethane sulfonate also demonstrated that testicular feedback regulation of LH is functional from e19.5 onward (145). Although early data using fetal rabbits hypophysectomized by decapitation on gestational d 19 reportedly resulted in absent or minimal virilization of the prostate and external genitalia (146), more recent experimentation has led to the general consensus that the onset of testosterone biosynthesis and resulting differentiation of the reproductive tract in rabbits occurs in the absence of gonadotropins (147–149) as it does in rats and mice.

These data suggest that the steroidogenic function of fetal mouse, rat, and rabbit testes is independent of fetal pituitary hormones, at least during the initial period of testosterone production (Fig. 3). The current evidence suggests that humans and rodents all seem to masculinize normally in the absence of a pituitary or pituitary LH, although there are some remaining uncertainties due to ambiguities in terminology used by earlier authors. These have largely been dispelled by more recent studies that have used naturally occurring models of LH (and FSH) deficiency or comparable models achieved via transgenesis.

B. GnRH and kisspeptin/GPR54 mutations

Hypogonadotropic hypogonadism encompasses a number of conditions typified by dysfunction of the hypothalamus and/or pituitary, which leads to an inadequate/absent production of gonadotropins, most importantly LH. Hypogonadotropic hypogonadism is characterized by a delay or absence of pubertal development secondary to gonadotropin deficiency (150). Although the majority of patients with hypogonadotropic hypogonadism have a GnRH deficiency (151), only one male has been identified as having an inactivating GnRH gene mutation, and he presented with normal masculinization at birth but with microphallus and absence of puberty (152). Kallmann syndrome is a disorder resulting from the failed migration of GnRH neurons from the olfactory placode to the hypothalamus; this failure of migration of GnRH neurons results in overt GnRH deficiency. In addition to hypogonadotropic hypogonadism, these patients usually present with anosmia. The phenotype of an individual depends on the gene that is mutated. Mutations in a number of genes have been associated with Kallmann syndrome and hypogonadotropic hypogonadism. Loss of function mutations in the X-linked KAL1 gene have been shown to cause severe hypogonadotropic hypogonadism (153–155), whereas loss of function mutations in the autosomal dominant fibroblast growth factor receptor type 1 (FGFR1) gene (KAL2) produce a far more variable hypogonadotropic phenotype (155–157). Mutations in either of these genes, however, have only been found in approximately 20% of affected individuals (158). More recently, loss of function mutations in the prokineticin 2 (PROK2) gene, which encodes a secreted peptide responsible for regulating the development and migration of the olfactory tract and GnRH neuron progenitors, have been associated with Kallmann syndrome and hypogonadotropic hypogonadism (159). Mutation of the PROK2 gene also results in a variable phenotype, with both nonanosmic and anosmic forms of hypogonadotropic hypogonadism (159). There is also evidence that mutations of the gene encoding the prokineticin receptor-2 may also be involved in Kallmann syndrome (160). As such, these two genes have been grouped together as KAL3.

Other genetic mutations associated with hypogonadotropic hypogonadism include GnRH receptor 1 mutations that are evident in 40% of patients with autosomal recessive, normosmic hypogonadotropic hypogonadism (161, 162) and a spectrum of loss-of-function mutations of the G protein-coupled receptor 54 (GPR54) (163–165). To date, no inactivating mutations of the ligand (Kisspeptin 1) for the GPR54 have been reported (166). Although

hypogonadotropic hypogonadism is characterized by a delay or absence of pubertal development, in many cases it can be diagnosed shortly after birth by the presence of a micropenis and/or cryptorchidism and subnormal levels of LH and FSH (163, 167). GnRH secretion commences in the second trimester of human fetal development (168), which is subsequent to the predicted masculinization programming window (Fig. 2), so masculinization is essentially complete by this time (Fig. 3). Because testosterone production before the start of GnRH secretion must be independent of GnRH stimulation, this explains why there are no reports of incomplete masculinization associated with hypogonadotropic hypogonadism (Fig. 3). However, in fetal males, a functional hypothalamic-pituitary-gonadal axis is necessary for penile growth (Fig. 4), and possibly also for testicular descent. Consequently, any defects in the hypothalamic-pituitary-gonadal axis during the second and/or third trimesters may result in micropenis (131, 169).

In mice, a naturally occurring deletion in the GnRH gene renders them hypogonadotropic (*hpg*), with a near-total deficiency of LH (170). Despite this LH deficiency, *hpg* mice are normally masculinized at birth (171), although there are no reports of whether the size of external genitalia is affected at this stage. ITT levels in *hpg* mice are normal throughout fetal life and on the day of birth (170), implying that LH is unnecessary to maintain testicular steroidogenesis even late in gestation. This does not necessarily mean that fetal testosterone production is LH-unresponsive until birth because LH and full-length LH receptor transcripts are expressed from e16 (170, 172) and LH has been shown to stimulate testicular testosterone production by fetal testes *in vitro* at this age (172, 173). This suggests that testosterone production in late gestation may be under dual control (170), but whether LH action is absolutely necessary for normal testosterone production is doubtful. As outlined above, recent findings have shown that as long as the fetus is exposed to sufficient testosterone during the masculinization programming window, it will masculinize normally (9, 10), and the masculinization programming window spans the fetal period before secretion of LH in rodents (Figs. 2 and 3). This implies that even if testosterone levels were reduced during late gestation in rats or mice due to absent/deficient LH secretion, this should not have any effect on masculinization *per se* (Fig. 3). This has been demonstrated in fetal rats decapitated at either 16.5 or 18.5 d post coitum, which despite having reduced testosterone concentrations (−56% in plasma and −67% in testes), displayed normal Wolffian duct growth and AGD elongation (144). It is likely, however, that a reduction in testosterone levels in late gestation may reduce penile elongation (10, 131) and reduce testicular size due to reduced Sertoli cell numbers because proliferation of these cells is driven by testosterone primarily during the late phase of gestation (Fig. 4) (8, 107).

Other mouse models of hypogonadotropic hypogonadism include at least four GPR54 transgenic mice lines (165, 166, 174, 175), and two Kiss1 transgenic mice lines (166, 176, 177). These mice are all normally masculinized at birth, although they have small external genitalia when evaluated at 7 wk of age (174). Mice with N-ethyl-N-nitrosurea-induced GnRH receptor mutagenesis are also reported to have micropenis and small testes in adulthood, indicating that although normal masculinization of the external genitalia occurred, due to adequate GnRH/LH-independent testosterone production during the masculinization programming window (Fig. 3) (9), the impairment of GnRH (and thus LH) secretion and subsequent reduction in testosterone secretion in late gestation and postnatal life reduced penile growth and development (178), as would be expected (Fig. 4) (131, 179).

C. LH and LH receptor mutations

1. Common α-subunit (LH, FSH) inactivating mutations

There are currently no humans identified with mutations of the common α-subunit gene. Because there has been no proven germ line mutation, it has been suggested that the mutation is possibly lethal in humans (180) because it would also affect production of hCG and TSH in addition to gonadotropins (180). Consequently, the human phenotype of a common α-subunit gene mutation is still unknown. On the other hand, mice with targeted disruption of the common α-subunit gene have a subsequent loss of LH, FSH, and TSH, which causes hypogonadism and hypothyroidism, and a comparable phenotype to mice with combined GnRH and TSH deficiency. These mice are viable, and masculinization is normal at birth (181), reinforcing the evidence that LH action is not required for the fetal period of masculinization in rodents (Fig. 3). However, because mice do not produce or rely on CG for maintenance of pregnancy, this may not be a suitable model to compare to the human situation.

2. LHβ-subunit inactivating mutations

There are currently four reports of five human males with LHβ gene mutations (182–185). All of these individuals had normal genitalia and descended testes at birth but then failed to undergo postnatal genital and pubertal development (182–185). Taken together, these five cases suggest that in the absence of pituitary LH, either hCG or paracrine factors are able to stimulate fetal Leydig cells to produce testosterone in the human male fetus (Fig. 3) so that normal intrauterine masculinization occurs; this agrees with other data discussed below. In a mouse model

with targeted inactivation of the LHβ-subunit gene, the males exhibit normal prenatal masculinization (186).

Therefore, several lines of evidence show unequivocally that, in the absence of LH, rodents masculinize normally, which shows that LH is not required for normal fetal testicular testosterone production during the period when masculinization is induced, now established to be during the masculinization programming window (Fig. 2). Consequently, fetal rodent testosterone production must be driven by unknown local (paracrine) factors because rodents do not produce CG. The evidence just described for one man lacking LHβ (and thus LH) suggests that masculinization-dependent testosterone production is also LH-independent in humans. However, in contrast to rodents, in the human this LH independence could be due either to the action of local paracrine factors (as in rodents) or to stimulation by hCG produced by the placenta. Evaluation of patients with inactivating mutations of the LH receptor points clearly to the latter as being of primary importance.

3. LH receptor-inactivating mutations

Inactivating mutations of the LH/CG receptor (LHCGR) in human males leads to an absence of, or incomplete, masculinization (pseudohermaphroditism) as well as cryptorchidism and Leydig cell hypoplasia (187). Despite displaying female external genitalia, these individuals still have epididymides and vasa deferentia (180, 188, 189), but they do not retain Müllerian duct structures (180). The human phenotype depends on the completeness of the receptor inactivation (187), and partial inactivation of the LHCGR results in micropenis, hypospadias, and cryptorchidism (180, 190), consistent with subnormal, but not absent, testosterone production during the masculinization programming window (Fig. 3) (9). The presence of epididymides and vasa deferentia implies that individuals with LH receptor inactivation must still be able to produce sufficient testicular testosterone to masculinize local Wolffian duct structures, and this has led to suggestions that the testosterone needed for Wolffian duct stabilization may be produced independently of LH/hCG action (189). Others have proposed that this may indicate that testosterone synthesis starts autonomously and then becomes dependent on placental hCG or that some components of steroidogenesis may be constitutively independent of LH/hCG stimulation (67, 191, 192). This scenario is comparable with the phenotype of male rats exposed to flutamide *in utero*. It has been shown that flutamide exposure prevents normal masculinization of the external genitalia but only has minor effects on the Wolffian duct, affecting differentiation but not stabilization (193). It has also been demonstrated that the critical window for Wolffian duct development is during the masculinization programming window (194). These data suggest that low (subnormal) levels of testosterone produced during the masculinization programming window could result in the maintenance of Wolffian duct structures, although masculinization of the external genitalia was absent or incomplete. Although this may possibly be explained by the degree of LHCGR inactivation, it seems more likely that the very earliest stages of testosterone production by the fetal human testis may be driven partially or completely by hCG/LH receptor-independent local mechanisms, although it would appear that this is rapidly superseded by hCG-driven control (Figs. 2 and 3). The identity of the paracrine factor(s) that might drive fetal testis steroidogenesis in humans or rodents is unclear but merits further study because of its importance; potential candidates have been identified in rodent studies (Table 1).

