Chapter 9
Orchestration of the Synthesis of Sex Hormones and their Roles in Establishing Sex Differences in Mammals

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Abstract This chapter presents classical knowledge as well as recent findings on sex hormones. First, the synthesis of hormones is reviewed, and a tight linkage with core energy metabolism is introduced. The reader can thus realize the presence of a sophisticated regulation system governed by a single transcription factor, Ad4BP/SF-1. Several examples of the important roles of sex hormones in reproduction and the establishment of sex differences in multiple organs/cell types are introduced. Furthermore, the latest knowledge on sex differences in skeletal muscle and the adrenal cortex is presented. Finally, the involvement of sex hormones in establishing the sex spectrum is discussed.

Keywords Sex hormones · Sex differences · Skeletal muscle · Adrenal cortex

9.1 Sex Hormones

9.1.1 Discovery of Sex Hormones

Sex hormones are key molecules in endocrine regulation that induce sex differences. The concept of a "sex hormone" was first provided by Berthold et al. in 1849 (& NA, 1996). They observed the disappearance of male-specific larger crests and male-specific behavior from roosters on the removal of their testes. These male-specific characteristics were recovered by the re-implantation of the testes in the castrated roosters. These observations revealed the presence of humoral factors secreted from the testis that were necessary for maintaining male-specific characteristics. Currently, androgens and estrogens are known to induce male and female characteristics.

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M. Tanaka, M. Tachibana (eds.), *Spectrum of Sex*,
https://doi.org/10.1007/978-981-19-5359-0_9

9.1.2 Regulation of Sex Hormone Synthesis

Sex hormones are primarily synthesized in the testis and ovary using cholesterol as a starting material. As CYP11A1 (P450 side-chain cleavage), an enzyme that mediates the first step of steroidogenesis, localizes in the inner mitochondrial membrane, cholesterol is transported to the specific location to be utilized for the synthesis. The transportation of cholesterol from the outer to the inner mitochondrial membrane has been shown to be mediated by steroid acute regulatory protein (STAR). The delivery of cholesterol from the endoplasmic reticulum, where cholesterol is synthesized de novo to the outer mitochondrial membrane, was recently shown to be mediated by MGARP (also known as OSAP or HUMMR) (Jinn et al. 2015). A potent androgen, testosterone, is synthesized in testicular Leydig cells from cholesterol through four enzymatic reactions, mediated by CYP11A1, HSD3B (3β-Hydroxysteroid dehydrogenase), CYP17A1 (17α-hydroxylase/17,20-lyase P450), and HSD17B3 (17-β-Hydroxysteroid dehydrogenase type III) (Miller 1988, 2013; Waterman and Bischof 1997; Morohashi et al. 2013) (Fig. 9.1). Synthesized testosterone is secreted into the blood to be delivered to the entire body; in target cells, testosterone is converted to a more potent androgen, 5α-DHT, by SRD5A1/2. In the ovary, androstane-3,17-dione produced in theca cells is transferred to granulosa cells, where CYP19A1 (aromatase P450) mediates the conversion of androstane-3,17-dione to estrone. Thereafter, HSD17B1 (17β-Hydroxysteroid dehydrogenase type I) mediates the conversion of estrone to a potent estrogen, 17β-estradiol.

9.1.3 Orchestration of Sex Hormone Synthesis by Ad4BP/SF-1

Interestingly, all genes encoding the above enzymes (*Cyp11a1*, *Hsd3b1*, *Cyp17a1*, *Hsd17b1/3*, and *Cyp19a1*) and *Star* genes have been reported as the targets of a transcription factor, Ad4BP/SF-1 (Ad4-binding protein/steroidogenic factor-1, NR5A1) (Morohashi et al. 1992; Val et al. 2003; Hammer et al. 2005; Hoivik et al. 2010). Thus, it is considered that Ad4BP/SF-1 is a master regulator of sex hormone synthesis in gonads, which governs the transcription of all genes involved. It should be noted that *Mgarp* genes are also directly regulated by Ad4BP/SF-1 (Baba et al. 2018).

