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X Chromosome Agents of Sexual Differentiation

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Abstract

Understanding sex differences in physiology and disease requires the identification of the molecular agents that cause phenotypic sex differences. Two groups of such agents are sex chromosome genes, and gonadal hormones. The former have coherent linkage to chromosomes that formed differently in the two sexes under the influence of genomic forces that are not related to reproductive function, whereas the latter share a direct or indirect relationship to reproduction. Recent evidence supports the identification of several X chromosome agents of sexual differentiation, including *Kdm5c*, *Kdm6a*, *Ogt* and *Xist*. These X chromosome agents have wide pleiotropic effects, potentially influencing sex differences in many different tissues, a characteristic shared with the gonadal hormones. The identification of X chromosome agents of sexual differentiation will facilitate understanding of complex intersecting gene pathways underlying sex differences in disease.

Many diseases have a different incidence or progression in females and males, suggesting that each sex possesses different inherent biological factors that protect from or exacerbate disease.[1] An important goal is to identify the sex-biased molecular forces modifying disease, the agents of sexual differentiation, in part because they are potential targets of novel therapies. Sex differences in disease occur when the tissues affected are already sexually different in the absence of disease, because the disease operates on an unequal substrate. But sex differences in disease also occur in physiological systems that show no overt sex difference in healthy individuals, if the sexual equivalence is achieved by mechanisms operating unequally in females and males that counterbalance each other[2]. In the latter case, the disease may uncover sex differences by changing the normal balance of sex-specific mechanisms. [3–7]

In mammals, the *primary* agents causing phenotypic sex differences are encoded by the sex chromosomes. The different numbers of X and Y chromosomes, which are present at the formation of the zygote, establish inherent inequalities that are the root genetic causes of all subsequent sex differences in phenotype. The sex chromosomes have the potential to influence development at the earliest stages, because the embryonic transcriptome is already sexually differentiated by the 8-cell stage.[8,9] We define a *primary* agent of sexual differentiation as an X or Y gene or genetic region that acts more or differently in one sex

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Competing Interests

I declare that I have no competing interests.

than the other, causing a reliable sex difference in phenotype, or causing sex differences in mechanisms underlying a sexually balanced phenotype, because of the inherent difference in the number and type of sex chromosomes. The primary agents regulate downstream pathways encoded by genes throughout the genome, which act together to produce sex differences in function of diverse tissues. Identifying X- or Y-linked primary agents has been a holy grail of the field of sexual differentiation,[3,10] but until recently few genes were proven to have this role.

This view of sexual differentiation contrasts with the main perspective of the 20th Century, when gonadal hormones were considered to be the proximate causes of phenotypic sex differences.[11] All sexual differentiation of non-gonadal tissues was thought to occur after differentiation of the testes and ovaries, and to depend on the sex-biased action of the different secretions of the two types of gonad. Identification of the precise hormonal agents controlling sex differences (androgens, estrogens, and progestins) was a breakthrough beginning in the 1920s and 1930s, leading to a great amount of subsequent research to manipulate these agents to ascertain where and how they act, and to understand their receptors, pathways of synthesis, and the downstream molecular pathways that they regulate. With the realization that many sex differences are not caused exclusively by gonadal hormones, but also by sex chromosome effects,[3,12–14] understanding sexual differentiation now requires the identification of X and Y chromosome agents that also cause sex differences in many non-gonadal tissues.

Like all phenotypic sex differences, the sex difference in secretion of gonadal hormones is traced back to the sex chromosomes. In therian mammals, the Y-linked gene *Sry* is upregulated in the undifferentiated gonadal ridge in XY embryos, causing testicular development and inhibiting ovarian development.[15] SRY's role as a primary genetic agent of sexual differentiation is held in special regard, because the sexual differentiation of the gonads leads to sex differences in gametogenesis and levels of gonadal hormones that control sexual dimorphisms of the internal and external genitalia, which are required for sexual reproduction and are closely tied to classic definitions of maleness and femaleness. Importantly, however, the gonadal steroids also act broadly on tissues throughout the body to induce many types of sex differences in physiology and disease.

Sex-chromosome-modified mice facilitate identification of X and Y genes that are agents of sexual differentiation.