In contrast to the human, genetically modified mice in which the LHCGR gene has been inactivated (LuRKO mice) are identical to wild-type mice at birth and display normal masculinization (195, 196). These data are wholly consistent with all of the other transgenic mouse models reviewed in this section and further emphasize that, in the rodent, signaling via the LHCGR is not required for masculinization of the reproductive tract and genitalia (195), whereas in the human a functional LHCGR is essential for normal masculinization (Fig. 3).

4. LH receptor-activating mutations

If LH/CG stimulation of steroidogenic cells is critical for the onset of androgen synthesis, it might be expected that LHCGR-activating mutations would initiate androgen production in females and result in masculinization of the female fetus. Human males with LHCGR-activating mutations have been identified and exhibit early-onset, gonadotropin-independent, precocious puberty (testotoxicosis) (197), yet human females with activating LHCGR mutations do not manifest an abnormal phenotype (198, 199). Why this is so is currently unexplained, but the most likely explanation is that a mature LHCGR protein is not expressed at this time in females, and probably not until a time during puberty based on the absence of females with precocious puberty resulting from activating mutations of the LHCGR.

D. P450 oxidoreductase (POR) deficiencies (PORD)

The enzyme POR is required for the catalytic activity of all of the microsomal P450 enzymes because it acts as the final electron donor to these enzymes (200, 201). Inactivation of the *Por* gene in mice leads to severe developmental defects and embryonic death at e9.5 (202). Consequently, (partially) inactivating mutations of POR in the autosomal recessive human POR disorder (PORD) could

potentially affect the activities of all microsomal P450 enzymes involved in cholesterol biosynthesis, steroidogenesis, and xenobiotic metabolism (201). The modulatory effects of the various POR mutations appear to have different hierarchical impact on the activities of the P450 enzyme families. The C17–20 lyase activity of human CYP17 *in vitro* appears strikingly more affected by POR mutations than is CYP17-associated 17-hydroxylase activity, a probable reflection of CYP17-associated lyase's particular dependence on the abundance of both its redox partners, POR and cytochrome b_5 (203). Because the relative 17-hydroxylase/C17–20 lyase activity and character varies not only across species but also between organs in any given species, the relative sensitivity of C19-steroid biosynthesis to POR mutations may differ between both species and organs. The rat 17-hydroxylase/lyase catalyzes a more concerted two-stage reaction than that of the human or the bovine enzyme (204), whereas remarkable changes also occur in adrenal CYP17-associated activities between the human fetal, preadrenarchal, and adult adrenal stages of development (205). The production of C-19 steroids (androgens) in male and female PORD patients is severely reduced, presumably because the 17,20-lyase activity of the CYP17 enzyme is particularly sensitive to POR impairment (206). As a result, male patients with inactivating mutations of POR can be undermasculinized, although this does not happen as frequently as would be predicted, probably because the degree of impairment of another P450 enzyme, placental aromatase (CYP19), is also dependent on the nature of the particular POR mutation. Defective CYP19 activity (because of the absence of normal POR), may result in the accumulation of testosterone and androstenedione (possibly of fetal adrenal rather than of fetal testis origin), which then enters the fetal (and maternal) circulation and goes part way to compensating for suppressed testicular testosterone production (207–209). This would follow if a differential effect of POR mutations on adrenal *vs.* testis CYP17-activities occurs. Little is presently known concerning the effect of POR mutations on CYP17-dependent activities in specific human and rodent fetal issues including the fetal adrenal and testis.

Although some male PORD patients are undermasculinized, nearly all females with PORD develop virilized external genitalia, despite having low androgen production after birth (206). Although the accumulation of placental testosterone and androstenedione (including maternal sources) also enters the circulation of female fetuses, compromised aromatase activity is not sufficient to explain female virilization because: 1) in classic CYP19 deficiency only females with near-complete loss of placental CYP19 activity are virilized (210); and 2) PORD also causes a reduction in the supply of DHEA, the precursor for both androstenedione and testosterone (209, 211).

A proposed explanation for this paradox is the production of androgens in PORD females via the "backdoor pathway" (206, 207). This appears to be the main pathway used by the tammar wallaby pouch young (212), neonatal mice (213), and neonatal rats (214) to produce dihydrotestosterone (DHT), but it bypasses the conventional intermediates androstenedione and testosterone (201). Under most circumstances, little 5α-reductase is expressed in the testis, so the conversion of testosterone to DHT occurs in androgen target tissues from circulating testosterone (206). In rare situations, including in the tammar wallaby pouch young and neonatal rodents, 5α-reductase is expressed in the testis, and as a consequence, 17α-hydroxyprogesterone is converted via 5α-reductase and reductive 3α-HSD to 5α-pregnane-3α,17α-diol-20-one (Pdiol). Pdiol is then converted to androsterone by CYP17, then androstanediol by 17β-HSD3, and is finally converted into DHT by oxidative 3α-HSD in target tissues (206). Although the specific pathways used in the backdoor pathway are slightly different in the wallaby pouch young and immature mouse testis, androstanediol is synthesized by two pathways in both cases (206).

In PORD, the particular impairment of the lyase aspect of the CYP17 enzyme results in an accumulation of 17α-hydroxyprogesterone, which may drive the flux of 5α-reduced 21-carbon steroids to androsterone via the backdoor pathway; this is supported by evidence showing that the 19-carbon steroids in PORD are derived primarily from 5α-reduced 21-carbon steroids rather than the traditional Δ^4-steroid pathway involving androstenedione and testosterone (206). Additionally, although CYP17 is required to convert 5α-pregnane-3α,17α-diol-20-one into androsterone, 5α-pregnane-3α,17α-diol-20-one has a much higher affinity for CYP17 than does 17α-hydroxyprogesterone (215), making the backdoor pathway a far more feasible route of androgen production in PORD, and urinary steroid profile analyses in a number of individuals with PORD concluded that this pathway is indeed operating (201).

E. Masculinization disorders when fetal testis function is normal

Failure or incomplete masculinization at birth is not always indicative of impaired testosterone production by the fetal testis. It can occur due to deficiencies in androgen action downstream of the testis, for example because of mutations in the AR (1) or because of 5α-reductase type 2 deficiency (pseudohermaphroditism) (216). The presenting genital phenotype of such individuals can be similar to those described above in which deficiency in testicular secretion of testosterone (for various reasons) is the root

cause. Therefore, although masculinization disorders are extremely informative of fetal testis function, correct interpretation requires assurance that deficiencies in androgen action are not the cause. Furthermore, human/rodent differences also need to be taken into consideration in such comparisons, especially those involving 5α-reductase type 2 deficiency because knockout of this gene in mice, with or without concomitant knockout of 5α-reductase type 1, does not affect masculinization (217). In contrast, blockade of 5α-reductase type 2 using the specific inhibitor finasteride in rats does impair masculinization (218). There are also numerous environmental chemicals that are effective AR antagonists at appropriately high levels of exposure in animal studies, and their resulting effects on masculinization can therefore also be misinterpreted as reflecting impaired testis steroidogenic function (219).

VI. Susceptibility of Fetal Leydig Cell Steroidogenesis to Disruption/Inhibition by Therapeutic Compounds and Environmental Chemicals

There is currently considerable concern about the potential for some environmental chemicals (endocrine disruptors) to impact on human male reproductive health, and this is centered around inhibition of fetal androgen production or action (117). Most of this concern stems from studies in laboratory animals, and comparison of the susceptibility of fetal Leydig cell steroidogenesis to disruption by such compounds may help to assess risk to the fetus as well as identifying (common) mechanisms of action. One criticism of these studies is that adverse effects on the fetal testis, or on masculinization, have only usually been shown at levels of exposure to individual chemicals that are probably far in excess of human exposure levels (7). However, this criticism has been effectively undermined by demonstration that there are additive effects of mixtures of individual chemicals when they target a similar process (220). For compounds that affect fetal testis steroidogenesis, such as certain phthalates (see below), the presumption is that effects in rodents are likely to predict effects in humans because of the near-identity of the steroidogenic process, as detailed above. Nevertheless, this presupposes that there are identical pathways of effect in human and rodent fetal testes, and testing of this presumption was a primary motivation for this review.

Additionally, in the human there is the potential that fetal exposure to certain therapeutic drugs, administered to the mother, may inhibit fetal Leydig cell steroidogenesis and thus potentially affect masculinization in the male. Comparison of such findings with those from experimental studies in rodents with the same compounds affords a further means of comparing human and rodent testis susceptibility. Finally, altered exposure to endogenous hormones (other than androgens), due to altered fetal growth (221), maternal disease (222), stress (223), or multiple pregnancy (224) could also potentially affect the fetal testis directly or indirectly in humans, and such effects may mimic those seen after therapeutic exposure to the same hormone (*e.g.*, estrogens, glucocorticoids). Because there is also abundant evidence from rodent studies for effects of some of these hormones, this provides a further point of comparison.

A. Estrogens

It is generally perceived that estrogens are inhibitory in the male or at least that they antagonize androgen action. The mechanism for such antagonism is not clear, but at its simplest could involve estrogen inhibition of Leydig cell testosterone secretion, for which there is some evidence (see below). Other studies suggest that it is the balance between androgen and estrogen action, rather than absolute levels of either hormone, that is critical in determining effects (225). The mechanisms via which the androgen-estrogen balance could induce effects is unclear, but it could involve interactions of androgen and estrogen receptors with Src (226–228) and bypassing of transcriptional regulation. Nothing is known about whether such mechanisms might operate in the fetus.