Cholesterol is delivered into peripheral cells, including the testis and ovary, through blood flow. In addition, cholesterol can be synthesized de novo using acetyl-CoA as a starting material. Twenty enzymatic reactions are necessary to produce cholesterol from acetyl-CoA. The transcription factor SREBP-2 is known to transactivate all genes involved in cholesterogenesis in response to lowered cholesterol content in the cytoplasm (Freeman and Ascoli 1982; Brown and Goldstein 1999; Horton et al. 2002). In addition, we recently found that *Ad4BP/SF-1* knockdown resulted in a decrease in the expression of all 20 cholesterogenic genes

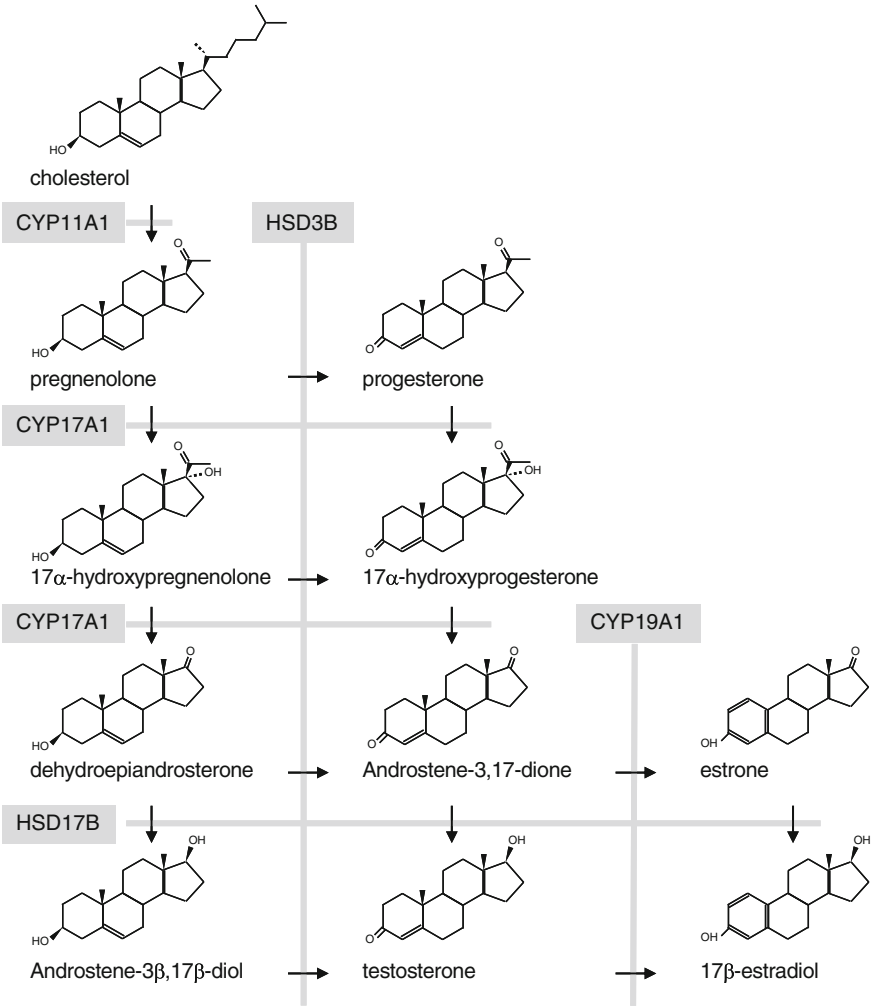

Fig. 9.1 Synthetic pathway of sex hormones from cholesterol

(Baba et al. 2018). Moreover, ChIP-sequencing revealed that Ad4BP/SF-1 bound to many cholesterogenic gene loci. As expected, Ad4BP/SF-1 was observed to physically interact with SREBP-2, and this interaction was shown to be necessary for the high-level expression of cholesterogenic genes. Together, these results indicate that Ad4BP/SF-1 is involved in the regulation of cholesterogenic genes in cooperation with SREBP-2, at least in steroidogenic cells.