Pregnancy is a time of high estrogen production, especially in the human (229), which has raised doubts as to how additional exposure of the fetus to exogenous estrogens could add effectively to the already high exposure to placental estrogens. This raises complex issues regarding the bioavailability of pregnancy estrogens, which are mainly bound to SHBG in the human (but not in rodents), whereas for example, synthetic estrogens such as diethylstilbestrol (DES) and ethinyl estradiol do not bind to SHBG and will therefore be more freely bioavailable (12). Additionally, even in humans, fetal exposure to pregnancy estrogens (as indicated by maternal estriol levels) may be much lower early in pregnancy during the period of the masculinization programming window (229, 230), so that exposure to exogenous estrogens at this time could potentially increase fetal estrogen exposure. This could be detrimental if this exposure resulted in antagonism of androgen action. The mechanism of action of estrogens in this situation may be complex and involve more than inhibition of steroidogenesis.

The evidence for adverse effects of estrogens on steroidogenesis in the fetal human testis and/or it having downstream consequences is rather weak, and the limited available evidence indicates no estrogen inhibition of steroidogenesis by the fetal human testis (231). It is commonly reported that fetal DES exposure in humans results

in increased occurrence of hypospadias in sons (232, 233), as is clearly the case in rodents (see below). However, this is not the case (12), and the cited studies in fact relate to the mother's exposure as a fetus to DES, which then increases the incidence of hypospadias in her sons, probably via effects related to DES-induced uterine maldevelopment in the mother (232, 233). However, hypospadias or other genital abnormalities have been reported in boys whose mothers were exposed to ethinyl estradiol or progestogens in pregnancy (234, 235), especially when this exposure occurred early in gestation (before 11 wk gestation) (236, 237). Recent epidemiological evidence shows approximately 2-fold increased risk of hypospadias in sons associated with maternal progestin exposure during the first trimester (238). Whether such an effect is due to alteration in steroidogenesis or to direct effects on the penis is presently unknown. Direct effects on the developing penis may provide a more logical explanation for both progestins and estrogens (see below). Microphallus and reduced testis size are associated with fetal estrogen exposure (12, 239), both of which might reflect reduced testosterone levels or androgen action in the second and third trimesters of pregnancy (Fig. 4).

Estrogen (estriol) levels in women having twins have been shown to be 1.8 to three times higher than in singleton pregnancies (224), and geometric mean estradiol levels have been reported to be 59% higher in women carrying twins (240). It has also been shown that there is a higher frequency of hypospadias in boys who have a twin (221). As fetal growth is often compromised in twins and this is a known risk factor for hypospadias (221), this may provide a more logical explanation for this association. In this regard, meta-analyses of epidemiological studies that investigated the relationship between *in utero* exposure to estrogens, including endogenous estrogens, the contraceptive pill, or environmental estrogens, have shown no association with increased risk of reproductive tract disorders, including hypospadias in humans (241–243). Therefore, in comparison with rodent studies (see below), the lack of evidence (direct or indirect) for estrogen inhibition of fetal testicular steroidogenesis in the human is reasonably consistent and fits with the reported absence of estrogen receptor (ER)-α in fetal human Leydig cells (indeed, its complete absence from the fetal testis) (244, 245) because all such effects in rodents appear to be mediated via ERα (see below). It still remains possible that effects could occur via ERβ, which is expressed in fetal human Leydig cells (245).

Experimental studies in both rats and mice demonstrate that estrogens can directly inhibit testicular steroidogenesis in the fetus. For example, administration of DES or estradiol to pregnant dams (e10.5–e17.5 in mice; e13.5–e17.5 in rats) results in major reductions in fetal ITT levels at e18.5 in mice (246) and at e19.5 in rats (247). Although it is uncertain from these studies whether suppression of testosterone levels would have occurred during the masculinization programming window (Fig. 2), studies using explant cultures of e14.5 fetal rat testes suggest that this is likely (248). Consistent with this, studies in male offspring of pregnant mice exposed *in utero* (e12-e17) to DES or a range of doses of ethinyl estradiol reported a 40–57% incidence of hypospadias (249), although these authors attributed this to direct effects of estrogen on the developing penis because other lines of evidence point to such a possibility (132, 250). Nevertheless, inhibition of fetal Leydig cell steroidogenesis by high estrogen exposure is proven unequivocally for rodents (251) and is associated with reduced expression of SF1, StAR, and CYP17 (246, 252, 253). Estrogen exposure in these situations *in vivo* in rodents does not appear to involve any change in LH secretion (247, 254). Moreover, it is clear that these effects occur via ERα because DES does not inhibit testosterone production *in vitro* by fetal testes from ERα knockout mice, nor does any suppression of CYP17 occur as in wild-type mice (252). These findings are also consistent with the strong expression of ERα in fetal Leydig cells in rodents (255, 256). The importance of timing of estrogen exposure was shown by a study in which rats were exposed to DES from e17 to e19 (257), which is after the masculinization programming window (Fig. 2). Although these animals had reduced Sertoli cell number and testis weight, as would be predicted because androgen-driven Sertoli cell proliferation occurs mainly late in gestation (8), AGD was unaffected, and there was no mention of any reproductive tract anomalies (257).

There is also another potential mechanism via which estrogens may interfere with androgen-dependent masculinization, based on studies in neonatal rats exposed to DES. These studies have shown that as well as suppressing testosterone production by the testis, a more profound effect involves almost complete loss of AR protein expression, and this is associated with impaired development of the reproductive tract (225, 258). It is unknown whether this mechanism can also be triggered by estrogen exposure during pregnancy, but if so, it could provide a mechanism via which estrogens could affect development of AR-dependent organs such as the penis, irrespective of any effect on testosterone production by the fetal testis, and this could apply to the human as well as to the rat.

The inhibitory effects of high fetal estrogen exposure on steroidogenesis in rodents may reflect a physiological role because ERα knockout mice show increased steroidogenic activity per Leydig cell and increased mRNA levels for StAR, CYP17, and CYP11 (254). Studies *in vitro* with

fetal rat testis explants cultured with ER antagonists point to a similar possibility (251). However, low-dose (oral) exposure of pregnant mice to 17β-estradiol levels more compatible with pregnancy levels failed to induce hypospadias in the male offspring (259).

In conclusion, high fetal exposure to estrogens can cause male reproductive tract anomalies in rodents, and it seems likely that this is due (at least in part) to the perturbation of androgen production by the fetal testis. The majority of evidence suggests that this effect does not happen in the human fetal testis, pointing to a fundamental difference between rodents and humans with regard to regulation/disruption of fetal testicular steroidogenesis.

B. Glucocorticoids

In the human, the main glucocorticoid is cortisol, and in the rat it is corticosterone. Glucocorticoids have been selected for discussion in this review because it is well recognized that elevated levels of glucocorticoids, in response to psychological or physical stress or disease, can result in suppression of testosterone levels in adult males (260), and there is tenuous evidence for a link between maternal therapeutic use of glucocorticoids and occurrence of hypospadias in the male offspring (261). Glucocorticoids are also used widely in the treatment of a range of disorders, including during pregnancy. The majority of data describing the effects of elevated levels of glucocorticoids on testosterone production and possible mechanisms come from studies in adults, and there are only limited data for fetal effects. Although the human fetal adrenal is not as active in glucocorticoid production as is the adult adrenal, it does produce cortisol at the start of the masculinization programming window, before becoming a principal producer of Δ^5-3β-hydroxysteroids (262). Cortisol production by the adrenal at this age may be important in the human female because it is thought to lead to suppression of adrenal androgen production via negative feedback on ACTH, which minimizes the potential for masculinization via adrenal androgen production (262).

The human fetus may be exposed to elevated levels of glucocorticoids via several possible routes. It may be exposed to elevated levels of maternal cortisol, resulting from maternal stress (263). Additionally, synthetic glucocorticoids such as dexamethasone are often administered to pregnant mothers for various reasons, but especially if they are at risk of preterm labor, to prevent respiratory distress syndrome (264). After fetal exposure to dexamethasone for this reason, no differences in cord blood or postnatal infant testosterone levels were reported between dexamethasone-exposed and control fetuses (264). However, in such cases the fetal glucocorticoid exposure is usually much later in gestation than the presumptive masculinization programming window and is at a time when the fetal testis is not especially steroidogenically active (Fig. 2). Two studies have investigated possible correlations between fetal levels of cortisol and testosterone, one in fetal plasma (265) and one in AF (266), and both reported a positive, as opposed to a negative, correlation between levels of the two hormones. The results therefore contrast with the inverse relationship seen between cortisol and testosterone levels in adult men (see below) (267). Although it can be difficult to interpret associations and determine what is cause and effect, these results may suggest that the mechanism of interrelated control of the hypothalamic-pituitary-adrenal axis and testosterone production is different in the human fetus compared with the adult (265). What is unclear is whether the potential positive effect of elevated glucocorticoids on testosterone concentrations in the human fetus is mediated by LH or by intratesticular effects; studies from adults do not indicate important changes to LH secretion in situations where glucocorticoids reduce testosterone levels (267). Studies using fetal adrenal tissue *in vitro* indicate that dexamethasone does not stimulate androgen secretion, at least during the first trimester (262).

There are numerous studies in adult men on the effects of increased exposure to cortisol or dexamethasone, all of which show reduced testosterone levels with no preceding change in LH levels. A study in which 60 mg cortisol was administered at 1000 h and then 30 mg every 3 h, or 6 mg dexamethasone was administered at 1000 h and 3 mg every 6 h over a 24-h period, showed that testosterone was not suppressed until night time and that a rise in LH and FSH did not occur until after testosterone levels had fallen (268). A study in which 8 mg dexamethasone per day was administered for 3 d reported a significant reduction in testosterone and no change in LH levels (269). Chronic glucocorticoid therapy in older men (average age 60 yr) with chronic pulmonary disease suppressed serum testosterone levels with no change in basal gonadotropin levels (270). Finally, elevated levels of circulating cortisol as a result of insulin-induced hypoglycemia or administration of cortisol resulted in a rapid reduction in serum testosterone levels with no change in LH levels (267). This is also the case in men with Cushing's syndrome, who have high levels of cortisol and low levels of testosterone with no alteration in LH levels (271–273). All of these studies point to direct inhibition of Leydig cell steroidogenesis by glucocorticoids in the adult human testis.

Studies investigating fetal rat exposure to the synthetic glucocorticoid dexamethasone all demonstrate a concurrent reduction in testosterone. When pregnant rats were treated with 100 μg/kg dexamethasone during the last week of gestation, the male offspring exhibited signifi-

cantly reduced levels of ITT and modest, but significant, reductions in AGD on e21.5 (274) or on the day of birth (275), indicative of slightly reduced testosterone levels during the masculinization programming window (Fig. 2); these rats showed no change in plasma LH concentrations (275). In contrast, a study exposing pregnant rats to dexamethasone via their drinking water (10 μg/ml, therefore exposed to no more than 200 μg/d) from e15–e21 demonstrated that plasma testosterone levels were significantly reduced on e19 and e20, but not e18 (276). Acute treatment with dexamethasone on gestational d 18 resulted in fetuses (killed on e19) with significantly reduced plasma testosterone and LH (276). A single injection of dexamethasone (10 μg) after cesarean section on e21 suppressed plasma LH and the testosterone surge normally seen after birth when examined 30, 60, and 90 min after administration (276). Fetal rats exposed to maternal stress from e14 to e21 were investigated on e17, e18, e19, or e21 to determine levels of corticosterone, progesterone, and testosterone. Levels of corticosterone were at their highest between e17 and e20. Testosterone levels were higher in the stressed male fetal rats at e17, but then decreased so that the normal testosterone surge that occurred in the control males between e17 and e19 was absent. Although LH levels were not measured in this study, the authors suggested that the suppression of LH in the fetus by stress could explain the loss of an LH-dependent testosterone surge (105).

The foregoing results strongly indicate that dexamethasone alters direct or GnRH-mediated LH secretion to suppress testosterone production in fetal rats (276), which contrasts in several ways with both the fetal and adult data for humans. It is also possible that glucocorticoid exposure of the fetal rodent reduces Leydig cell steroidogenesis by inhibiting ACTH secretion (via negative feedback) because the fetal (but not adult) Leydig cells in the mouse contain active ACTH receptors and ACTH stimulates their testosterone secretion (277). However, it seems unlikely that ACTH is an important regulator of fetal Leydig cell steroidogenesis because mice lacking ACTH have normal fetal testosterone levels (277). Because the main mechanism for glucocorticoid suppression of fetal testosterone in the rat appears to involve suppression of LH, this means that glucocorticoids are unlikely to have major effects within the masculinization programming window (Figs. 2 and 3), and this fits with the modest reported reduction in AGD in dexamethasone-exposed fetal male rats (274, 275).

Several studies have investigated the effects of glucocorticoids on testosterone production in postnatal and adult rodents or in cell lines/cultures, and the majority of these data show, as in adult humans, that elevated glucocorticoid exposure inhibits testosterone production via a direct effect on Leydig cell steroidogenesis. For example, studies in immature (278, 279) or adult (280) rats showed that dexamethasone or corticosterone reduced testicular testosterone production, and similar effects can be induced in either adult mice (281) or rats (282) via immobilization stress, which increases endogenous corticosterone levels. The latter study in rats showed that the likely mechanism for this effect is reduced CYP17 enzyme activity (282), and this has been confirmed using primary cultures of rat Leydig cells isolated from hypophysectomized rats (283). Other studies using adult rat Leydig cell cultures have shown inhibition of 3β-HSD activity (284) or 3β-HSD and 17β-HSD mRNA expression and activity (285), and data in mice support this also (70). Additionally, two studies using MA-10 mouse tumor Leydig cells have shown that StAR expression is suppressed by glucocorticoids (286, 287), in a glucocorticoid receptor dependent manner (286). The adult rat Leydig cell is known to contain high levels of 11β-HSD type 1 (288), whereas it is widely accepted that it is 11β-HSD type 2 that inactivates glucocorticoids (289). Therefore, although it has been postulated that 11β-HSD expression may protect Leydig cell steroidogenesis from inhibition by glucocorticoids (290), this does not fit straightforwardly with the low expression of 11β-HSD type 2. Although fetal rat Leydig cells lack type 2 11β-HSD immunoreactivity, it is thought that fetal androgen production is probably protected from glucocorticoid-mediated inhibition by the high levels of 11β-HSD type 2 in the placenta (289). Presumably, dexamethasone reduces fetal testosterone levels not only because it is a poor substrate for rat 11β-HSD type 2, and consequently is not inactivated during passage across the placenta (291), but also because any 11-dehydrodexamethasone formed remains an active glucocorticoid.

Although the data in the fetal human is limited in comparison to rodents and is mainly indirect, it is apparent that elevated glucocorticoid levels may have opposing effects on human and rat fetal Leydig cells. Human fetal exposure to increased glucocorticoid concentrations appears to have no effect on testosterone production or may even increase it, whereas in fetal rats it suppresses testosterone production, perhaps via effects on LH; such a mechanism would only be able to operate later in gestation, after the masculinization programming window, because LH is the driver of steroidogenesis before this in the rat (Fig. 2).

C. Glitazones

The glitazones (also known as the thiazolidinediones) include rosiglitazone, pioglitazone, and troglitazone (the latter was withdrawn from the market in 2000 due to high risk of hepatotoxicity) (292). Through the activation of

peroxisome proliferator-activated receptors (PPARs), specifically PPARγ (293), the glitazones act to reduce insulin resistance and as such are used to treat type 2 diabetes (293). Glitazones are of interest in the context of this review because they can inhibit the steroidogenic enzymes CYP17 and 3β-HSD and reduce testosterone production in certain circumstances (294–296). An additional feature of interest is that interaction of glitazones with PPARs (297) is a feature that they share with phthalates (discussed below) (298), and some phthalates also interfere with testicular steroidogenesis. It has been shown that rosiglitazone transfers across the human placenta in the first trimester (299). Glitazones are also used to treat insulin resistance in women with polycystic ovarian syndrome (300), and in addition they reduce hyperandrogenemia (301–303). The potential mechanisms via which the glitazones exert their effects are most clearly established for the adult testis, so these are also considered as they may apply to the fetus.

A number of studies have reported human fetal exposure to rosiglitazone during pregnancy. The most recent study examined eight women with polycystic ovarian syndrome who used rosiglitazone before and during the first 12 wk gestation. All eight women gave birth to healthy babies at term, none of whom had congenital abnormalities (304). There have been two other cases of human fetal exposure to rosiglitazone during the first trimester, both taking rosiglitazone to control type 2 diabetes. Both women became pregnant and continued with rosiglitazone unaware of their pregnancy until 5 (305) or 7 (306) wk gestation. Both individuals discontinued the use of rosiglitazone upon discovering their pregnancies, and both gave birth to normal, healthy babies, one of whom was a male (305). Another case has been reported in which the individual knowingly took rosiglitazone during the second trimester of pregnancy. This woman had a history of type 2 diabetes that was controlled by diet and exercise until the 13th week of gestation, when she began treatment with rosiglitazone. This continued until the 17th week of gestation. The woman gave birth to a healthy male, with no major or minor malformations (307).

The aforementioned evidence suggests (indirectly) that human fetal steroidogenesis is not disrupted by exposure to rosiglitazone administered at the therapeutic dose of 4 mg/d (on average) because no reproductive abnormalities associated with suppressed androgen production were reported. However, this is based on small numbers of subjects (and male offspring), and not all male offspring were exposed during the relevant stage of gestation in which adverse (indirect) effects on testicular steroidogenesis might become evident. Evidence from studies in adult men or using *in vitro* systems suggests that glitazones can inhibit testicular steroidogenesis under certain circumstances.

Two studies have assessed the effect of rosiglitazone on testosterone levels in adult men, but the results are conflicting. Thus, both plasma testosterone and DHT levels were significantly reduced in 10 healthy men administered 8 mg/d (clinically relevant dose) of rosiglitazone for 7 d (296). In contrast, chronic treatment of 20 hypogonadal men with type 2 diabetes for 6 months with the same dose of rosiglitazone increased levels of total testosterone, free testosterone, and bioavailable testosterone, as well as increasing levels of SHBG (308). These apparently contradictory results are probably explained by the other effects of rosiglitazone on adiposity, insulin, and leptin levels after chronic treatment in the diabetic men. It is generally considered that the low levels of total testosterone observed in diabetic men (309) are a consequence of low levels of SHBG, which result from negative regulation by insulin (310). As well as increasing levels of SHBG in diabetic men, presumably by increasing insulin sensitivity, rosiglitazone may also increase levels of total testosterone by reducing visceral fat and thus lowering breakdown of testosterone by aromatase in adipose tissue (308). Obese men frequently exhibit higher levels of circulating leptin (311), and evidence from *in vitro* studies with cultured adult rat testicular tissue indicate that leptin inhibits basal and hCG-stimulated testosterone production through the inhibition of StAR and CYP11A (312).

In humanized yeast cells that express steroidogenic enzymes in a microsomal environment, it was shown that troglitazone, rosiglitazone, and pioglitazone all competitively inhibited CYP17 and noncompetitively inhibited 3β-HSD (294), and similar results were obtained with pioglitazone and rosiglitazone using human adrenal NCI-H295R cells (295). It was thought that the glitazones may inhibit testosterone production via PPARγ, as they exert their effect on insulin sensitivity via this receptor, because PPARγ is expressed in the human testis (313) but also because PPARγ has been shown to be activated by phthalates, which also act to reduce testosterone production (314). However, inhibition of PPARγ using small interfering RNA did not prevent the inhibition of CYP17 and 3β-HSD by pioglitazone (295), indicating lack of involvement of PPARγ in steroidogenic inhibition by glitazones. This is also supported by evidence that the ability of each of the glitazones to inhibit CYP17 and 3β-HSD does not correlate with their ability to increase insulin sensitivity (which is mediated via PPARγ), implying that they reduce testosterone and increase insulin sensitivity via two separate mechanisms (294). Evidence indicates that the MAPK kinase/ERK signaling pathways may be involved in the suppression of androgen biosynthesis by pioglitazone (295).