How is acetyl-CoA supplied for cholesterogenesis? In addition to fatty acid β-oxidation, glycolysis is considered a major acetyl-CoA-supplying pathway. The final product of glycolysis, pyruvate, is converted to acetyl-CoA by the pyruvate dehydrogenase complex in the mitochondrial matrix; thereafter, it is used as a carbon

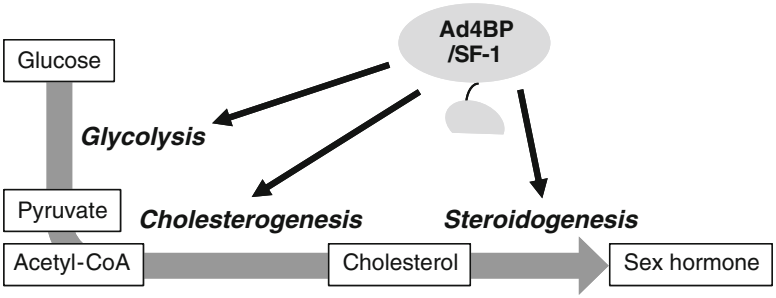

Fig. 9.2 Orchestration of multiple metabolic pathways by Ad4BP/SF-1. Ad4BP/SF-1 regulates expression of all genes involved in glycolysis, cholesterogenesis, and steroidogenesis, thereby governs the synthesis of sex hormones from glucose

source in the TCA cycle. One of the intermediates of the TCA cycle, citrate, can be translocated to the cytosol, where it is converted back into acetyl-CoA by the function of ACLY (Chypre et al. 2012). We demonstrated that *Acly* is directly regulated by Ad4BP/SF-1. We also examined the involvement of Ad4BP/SF-1 in glycolysis. Consequently, transcriptome analysis and ChIP-sequencing showed that Ad4BP/SF-1 regulates all glycolytic genes (Baba et al. 2014). Furthermore, *Ad4BP/SF-1* knockdown lowered glycolytic activity. Taken together, these findings suggest that Ad4BP/SF-1 governs sex hormone synthesis by orchestrating multiple metabolic pathways from glucose to sex hormones (Fig. 9.2). Notably, the synthesis of sex hormones is tightly linked to core energy metabolism.

9.2 Sex Hormone Receptors

How do sex hormones exert their functions? Unlike other hormones for cell membrane receptors, sex hormones are lipophilic and can transverse the cell membrane to reach the cytoplasm. In the cytoplasm, sex hormones bind to their specific receptors, androgen receptor (AR) and estrogen receptors (ERα and ERβ), to transduce signaling. These sex hormone receptors belong to the nuclear receptor superfamily (Olefsky 2001). Nuclear receptors have a common structure with five functional domains: an N-terminal regulatory domain, a DNA-binding domain, a hinge region, a ligand-binding domain (LBD), and a C-terminal regulatory domain. Upon the binding of ligands, including sex hormones, to the LBD, the receptors undergo conformational changes to become active. The activated receptors translocate into the nucleus where they bind to their cognate DNA sequences and induce the transcription of adjacent genes. The cDNA of the estrogen receptor was cloned by Pierre Chambon in 1985, and the gene product is presently known as ERα (Walter et al. 1985). Another gene encoding β type of the receptor (ERβ) was cloned from rat prostate, human testis, and mouse ovary in 1996–1997 (Kuiper et al. 1996; Mosselman et al. 1996; Tremblay et al. 1997). The cDNA of AR was cloned in

1988 (Faber et al. 1991). cDNA cloning and determination of the structural organization of the genes have provided valuable tools to elucidate the functions of sex hormone receptors in the androgen and estrogen signaling pathways. Indeed, a number of knockout mice have been generated to elucidate the functions of sex hormones in multiple organs/cells.