Exposure of pregnant rats to rosiglitazone (up to 600 mg/kg/d) from e7–e21 had no effect on testicular testosterone production or testosterone levels in male fetuses or on AGD, although plasma leptin and insulin levels were both reduced (315). Therefore, this finding is consistent with the human studies discussed above, but contrasts with the evidence pointing to inhibition of steroidogenesis by glitazones in adulthood in humans via inhibition of the steroidogenic enzymes CYP17 and 3β-HSD. There are, however, confounding factors involved in many adults using glitazones for the treatment of insulin resistance (obesity, increased leptin, and increased aromatase), and these may also affect steroidogenesis. The current data regarding rodent exposure to glitazones is limited and does not provide conclusive evidence that these compounds affect steroidogenesis.

D. GnRH/GnRH analogs

There has been considerable interest in the potential direct effects of GnRH and its agonists on Leydig cell steroidogenesis, largely because of the therapeutic use of these compounds for suppression of testicular steroidogenesis in adult men with prostate cancer (316). The latter suppression is thought to result from down-regulation of pituitary gonadotropin secretion due to overstimulation by GnRH agonists, but the possibility of direct Leydig cell effects emerged because studies in rats (see below) had clearly shown both stimulatory and inhibitory effects of GnRH agonists on fetal and adult Leydig cell function. There have been no studies of expression of GnRH receptors or GnRH agonist action on human fetal Leydig cells/testes, and initial studies in adult testes pointed to absence of GnRH receptors (317). However, more recent studies indicate that at least the mRNA for GnRH receptors is expressed in the adult human testis (318, 319). Despite this, the majority of the available data, based on various *in vivo* (320) and *in vitro* (321) approaches, suggest that there are no significant effects, either stimulatory or inhibitory, of GnRH agonists on Leydig cell steroidogenesis in adult humans (316). Similar conclusions were reached from studies in nonhuman primates (322, 323). Those studies that have suggested the possibility of direct effects of GnRH agonists on human testicular steroidogenesis have provided only tentative evidence and only for positive, not negative, effects (316, 324).

In contrast to the human, it is clear that, in the rat, fetal (325), neonatal (326), and adult (326, 327) Leydig cells all express GnRH receptors and that GnRH agonists can modulate steroidogenesis via interaction with these receptors in either stimulatory or inhibitory directions depending on age, the duration of exposure to GnRH agonist, and whether or not there is concomitant LH/hCG exposure. Of particular interest is that the stimulatory effect of GnRH agonists on Leydig cell testosterone secretion does not emerge until near the end of gestation (328) and becomes much more obvious for adult Leydig cells (329). Instead, the initial effect (e16.5–e18.5) of GnRH agonists on fetal testicular steroidogenesis in rats is uniformly negative (86, 328). Although potential stimulatory effects on basal testosterone secretion then emerge by e20.5 (328), in the presence of LH stimulation (*i.e.*, the normal physiological situation at this age; Fig. 2), the effect of GnRH agonist continues to be inhibitory in fetal (328, 330, 331) life through to adulthood (332) and results primarily from inhibition of CYP17 (330, 332). Although most of these data were obtained from studies *in vitro*, studies *in vivo* in hypophysectomized fetal (333), immature (334), and adult (334, 335) rats show similar effects to those *in vitro*. In contrast to these findings in rats, purified fetal (336) and adult (329) mouse Leydig cells are unaffected by GnRH agonists *in vitro*, including absence of attenuation of LH/hCG-stimulated testosterone secretion, a finding consistent with the reported absence of GnRH receptors on Leydig cells in this species (335). However, *in vivo* studies in adult hypophysectomized mice are conflicting with one study showing absence of effect of GnRH agonist on testosterone levels (335) and one showing a negative effect (337) after chronic exposure; it is difficult to reconcile the latter finding with the aforementioned *in vitro* studies, and it is possible that the effects observed were due to incompleteness of the hypophysectomy. Otherwise, it seems likely that mouse Leydig cells, including fetal Leydig cells, are resistant to inhibitory effects of GnRH agonists, in marked contrast to rats, a contrast that echoes that noted later for the effect of certain phthalates on fetal Leydig cell steroidogenesis.

E. Ketoconazole

Ketoconazole is an orally active broad-spectrum antifungal agent used in the treatment of skin and fungal infections. Ketoconazole inhibits the cytochrome P450 dependent 14-demethylase, which catalyzes the conversion of lanesterol to ergosterol in yeast and fungi (338). In addition to its antifungal activity, ketoconazole also inhibits several mammalian cytochrome P450-dependent enzymes, interfering with steroidogenesis in the testes, adrenals, and placenta (339–341). Because fungal infections are not uncommon in pregnant women (some infections such as candida vaginitis are more prevalent in pregnant women) (342), it is important to consider the risk of inhibiting steroidogenesis in the pregnant woman and fetus.

In mammalian systems, the P450 enzyme particularly sensitive to ketoconazole is CYP17. This enzyme catalyzes two reactions in testicular steroidogenesis, 17α-hydroxylation and the 17,20-lyase reaction (Fig. 1) (343, 344).

Both of these reactions are key steps in the Δ^4 and Δ^5 pathways (345), and it has been reported that the 17,20-lyase reaction is preferentially blocked by ketoconazole (346). Investigations into the effects of ketoconazole on fetal testicular testosterone biosynthesis have been undertaken in both humans and rats, and the difference in response highlights an intriguing difference in the susceptibility to inhibition between these two species.

Ketoconazole has been used in the treatment of Cushing's syndrome, an endocrine disorder caused by elevated blood levels of cortisol. Cushing's syndrome can have a variety of causes, including pituitary adenoma (known as Cushing's disease) and adrenal hyperplasia or neoplasia. Ketoconazole predominantly inhibits the 17,20-lyase reaction and inhibits 17α-hydroxylase and 11β-hydroxylase to a lesser extent, so although ketoconazole mainly blocks androgen synthesis, it also inhibits cortisol production (346). There have been at least two reports of ketoconazole being administered to pregnant women with Cushing's syndrome (347, 348), situations that cause high risk for both the mother and fetus. In the first case, ketoconazole was administered from the 32nd to the 35th week of gestation, and the infant was born by elective cesarean section at 37 wk. The infant was female and was normally developed at birth (347). This is not surprising because ketoconazole was administered during the third trimester, after organogenesis and, had the infant been male, after the predicted masculinization programming window (9). In the second case, however, ketoconazole was administered throughout pregnancy, apart from between wk 3 and 7, and the male infant was born normally, with no congenital abnormalities and with normal masculinization (348). Because the fetus was exposed to ketoconazole during the presumptive masculinization programming window (Fig. 2), this implies that perhaps the dose of ketoconazole used (600–1000 mg/d) was not high enough to inhibit testosterone production sufficient to perturb masculinization. There has also been a population-based study of fetal exposure to ketoconazole during the second and third trimesters (349). It was concluded that the data did not demonstrate a higher rate of congenital abnormalities after *in utero* exposure to ketoconazole (349), although in this instance exposure was later in gestation than the presumptive masculinization programming window.

In contrast to the *in vivo* studies above, *in vitro* studies involving exposure of human (14–20 wk gestation) or rat (e19.5) fetal testis explants to 100 μM ketoconazole demonstrated that, whereas human testicular testosterone production was significantly reduced, ketoconazole appeared to have no or highly variable effects on the fetal rat testis (350). Another *in vitro* study also demonstrated that human fetal testes (7–12 wk gestation) exposed to 4 μM ketoconazole for 4 d caused significantly reduced testicular testosterone production from d 2 onward (99). The latter finding suggests that sufficient exposure of the human fetus to ketoconazole during the masculinization programming window would be likely to impair steroidogenesis and potentially affect masculinization. The absence of any such effects in human males exposed *in utero* to ketoconazole is presumably because in such cases exposure did not coincide with the presumptive masculinization programming window (Fig. 2) or that exposure was too low to cause significant steroidogenic effects.

Other studies have administered ketoconazole to pregnant rats; however, the reported outcomes are contradictory. Two separate studies exposed pregnant rats to up to 50 mg/kg/d ketoconazole, one study from e14 to lactational d 3 (351), and the other from e7 to e21 (352), and both reported a high rate of litter loss. However, only one of the studies reported a significant reduction in testicular testosterone production and an effect on masculinization, namely a reduction in male AGD (352). It is possible that the difference in response to ketoconazole in these two studies reflects the use of different rat strains (Wistar *vs.* Sprague Dawley). It has been reported that ketoconazole crosses the rat placental barrier and that elimination is very slow from fetal membranes (www.apotex.com/ca/en/products/downloads/di/0105_PIL.pdf). However, it is equally likely that the particularly high rates of fetal death and growth seen in these rat studies have confounded the results. Either way, the available evidence indicates that human fetal testicular steroidogenesis is more susceptible to perturbation by ketoconazole than that of the fetal rat. Why this is the case remains to be answered, but a logical explanation could be that ketoconazole has a preference for inhibiting the human CYP17 activities involved in the Δ^5 pathway, which is the preferred steroidogenic pathway in the human fetal testis but not in the rat (Fig. 1), making human testicular steroidogenesis more vulnerable to inhibition by ketoconazole.

As just noted for the fetus, it is also apparent that adult human testicular steroidogenesis is more sensitive than rodent steroidogenesis to inhibition by ketoconazole. A study comparing the sensitivity of adult human, rat (Wistar), and dog testicular cells to ketoconazole reported that human cells were more sensitive to ketoconazole than rat and dog cells (343). This was confirmed by *in vivo* studies that showed that the dose of ketoconazole required to reduce plasma testosterone levels in humans was only 5 mg/kg (339), compared with 10–15 mg/kg in the dog (353, 354) and 24 mg/kg in the rat (355). It has also been stated that the affinity of ketoconazole for testicular P450 enzymes is higher in humans than in rats (356). As for fetal

steroidogenesis, it seems plausible that the species differences in susceptibility to inhibition by ketoconazole in the adult testis reflect the differences in preferred pathways via which testosterone is produced in the human (Δ^4 pathway) *vs.* the rat (Δ^5 pathway) (46), with the former being more susceptible to inhibition (Fig. 1).

F. Prochloraz

Prochloraz, like ketoconazole, is an imidazole fungicide. Prochloraz is used for crop protection because it inhibits CYP51 and thus weakens the fungal cell membrane (357, 358). Consequently, prochloraz was chosen for consideration in this review due to the high probability that it will work through similar mechanisms to ketoconazole, plus existing evidence that prochloraz can affect steroidogenesis in the rat and that human exposure is highly likely because it is registered for use in Europe and Australasia (http://pesticideinfo.org/Detail_Chemical.jsp?Rec_Id=PC36352).

There are no available data for human exposure to prochloraz during pregnancy, but two *in vitro* studies have exposed H295R cells (human adrenocortical carcinoma cells) to prochloraz (359, 360). The results from both of these studies match the results after exposure of H295R cells to ketoconazole (see *Section VI. E*), namely progesterone levels were significantly increased, and testosterone levels were significantly reduced (359, 360). Evidence from fetal studies with ketoconazole and prochloraz in rats (see below) suggests that this occurs via inhibition of CYP17 activity.

There have been several studies on the effects of prochloraz on fetal rodent steroidogenesis. Pregnant Wistar rats exposed to 30, 50, or 150 mg/kg/d prochloraz from e7–e21 all exhibited a significant reduction in plasma and testicular testosterone production and an increase in progesterone at e21 (360, 361). Prenatal exposure of Sprague Dawley rats to 62.5, 125, or 250 mg/kg/d prochloraz from e14–e18 also resulted in a significant reduction in *ex vivo* testosterone production (362–364) and increased progesterone (362, 363). Exposed male pups also presented with reduced AGD, nipple retention, and severe hypospadias (363), resulting not only from prochloraz-induced suppression of androgen production, but because prochloraz is also an AR antagonist and competes with testosterone and DHT for binding to the AR (360, 363, 365).

The reduction of testosterone and increase in progesterone indicate that prochloraz impairs steroidogenesis by inhibiting CYP17 conversion of progesterone to testosterone (362). However, analysis of gene expression after *in utero* exposure to prochloraz has suggested that there are no effects on SRB1, StAR, CYP11a, or CYP17 expression in testicular tissue (360, 362). However, microsomal CYP17 hydroxylase activity was significantly reduced, indicating that although prochloraz does not down-regulate expression of any of the genes involved in steroidogenesis, it does directly inhibit CYP17 enzyme activity (362). The increase in 17α-hydroxyprogesterone indicates that in addition to inhibition of CYP17 hydroxylase activity, CYP17 lyase activity is also inhibited by prochloraz, although at high doses (125 mg/kg/d) evidence suggests that the inhibition of hydroxylase activity is more pronounced than is inhibition of lyase activity (366); this might indicate that the human will be less susceptible than the rat to inhibition of steroidogenesis (Fig. 1). The rodent data on postnatal prochloraz exposure points in a similar direction. A study exposing rats from postnatal d 23–42 or 23–51 showed that both serum and *ex vivo* testicular testosterone levels were significantly reduced and that serum levels of progesterone and 17α-hydroxyprogesterone were increased (366), consistent with inhibition of CYP17 activity. Prochloraz reduces testosterone production at a lower dose in the postnatal rat (15.6 mg/kg/d) (366) than in the fetal rat (31.3 mg/kg/d) (362). It remains a matter for speculation as to whether prochloraz is capable of inhibiting testicular steroidogenesis in the human fetus, especially because it is evident that the CYP17 enzyme in humans and rodents may exhibit somewhat different catalytic preferences, as described earlier.

On the whole, the data indicate that prochloraz can suppress testosterone production in the postnatal human and in the fetal and postnatal rat by inhibiting CYP17 enzyme activity. The rodent data suggest that adult steroidogenesis is more susceptible to prochloraz than is fetal steroidogenesis.

G. Statins

Statins are used not only for the treatment of hypercholesterolemia (367), but also for their ability to decrease cardiovascular risk (368). They reduce plasma cholesterol levels by interfering with cholesterol synthesis, inhibiting the HMG-CoA reductase enzyme that catalyzes the rate-limiting step in the biosynthesis of cholesterol, the conversion of HMG-CoA to mevalonate (369). They also reduce circulating LDL cholesterol by increasing the expression of LDL receptors in hepatocytes (370). Because cholesterol is the precursor of all steroid hormones, it is recognized that HMG-CoA reductase inhibitors (statins), which act to reduce intracellular free cholesterol levels, could have a negative effect on steroidogenesis (371, 372).

The data available for humans regarding inadvertent exposure to statins during pregnancy is limited (373). A handful of studies have investigated the effects of *in utero* exposure to statins and, although teratogenic effects have been reported, most notably affecting the central nervous system and limbs (374), there is no conclusive evidence that exposure to statins increases the risk of congenital abnormalities. One study reported that the rate of con-

genital abnormalities in children exposed to statins (simvastatin or lovastatin) *in utero* (3.8%) was comparable to that in the general population (3%) (375). More importantly regarding this review, it was also reported that in this study of 477 individuals exposed to statins during fetal life, only one was reported to have hypospadias (375), an incidence similar to that in the normal population (5).

Data from animal studies on the effect of exposure to statins during pregnancy is equally lacking in evidence for inhibition of fetal testicular steroidogenesis. Studies in rats and rabbits exposed to atorvastatin during gestation only showed developmental toxicity at doses high enough to induce maternal toxicity (376). Although pups and litter sizes were smaller (maternal body weight and food consumption were also reduced), there were no incidences of fetal malformations, which would presumably include disorders of the reproductive tract. A more recent study investigated exposure of rats to statins from e6 to lactation d 20. Despite being exposed to statins throughout the masculinization programming window, there were no reports of genital abnormalities or effects on fertility of the offspring (369).

Whether statins interfere with human gonadal steroidogenesis during adult life has also been investigated in a number of studies. Although two studies have described a decline in free testosterone (although levels were still in the normal range) (377) or in total and bioavailable testosterone (378), a greater number of studies have concluded that therapeutic doses of statins do not affect testicular steroidogenesis. Individuals have been exposed to provastatin, simvastatin, locastatin, or atorvastatin from 3–36 months, and all have reported no change in plasma total, free or hCG-stimulated testosterone, or plasma LH and FSH (379–386). Similarly, in adult rats exposed to simvastatin for 12 wk, no effects on basal plasma testosterone, LH, or FSH were reported (387), and *in vitro* no alterations in testosterone production by mouse Leydig cells were found after culture with lovastatin for 12 h (388). Therefore, based on analysis of studies involving fetal or adult exposure to statins, it appears that they do not significantly impair steroidogenesis in either humans or rodents, at least at therapeutic levels.

H. Phthalate esters (phthalates)

Phthalates, or phthalate esters, are a group of chemicals used principally as plasticizers and in the manufacture of a variety of products, including cosmetics and perfumes, medical equipment, pharmaceuticals, and building materials, although their use in other products (*e.g.*, toys) is now restricted in many countries (*e.g.*, the European Union). Indirect human exposure to phthalates is widespread because they are the most ubiquitous of all environmental contaminants, and additionally there may be direct exposure via personal care products, certain medicines, and polyvinyl chloride (PVC)-containing devices (389). The effects of phthalate exposure on perinatal testosterone production (assessed as AGD) in humans has been investigated in several studies, the results of which are conflicting.

The first cross-sectional study (in the United States) examined 85 boys at 2–36 months of age and found a negative correlation between AGD (corrected for body weight) and the level of certain phthalate metabolites, including monobutyl phthalate (MBP) and monoethyl phthalate (MEP), found in maternal urine during pregnancy (390). A recent expansion of this study to include a total of 106 boys has confirmed the negative correlation between AGD and maternal (urinary) levels of phthalates, including MEP, MBP, monoethyl hexyl phthalate (MEHP) and the further MEHP metabolites, monoethyl hydroxyhexyl phthalate and monoethyl oxohexyl phthalate (391). Both of these studies also demonstrated that AGD correlated to penile volume/length (390, 391) and the incidence of cryptorchidism (390), similar to rat studies (9). Another study of 73 pregnant Mexican women in a hospital-based cohort investigated the association between exposure to MEHP, monobenzyl phthalate, MEP, and MBP during pregnancy and AGD in male newborns (392). This study found a statistically significant association between MEP exposure and reduced AGD and also between monobenzyl phthalate exposure and reduced penis length and width. These studies are consistent with the possibility that perinatal phthalate exposure can inhibit testosterone production in the male fetus (during the masculinization programming window), resulting in reduced AGD and penile volume/length, as well as inhibiting normal testis descent; such effects are in broad, but not total, agreement with studies in rats detailed below. However, one point of disagreement is with regard to diethyl phthalate and its metabolite MEP. In the cited human studies, MEP was negatively associated with AGD in boys, whereas rats exposed *in utero* to MEP, at even very high doses, show no effect on either fetal testosterone levels or AGD (393, 394).

In contrast to the above-mentioned studies, a smaller (prospective) study in Taiwan, involving 33 boys, found no relationship between levels of MBP or MEHP, measured in AF or maternal urine, during pregnancy, and AGD of the male offspring (395). This finding fits with two *in vitro* studies that have investigated the effects of MBP or MEHP on human fetal testis testosterone production. In the first study, second-trimester human fetal testis explants were cultured with MBP in short-term culture, but there was no effect on basal or hCG-stimulated tes-

tosterone production (350). In the second study, exposure of first-trimester human fetal testis explants to MEHP in the presence or absence of LH/hCG found no effect on testosterone production or on steroidogenic enzyme expression (99). The latter study did, however, demonstrate that MEHP has negative effects on germ cells, as it did in fetal rat testis explants, which makes the lack of effect on testosterone production more convincing. There is, however, always concern with the reliability of *in vitro* systems, especially given that e19.5 fetal rat testis explants cultured with MBP exhibited no effect on testosterone production, in sharp contrast to the inhibition demonstrated *in vivo*, at this age (350). Therefore, at present, it is unclear whether or not phthalates exert inhibitory effects on steroidogenesis by the human fetal testis because the available data are conflicting, and the only supporting evidence is indirect (AGD). However, a recent study that involved administration of high doses of MBP [the active metabolite of dibutyl phthalate (DBP)] to pregnant marmosets for a 7-wk period, which included the presumptive masculinization programming window, found no evidence for masculinization disorders or altered reproductive organ size at birth or in adulthood (396). This finding is consistent with the absence of effect of MBP on steroidogenesis *in vitro* by fetal human testis explants (350).