9.3 Roles of Sex Hormones in Reproduction

9.3.1 Role of Testosterone in Male Secondary Reproductive Organs

Testosterone is produced by testicular Leydig cells in adult males. In contrast, in fetal males, androstane-3,17-dione produced by Leydig cells is transferred to Sertoli cells and converted to testosterone (O'Shaughnessy et al. 2000; Shima et al. 2013). Thus, both Leydig cells and Sertoli cells are necessary for testosterone production during the fetal stage. In mice, Sertoli and Leydig cells differentiate at embryonic day 11.5 (E11.5) and E12.5, respectively. The produced testosterone plays a role in the development of secondary reproductive organs of the male type, such as the epididymis, vas deferens, and seminal vesicles. All these organs are derived from the Wolffian ducts. Originally, both fetal males and females have two ducts: the Wolffian duct and Mullerian duct. When Sertoli and Leydig cells differentiate, the anti-Mullerian hormone (AMH) secreted from Sertoli cells induces the regression of Mullerian ducts, and testosterone helps the development of Wolffian ducts into secondary reproductive tissues. Consequently, Wolffian duct-derived organs develop in males, and Mullerian duct-derived organs, such as the uterus, develop in females. Thus, testosterone plays an important role in the development of sexually dimorphic secondary reproductive organs.

Testosterone is also necessary for sex determination of external genitalia, that is, a penis. The external genitalia of both sexes are derived from a common undifferentiated primordium, the genital tubercle (GT). In mice, GT develops from the cloacal region at E10.5. The conspicuous morphological sexual differences in GT appear at E16.5. The masculinization of GT is regulated by androgen. Indeed, when male fetuses were treated with antiandrogenic chemicals such as flutamide at E15.5–E17.5, their GTs would be demasculinized, and their morphologies would be similar to those of female GTs (Genitalia et al. 2009; Welsh et al. 2010; Matsushita et al. 2018). Considering the ability of androgen to cause the development of male-type GT, what happens when androgens are ectopically administered into female mice during the fetal stage? When female fetuses were treated with androgen at E15.5–E16.5, their GTs would masculinize. Based on these observations, it has been established that androgen plays a crucial role in the normal development of male external genitalia and that the critical time window of androgen-induced GT masculinization is E15.5–E16.5.

9.3.2 Role of Testosterone in Spermatogenesis

In addition to its indispensable role in the sex determination of somatic cells, androgen plays an important role in germ cells, namely spermatogenesis. Spermatogenesis occurs in the seminiferous tubules and involves three major cell types: peritubular myoid cells (PTM), Sertoli cells, and germ cells. To investigate the role of androgen in each cell type, cell-specific *Ar*-knockout mice were generated and their phenotypes are described briefly below. PTM-specific KO mice showed nearly no elongated spermatids and were infertile, indicating that AR in PTM is necessary for sperm maturation (Welsh et al. 2009). Sertoli cell-specific KO mice showed reduced testicular weight and were infertile. The number of postmeiotic spermatids were significantly reduced or absent by the elimination of AR in Sertoli cells, indicating that AR is necessary for germ cell meiosis (Chang et al. 2004; De Gendt et al. 2004; Holdcraft and Braun 2004). Germ cell-specific KO mice revealed normal testes, spermatogenesis, and fertility, revealing that AR expression in germ cells is dispensable (Zhou et al. 2002). This observation is consistent with the finding that *Ar* is not expressed or has low expression in germ cells. The aforementioned and other knockout mouse models for *Ar* revealed the following roles of AR in spermatogenesis: (1) maintenance of the blood–testis barrier; (2) regulation of meiosis; (3) maintenance of Sertoli cell-spermatid adhesion; and (4) release of sperm. Through the regulation of these multiple events, AR-mediated androgen signaling plays an indispensable role in achieving normal spermatogenesis and male fertility (Smith and Walker 2014).