Testosterone levels are elevated also in the first 3–5 months after birth in boys, with the source being fetal-type Leydig cells (169, 397). One study investigated whether neonatal exposure to phthalate metabolites in breast milk was associated with any change in blood hormone levels in corresponding sons at 1–3 months of age (398). Breast milk levels of MBP were found to be negatively correlated with free (biologically active) testosterone, and a positive relationship was also found between breast milk levels of monomethyl phthalate, MEP, and MBP and the LH:free testosterone ratio. This could indicate that testosterone production is impaired but has been compensated for by increased LH. Although this would be in general agreement with the findings described above for gestational phthalate exposure and AGD in sons, there is another potential explanation because a positive association was found between breast milk phthalate levels and SHBG levels in this study (398). An increase in SHBG would result in less free testosterone because more would be able to bind to SHBG, and this would result in an increase in LH because of reduced negative feedback, thus explaining the increased LH:free testosterone ratio observed without the need to invoke direct inhibition of steroidogenesis by the phthalates. Phthalates are known to act on the liver, which is also the source of SHBG, but it is unknown whether phthalate exposure affects SHBG production. Only one other study has previously investigated phthalate exposure and SHBG levels (in adult men), and it found no effect (399).

Perhaps the best data to support the view that phthalates can directly inhibit steroidogenesis by the neonatal (human) testis come from a study in which neonatal male marmosets were administered a high dose (500 mg/kg/d) of MBP (350). This resulted in an initial approximately 50% reduction in testosterone levels 5 h later, followed by compensation, presumably by increased LH secretion, because increased Leydig cell numbers/volume were found in all MBP-exposed infants after 14 d of MBP treatment (350). The marmoset produces SHBG (400), but there have been no investigations into the effects of phthalates on SHBG levels in this species, so it is not possible to exclude an effect of MBP on SHBG levels in this study. However, it seems intuitively unlikely that an approximately 50% decrease in testosterone levels 5 h after MBP exposure could be explained by an effect on SHBG because changes in SHBG tend to be sluggish, not rapid. Studies utilizing rodent models of neonatal phthalate exposure cannot be used to clarify this issue because rodents do not produce SHBG. Demonstration of a (transient) inhibitory effect of MBP on testosterone production in neonatal male marmosets may appear at odds with the absence of any evidence for masculinization defects after similar exposure *in utero* (396). However, these findings would be reconciled if, in both situations, high exposure to either CG (pregnancy) or LH (neonatal-compensatory increase) was able to overcome any MBP-induced inhibition of steroidogenesis. If so, the same would be likely in the human, although none of the limited human *in vivo* or *in vitro* studies described above support this interpretation.

Studies that have investigated exposure of adult men to phthalates also show conflicting results with regard to possible effects on steroidogenesis. A study of men working in a PVC factory, and thus occupationally exposed to phthalates, showed a modest and significant reduction in serum free testosterone in workers with high levels of urinary MBP and MEHP, compared with unexposed workers (401). However, a cross-sectional study of 295 men attending an andrology clinic in Massachusetts found no association between phthalate levels in urine and serum levels of testosterone (399), although in this instance phthalate exposure would have been notably lower than for the PVC workers. Moreover, if the rat is any guide, then adult human Leydig cells may be relatively insensitive to the effect of phthalates. Thus, treatment of prepubertal rats with 200 mg/kg/d diethyl hexyl phthalate (DEHP) from postnatal d 21–35 caused a 50% reduction in serum testosterone levels (402), whereas the same or higher doses administered to adult rats had little or no effect (402, 403). Although one study has shown a massive inhibitory effect

(>90%) of MEHP on LH-stimulated testosterone production by adult rat primary Leydig cells over 2 h of culture, this was found only after exposure to 1 mM MEHP, and no effect was found with a 10-fold lower dose (404).

In contrast to the data in humans, there is unequivocal evidence that certain phthalates can profoundly inhibit testosterone production by the fetal rat testis. Thus, administration of phthalates to pregnant rats during the last week or so of gestation results in reduced AGD and reproductive malformations, including hypospadias, in the Long Evans (351), Sprague-Dawley (405), and Wistar (406) strains of rat. These changes are consistent with reduced fetal androgen exposure, and this has been demonstrated directly after *in utero* exposure to a number of phthalates, including DEHP (364, 393, 407), DBP (364, 408–410), butyl benzyl phthalate (364), diisobutyl phthalate (315), diisononyl phthalate (393), and diisoheptyl phthalate (411). Several of these compounds have been shown to cause dose-dependent suppression of fetal testicular testosterone production, and for the most potent (DEHP and DBP), this occurs at doses above 100–250 mg/kg/d. Other phthalates, such as diethyl phthalate, dimethyl phthalate, dioctyl phthalate, and diisodecyl phthalate, do not affect fetal rat testicular testosterone production or AGD (393, 394). Molecular analyses in fetal rat testes after *in utero* exposure to phthalates has shed light on the potential mechanisms via which phthalates suppress testicular testosterone production. Several of the key genes involved in steroidogenesis (Fig. 1) are down-regulated after *in utero* exposure to DBP or MEHP. These genes are StAR, HMG-CoA synthase, and SRB1 (all involved in cholesterol uptake/transport), and the steroidogenic enzymes *Cyp11a*, *3β-Hsd*, and *Cyp17* (410, 412–414). Suppression of these various enzymes provides a convincing explanation for the phthalate-induced reduction in fetal testicular testosterone production.

Based on present evidence, it appears that all of the phthalates that affect testosterone production by the fetal testis do so by similar mechanisms, although the dose-response characteristics may differ; in this regard, DBP and DEHP are the most potent and are approximately equipotent, based on the above cited studies.

In contrast to the consistent effects of DBP and DEHP on fetal testicular testosterone production in the rat, data for exposure of fetal mice to DBP or DEHP have produced equivocal results. A detailed study showed that administration of single or multiple doses of DBP (up to 1500 mg/kg/d) or MEHP (up to 1000 mg/kg/d) to pregnant mice did not reduce testicular testosterone levels or affect the expression of the steroidogenic enzyme genes, as seen in the rat (415); this was shown in two strains of mice (C57Bl6, G3H/HeJ). In contrast, a recent study in C57Bl6 mice treated with 100, 200, or 500 mg/kg/d DEHP from e12–e17 reported that this dose-dependently induced hypospadias on e19, with males from the top dose group exhibiting a 75.7% incidence of hypospadias and a 13% reduction in AGD (416). In addition to conflicting with the study by Gaido *et al.* (415), this study also conflicts with data for the rat exposed to similar levels of DBP or DEHP because effects on AGD (measured postnatally) are of similar magnitude to those reported for the mice, but the rates of hypospadias reported are considerably less in the rats, ranging from 12–37% (393, 408, 417), an observation that is explained by the relatively poor suppression of testosterone levels by DBP in rats during the masculinization programming window (8). No other toxicological studies involving fetal exposure of mice to phthalates that cause fetal testis effects in rats have reported hypospadias (418, 419), although it is uncertain whether or not this was specifically sought. Another study has reported that DEHP can reduce insulin-like factor 3 mRNA expression by fetal mouse Leydig cells *in vivo* and *in vitro*, but effects on steroidogenesis were not studied (420). The conflict over fetal testis effects of phthalates in mice is not clarified by studies on isolated Leydig cells from postnatal mice because positive effects on steroidogenesis have been reported (421), whereas negative effects of MEHP have been reported on MA-10 tumor Leydig cells (422). However, a very recent study (423) perhaps reconciles these disparate findings for phthalate effects in mice. It shows that whether MEHP has inhibitory or stimulatory effects on steroidogenesis in fetal mouse testes cultured over 1–3 d depends on fetal age, culture duration, and the presence/absence of LH. Notably, no inhibition of basal testosterone production was observed at any age, but in particular at e13.5 (corresponding to part of the masculinization programming window), and this is in marked contrast to the studies in rats.

I. Linuron

Linuron is a urea-based herbicide that acts as an antiandrogen via dual mechanistic actions. Linuron was first acknowledged to competitively antagonize rat and human AR and inhibit androgen-induced gene expression (365, 424, 425). However, more recent studies have shown that linuron also reduces fetal testosterone levels in the rat (364, 426), although there are no relevant data on human fetal exposure to linuron. In rats exposed *in utero* to doses ranging from 12.5–100 mg/kg/d linuron from e13–e18 or e14–e18, significant reductions in whole body testosterone levels (426), testicular testosterone concentrations, and testicular testosterone production (426) were reported at e18. Furthermore, one of the studies showed that linuron could similarly inhibit testosterone production by fetal rat testes *in vitro* (427). Unlike phthalates, this sup-

pression of steroidogenesis did not involve altered mRNA expression of StAR, Cyp11a, or Cyp17a expression (427). Unlike fetal exposure to prochloraz (364), however, progesterone levels are not increased after fetal exposure to linuron, indicating that inhibition of steroidogenesis must occur before CYP17 activity (364) and, indeed before StAR, based on the mRNA expression profile (427). Therefore, the mechanism via which linuron interferes with fetal testosterone production remains unknown, but it could involve effects on LH receptor expression or activation. As was the case with human fetal exposure, there are currently no data on human adults after exposure to linuron. There are, however, a small number of studies that have exposed postnatal rats to linuron, although the results from these studies are not in full agreement with each other. One study exposed immature and mature rats to 200 mg/kg linuron for 2 wk and reported increased LH and estradiol levels but no change in testosterone levels (424). However, another study in which 150 mg/kg linuron was administered to adult male rats for 15 d reported reduced serum levels of testosterone, DHT, and LH and increased estradiol (428). Linuron has also been administered chronically to rats postnatally at doses of 10, 20, and 40 mg/kg, and in these animals, mean serum testosterone was reduced by 44%, but this was not statistically significant (351).

J. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD; dioxin) and related compounds

Dioxin is a highly toxic by-product of many combustion processes (*e.g.*, by industrial incinerators) to which humans are exposed, and it has been shown in animal studies to affect development and function of the male reproductive system (see below). Two major accidents (Seveso, Italy, 1976; and Yucheng, Taiwan, 1979) have occurred that resulted in especially high exposure to polychlorinated dibenzofurans or polychlorinated dibenzo-*p*-dioxins (dioxin) in humans, and both included pregnant women. Subsequent evaluations of males exposed *in utero* in these situations have not reported any masculinization disorders, although one male showed reduced sperm motility/morphology (429) and another showed significantly reduced sperm counts (430). The latter could reflect reduced Sertoli cell number as a result of reduced androgen action perinatally (Fig. 4), but presumably this did not include any major inhibition of testosterone production during the masculinization programming window because of the absence of genital disorders.

Several studies in rats have used a single (maternal) administration of a range of doses of dioxin on e15.5 (*i.e.*, at the start of the masculinization programming window) and variously reported small decreases in adult testis size/sperm counts and reductions in size of the penis, prostate, seminal vesicles, and AGD (431–434). The latter effects are all consistent with suppression of androgen production/action during the masculinization programming window (9), although this has not been demonstrated directly. One study (433) has shown dioxin-induced suppression of fetal plasma testosterone, and others have shown suppression of StAR (435) or CYP17 (436) mRNA expression later in gestation (e18.5 or e20.5), but these later gestation changes may be secondary to dioxin-induced suppression of LH secretion (437) and can be prevented by *in utero* administration of equine CG (435, 436). Although the dioxin effects appear repeatable, there are notable strain differences in the response of rats that may explain why one study reported that dioxin exposure increased fetal plasma testosterone levels (247). Moreover, in contrast to rats, mice appear largely unaffected by fetal dioxin exposure (438).

The much milder phenotypic consequences of fetal dioxin exposure in humans compared with rats could reflect species differences or could be because the human studies did not segregate men according to the gestational age at which their dioxin exposure occurred (most were exposed too late for any effect in the masculinization programming window). Alternatively, even the accidentally high dioxin exposure in the human studies may have been below that necessary to trigger any effects. Irrespective of the explanation, the animal experimental studies illustrate that both species and gestational age are important determinants of effects on steroidogenesis by the fetal testis and the mechanisms of effect.

VII. Conclusions and Unanswered Questions

The masculinization process in humans and rodents is remarkably similar, and, insofar as we understand them, the mechanisms involved and their complete dependence on androgen production/action are more or less the same (9). Despite this, there are notable differences in the stimulatory mechanisms for testicular steroidogenesis between humans (CG/LH-dependent) and rodents (CG/LH-independent) during the period when masculinization of the reproductive tract is being programmed, and this is reflected in differing consequences when LH receptor function is abnormal. Such contrasts highlight the crucial dependence of steroidogenesis by the fetal rodent testis on paracrine factors, whereas such factors are either unnecessary or of minor or transient importance in the human. It is remarkable that the identity of the "physiological" paracrine regulators of fetal testis steroidogenesis in rodents remains so poorly understood, and this is an important issue to resolve. Identification of these factors would make it easier to identify what factors can disrupt their

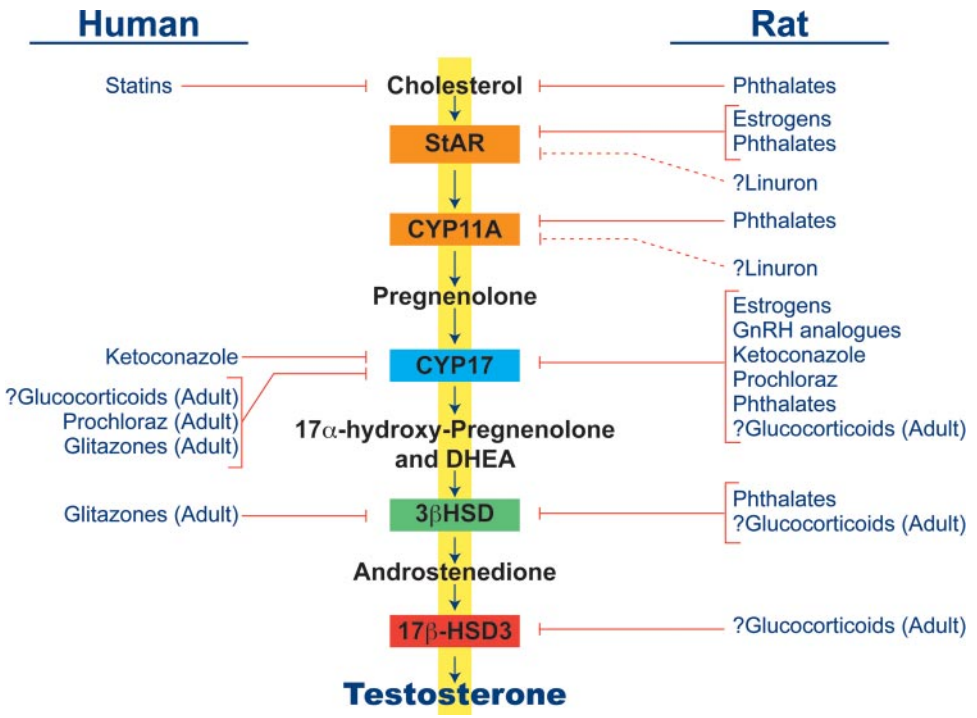

FIG. 5. Summary diagram that compares the known points in the steroidogenic cascade at which therapeutic and environmental compounds can impact negatively on testosterone production by the fetal testis in the human and rat. Where the only information available derives from study of the adult testis, this is indicated in *parentheses*. A site of action for dioxins in rats is not shown because its inhibition of StAR and CYP17 may be secondary to LH suppression. Note that Δ^4 and Δ^5 pathways are not shown in this diagram for the sake of clarity but are illustrated in Fig. 1 for the human and rats/mice. Note that this summary diagram does not apply in every instance to mice because GnRH agonists, phthalates (and dioxins) are largely without effect in this species (see *Sections VI. D, VI. H, and VI. J*).

function as well as paving the way for evaluating their importance in the human, and if so, when they occur in fetal life.

The process of steroidogenesis in humans and rodents is not identical with, for example, the Δ^5 pathway being preferred in the human and the Δ^4 pathway in rodents (Fig. 1), and this can affect sensitivity to inhibition by compounds such as ketoconazole and prochloraz (Fig. 5). Much starker differences are evident in the susceptibility of testicular steroidogenesis to inhibition by other factors, such as estrogens and GnRH analogs, both of which markedly inhibit in the rat but appear to be devoid of effect on the fetal human testis (Fig. 5), although this is based on rather few studies for the human. Nevertheless, the coincidence of absence of effect with absence of demonstrable receptors for GnRH and for ERα in fetal human Leydig cells is consistent and suggests that, for example, susceptibility of masculinization to disruption by environmental estrogenic chemicals may not be a concern, at least via suppression of steroidogenesis.

Arguably the most important unresolved issue is whether phthalates or their metabolites can inhibit testicular steroidogenesis by the fetal human testis as occurs in rats. Present conclusions based on (indirect) associations between maternal phthalate exposure and lower AGD in resulting sons, indicating potential effects at environmental phthalate levels in humans (and by a wider range of phthalates than in rats), contrasts with the absence of effect of MBP/MEHP on steroidogenesis *in vitro* by human fetal testis explants. Straightforward interpretation of these conflicting results is not currently possible, but because of the substantial human health implications, it is vital that further studies are able to resolve which of the findings is correct. However, this uncertainty over phthalate effects also illustrates the practical difficulties inherent in assessing whether compounds affect the human fetal testis because this has to depend either on indirect assessment (association of maternal exposures with masculinization disorders, such as reduced AGD, in resulting sons) or on studies using fetal testis cultures, both of which present interpretation problems. In the case of indirect associations with AGD, account has to be taken of the fact that humans are exposed simultaneously to many environmental chemicals, including multiple compounds that may interfere additively with androgen action/masculinization according to animal studies (394). Therefore, separating phthalate effects from those of these other compounds poses difficulties. Conversely, although fetal human testis cultures have the merit that direct effects of phthalates and their metabolites can be studied, confidence in negative data derived from such cultures is only complete if the *in vitro* system can be shown to accurately

reflect what occurs *in vivo*. Studies using grafts of fetal testis tissue into nude mice that are then treated with phthalates may represent a compromise approach for future studies but may still leave room for uncertainty.

Perhaps one of the most surprising outcomes of the present comparison is the fundamentally different response of rat and mouse fetal Leydig cells to certain compounds. Thus, in rats, GnRH agonists, certain phthalates, and dioxins all impair steroidogenesis by fetal Leydig cells, whereas these same factors have minimal or no effects on mouse fetal Leydig cells. In other respects, for example their response to estrogens, Leydig cells from both species show a similar response. Considering also the fundamental differences in response of rat and human fetal Leydig cells to some of the evaluated compounds, it is evident that considerable care should be exercised in extrapolating effects from one species to another if no direct data are available.

It is apparent from the lack of effect of statins (at therapeutic doses) on fetal testicular steroidogenesis in either rodents or humans that compounds that target cholesterol supply are probably benign in their steroidogenic effects because alternative sources of cholesterol can be used. However, once the supply route narrows down to just one pathway, as with the transport of cholesterol to the inner mitochondrial membrane via StAR, any disruption has marked effects, as illustrated by mutations in the StAR gene. A similar remark probably applies also to the enzyme cascade downstream from StAR because there are no alternative pathways. More compounds target CYP17 and impair its expression/function than any other step in testicular steroidogenesis, although it is unclear why this is so, apart from the fact that, physiologically, it is also a highly regulated step.

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