9.4 Sex Hormone-Induced Sex Differences

9.4.1 Sex Determination of the Brain

In addition to the sex determination of reproductive tissues, sex hormones are known to function in the sex determination of the brain. The female sex hormone 17-β-estradiol is produced by the conversion of testosterone, which is mediated by aromatase P450 (CYP19A1). The mouse *Cyp19a1* gene has a unique structure, where the gene has multiple untranslated first exons driven by multiple promoters. The promoters are activated in a tissue-specific manner such that the gene is expressed in multiple tissues, including the gonads, adipose tissues, chondrocytes, bone, brain, skin, fetal liver, and placenta (Simpson et al. 2000). The expression of *Cyp19a1* in multiple tissues suggests that estrogen can be locally produced, and that local estrogen plays a role in the tissues where it is produced. For example, during the perinatal period, testosterone produced by testicular Leydig cells in male mice is transferred to the brain and converted to estradiol by CYP19A1. Estradiol produced in the brain is known to be essential for the development of the brain to the male type. Indeed, it has been reported that the sexual behavior of *Cyp19a1*-knockout

mice is severely impaired (Honda et al. 1998; Ogawa et al. 1999; Robertson et al. 2001; Toda et al. 2001).

9.4.2 Role of Estrogen in Bone

The role of the female sex hormone, estrogen, in bone cells has been well understood by observing postmenopausal females. As osteoporosis is one of the most evident symptoms in postmenopausal women, it has been accepted that estrogen has osteoprotective effects. Bone tissue is composed of three types of cells: osteoblasts, osteoclasts, and osteocytes. Osteoblasts and osteocytes are involved in the formation and mineralization of bone tissue, while osteoclasts are involved in tissue absorption. Estrogen exerts osteoprotective effects through direct action on bone tissue as well as through indirect actions, such as the modulation of cytokine production by immune cells and the increased induction of pituitary gland hormones. The direct action of estrogen on each type of bone cell was examined in bone cell type-specific *Esr1* (ERα)-knockout mice. ERα deletion in cells of the osteoblast lineage suggests that ERα plays an important role in the maintenance of bone metabolism (Almeida et al. 2013; Määttä et al. 2013; Melville et al. 2014). Osteoclastic ERα-null mice showed that osteoclastic ERα shortens the lifespan of osteoclasts by promoting apoptosis (Martin-Millan et al. 2010). In osteocyte ERα-knockout mice, ERα in osteocytes was found to play a significant role in maintaining bone mass by regulating osteoblastic bone formation only in females (Kato et al. 2009; Kondoh et al. 2014).

9.4.3 Roles of Sex Hormones in Establishing Sex Differences in Skeletal Muscles

Skeletal muscle size is defined by its weight and cross-sectional area (CSA). It has been shown that both muscle weight and CSA are sexually dimorphic: skeletal muscles are heavier and larger in males and lighter and smaller in females (Haizlip et al. 2015). What establishes this sex difference in muscles? A clear answer was provided by conventional experiments with gonadectomized mice, followed by hormone replenishment/replacement. Male muscles became lighter and smaller on the removal of the testes, whereas female muscles were not significantly affected by the removal of ovaries. The sizes of gonadectomized male muscles are recovered by the replenishment of testosterone, while female muscles become heavier and larger by the replacement of estradiol by testosterone. These observations indicate that testosterone has the ability to make skeletal muscles larger and that sex hormones are one of the major determinants of skeletal muscle size (Aguet et al. 2020). Currently, skeletal muscles are widely recognized to be one of the most sensitive target organs for sex hormones. Notably, because testosterone has anabolic effects on skeletal

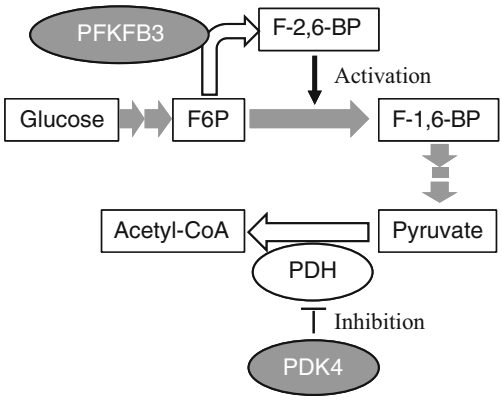

Fig. 9.3 Sexually dimorphic metabolic modulation underlying male-predominant glycolysis and female-predominant fatty acid utilization

muscles as described above, the use of sex hormones and their derivatives has been strictly prohibited in athletes.

In addition to muscle size, muscle performance is known to be different between males and females: muscles in males can generate more force, while muscles in female demonstrate greater endurance. What causes this sex difference? This difference may be due to the sexually dimorphic content of different types of muscle fibers. Skeletal muscles comprise multiple types of muscle fibers. In mice, there are four types of fibers: type I, type IIa, type IIx, and type IIb (Schiaffino et al. 1989; Pette and Staront 1997). Only type I fibers are classified as slow-twitch fibers, where energy production is highly dependent on mitochondrial oxidative phosphorylation. Type IIa, IIx, and IIb fibers are classified as fast-twitch fibers, where energy production primarily depends on glycolysis, although the extent of dependency varies among the three types of fibers. Because fast-twitch fibers are good at generating force and slow-twitch fibers are good at endurance, sexually dimorphic muscle performance is supposed to be due to the difference in the ratio of the fast-twitch and slow-twitch fibers. However, it is not known whether there are any intrinsic differences in the metabolism in the same type of fibers between males and females (i.e., male type IIB fibers vs. female type IIB fibers).

Our study on isolated type IIb fibers recently provided an answer to this question. We attempted to identify male-biased and female-biased genes using transcriptome analysis and found 68 male-biased and 60 female-biased genes, with >1.5-fold higher expression than that in the opposite sex (Christianto et al. 2021). Among the identified sex-biased genes, we focused on *Pfkfb3*, whose expression in type IIb fibers was three-fold higher in males than in females. PFKFB3 plays a crucial role in glycolytic regulation by producing fructose-2,6-bisphosphate, which robustly activates PFKM (a type of phosphofructokinase-1 found in muscles) (Obach et al. 2004; Ma et al. 2020) (Fig. 9.3). Therefore, the male-enriched expression of *Pfkfb3* suggested that the glycolytic activity in type IIb fibers in the quadriceps would be higher in males than in females. As expected, the glycolytic activity was indeed higher in males than in females, and *Pfkfb3* knockdown in male type IIb fibers decreased the activity of glycolysis to a level similar to that in female type IIb fibers.

In addition to *Pfkfb3*, we identified *Pdk4* as a potentially interesting sex-biased gene, whose expression was higher in females than in males in the presence of the female sex hormone, 17β-estradiol. PDK4 phosphorylates pyruvate dehydrogenase to inhibit its activity. As pyruvate dehydrogenase converts pyruvate to acetyl-CoA, its inhibition limits the supply of acetyl-CoA from glycolysis to the TCA cycle (Pettersen et al. 2019) (Fig. 9.3). In response to the inhibition of pyruvate dehydrogenase, fatty-acid β-oxidation is activated to provide acetyl-CoA to the TCA cycle as well as NADH to mitochondrial oxidative phosphorylation. Indeed, *Pdk4* knockdown in female type IIb fibers attenuated the dependency of oxidative phosphorylation on fatty acid β-oxidation. These results showed a clear difference in the metabolism between male and female type IIb fibers: glycolysis was preferred in male type IIb fibers and β-oxidation was preferred in female type IIb fibers.

The basis of the sex difference in metabolism in muscle fibers is the sexually dimorphic expression of two key metabolic genes, *Pfkfb3* and *Pdk4*. *Pdk4* expression is regulated by estrogen. In contrast, *Pfkfb3* expression is not controlled by androgen, but by some yet unknown testis-derived factors. It is necessary to identify these testis-derived factors to further understand the molecular mechanism underlying sexually dimorphic metabolism in the same types of muscle fibers. Further, the investigation of whether the difference in metabolism can be found in other types of fibers, especially in slow-twitch fibers, would provide valuable information.

9.4.4 *Roles of Androgen in Establishing Sex Differences in the Adrenal Cortex*

Sexually dimorphic organ size can also be observed in the mouse adrenal gland. The female adrenal gland is larger than the male adrenal gland, and these size differences are observed from 3 weeks after birth. As the secretion of a sufficient amount of testosterone from the testis to the circulation is initiated approximately 3 weeks after birth, the involvement of sex hormones has been suggested. Indeed, castration increases the adrenal size, whereas testosterone replenishment in females reduces the adrenal size, indicating that the male sex hormone, testosterone, is responsible for the sexually dimorphic size of the adrenal gland.

The adrenal gland is composed of the medulla and the cortex. The adrenal cortex consists of three layers: the zone glomerulosa (zG), zona fasciculata (zF), and zona reticularis. Which part of the adrenal gland is affected by testosterone and contributes to the difference in gland size? Although the testosterone receptor, AR, is expressed in all cell types in the adrenal gland, including in blood vessel cells, testosterone affects only the number of zF cells by regulating cell proliferation (Grabek et al. 2019). It should be noted that zF cells differentiate from zG cells. The rate of differentiation of zF cells from zG cells is reduced by testosterone, but the detailed mechanism underlying the action of testosterone is yet to be elucidated. To

understand the mechanism of the action of testosterone in zF cells, genome-wide analysis, such as ChIP-seq using AR antibody, is necessary.

Cortisol is produced and secreted by zF cells. In rodents, the plasma cortisol concentration reportedly exhibits a sex difference and is higher in females than in males. In contrast, a similar difference in plasma cortisol levels has not been reported in humans. However, Cushing's disease, which accompanies an excess secretion of cortisol, is reported more frequently in women than in men (Levasseur et al. 2019). Cushing's disease is caused by the excess secretion of ACTH from ACTH-producing tumors in the pituitary gland. As sex differences in the frequency of tumor occurrence have not been reported, differences in the sensitivity of zF cells to ACTH might be the cause of the high frequency of Cushing's disease in women.

9.5 Sex Spectrum

Male individuals have XY sex chromosomes in all cells, while female individuals have XX chromosomes. This is one of the most fundamental differences between males and females; in other words, cells have their own sex. A well-known significance of having the Y chromosome is in determining the gonadal sex as male by the function of sex determining factor on Y (SRY) (Koopman et al. 1990; Kashimada and Koopman 2010). However, even after sex determination, sex chromosome composition is sexually dimorphic throughout life. One may wonder whether different compositions of sex chromosomes may result in sexually dimorphic outputs in each cell. From this point of view, it may be interesting to focus on X- or Y-linked histone-modifying enzyme genes. It is established that there are four such genes: *Utx* and *Smcx* on the X chromosome, and *Uty* and *Smcy* on the Y chromosome. Gene products of all four genes are histone lysine demethylases that target H3K27 (UTX/Y) or H3K4 (SMCX/Y). The methylation of H3K27 and H3K4 is closely related to gene suppression and activation, respectively. Therefore, it can be hypothesized that these sex chromosome-linked demethylases establish sexually dimorphic epigenome landscapes to induce sex-biased gene expression profiles.

Another important sexual dimorphism is the production and secretion of sex hormones. Previous studies have shown that sex hormones function in the induction and, in some cases, the modulation of sexual characteristics such as the size of skeletal muscle fibers. As described above, sex hormones exert their roles by binding and activating specific receptors that function as transcription factors. Whether transcription factors can bind to their target sites is highly dependent on the epigenetic status of the target locus. For example, a transcription factor cannot bind to a target genomic region if the locus is heterochromatinized by the tri-methylation of histone H3K27. Thus, it is possible to expect crosstalk between the epigenome established by sex chromosome-linked genes and the transcription factors activated by sex hormones to establish sex-biased gene expression. The hypothetical concept of the "sex spectrum" is that all cells possess their own sex that can be located at any intermediate position between a typical male and a typical female. It is important to

consider that crosstalk between sex chromosomes and sex hormones provides an approximate position of sex on the spectrum, and if either is unexpectedly disturbed for some reason, this position can also be shifted.

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