Sex chromosome genes other than *Sry* are agents of sexual differentiation, a conclusion that comes from an improved understanding of the sex chromosomes and the genes that they encode,[16–20] from studies linking sex chromosomes and sex differences in disease,[21,22] as well as from research that has discovered phenotypic sex differences in animals with different sex chromosomes under experimental conditions in which the group differences cannot be attributed to effects of gonadal hormones.[12,23] Indeed, disentangling the hormonal and sex chromosome effects is an ongoing challenge, because the two effects are typically confounded when comparing normal XY gonadal males and XX gonadal females, and because manipulations of sex chromosomes often lead to changes in levels gonadal

hormones. One fruitful experimental approach uses the Four Core Genotypes (FCG) and XY* mouse models.[24] In the FCG model, the *Sry* gene is "moved" from the Y chromosome to Chromosome 3, so that XX and XY mice are produced that have the same type of gonad, either testes or ovaries. These are compared to uncover XX vs. XY differences that are not attributable to gonadal hormones. The XY* model produces mice that have one or two X chromosomes (XO or XX females, XY or XXY males), which can be compared to measure X chromosome effects, and mice with or without the Y chromosome (XO vs. XY, XX vs. XXY) to measure Y chromosome effects. In the last 25 years, the FCG model has uncovered sex chromosome effects (XX vs. XY) that cause sex differences in a wide variety of murine disease models, including autoimmune diseases, metabolism and obesity, neurodegenerative disease, Alzheimer's disease, aging, bladder cancer, neural tube closure defects, behavior, and numerous cardiovascular diseases including ischemia/reperfusion injury, stroke, hypertension, and atherosclerosis.[23] When a sex chromosome effect (XX not equal to XY) is found using FCG mice, the effect can be attributed to action of either X or Y genes using the XY* mouse model. Subsequent studies vary the number of copies of one candidate gene at a time, to identify that gene as a primary agent of sexual differentiation. Most (but not all) sex chromosome effects studied using the XY* mouse model have been found to be attributed to the X chromosome.[23] Most sex chromosome effects also occur in systems in which gonadal hormones also significantly contribute to sexual differentiation. The effects of sex chromosomes and gonadal hormones can synergize with or counteract each other. In addition, the relative effects of gonadal hormones and sex chromosomes can vary depending on modifying environmental conditions.[25]

Sex differences in incidence of X-linked diseases have long been recognized in genetically heterogeneous populations of individuals because of *hemizygous exposure* of specific X genetic variants in XY males. Males, with only one X chromosome, more often experience the phenotypic effects of X-linked variations because no other X allele is present, whereas fewer females are affected because of the buffering effects of a different allele on the second X chromosome, and the possibility of skewing of X inactivation in favor of the normal allele. For example, X-linked disease alleles have greater effects among males in X-linked hemophilia, Fragile-X Syndrome, and Rett's Syndrome, to name a few.[26] The protection of females in this type of X-linked disease occurs even if the X gene is typically expressed at the same level in males and females and produces no inherent difference in XX and XY cell function. Moreover, the sex difference can occur both for X genes that are subject to X chromosome inactivation (XCI) or escape inactivation. The protection is at the level of populations of individuals, affecting the frequency of cases in each sex within the population. We focus here on a different mechanism of X gene action, in which the number of X chromosomes makes XX and XY cells function differently in virtually all individuals of a population, often because of inherent sex differences in the level of expression or regulation of the X gene. This type of effect does not require allelic diversity, and can be modeled in inbred mouse lines such as FCG and XY*. One advantage of the FCG and XY* mouse models is that they identify phenotypes showing a sex difference caused by sex chromosome effects, establishing a foundation for manipulations of one X gene at a time to find X agents of sexual differentiation causing that specific phenotypic sex difference.

Classes of X genes causing sexual differentiation

The present-day X and Y chromosomes evolved from an ancient autosomal pair.[17,20,27–29] The emergence of the testis-determinant *Sry* on one chromosome eventually limited the inheritance of most of that chromosome to males. Inevitable forces of genome evolution (Hill-Robertson effects, Muller's ratchet, etc.) made the Y chromosome deviate in sequence from its former autosomal partner, reducing recombination between the two, and producing different sex-specific selection pressures on the two chromosomes.[20] The lack of recombination of the Y chromosome led to loss of almost all Y genes,[29] leaving their X gene counterparts expressed in one dose in XY cells but two doses in XX cells. The sexual inequality of X gene dose relative to autosomal dose was sometimes disadvantageous, leading to evolution of diverse sex-specific compensation mechanisms that reestablished sexual equality of expression.[16] X chromosome inactivation (XCI) is a female-specific mechanism by which most X genes are transcriptionally silenced on one X chromosome in somatic cells, making expression of inactivated X genes nearly equal in males and females. Some X genes escape inactivation and are expressed from both X chromosomes in XX cells, either because the sexual inequality of expression was advantageous, or had little effect on fitness, or was not subject to efficient selection. The X escapee genes have higher expression in XX than XY cells.[30,31] For a small minority of Y genes, mutational loss was catastrophic because the X partner gene was haploinsufficient, leading to the apparent extinction of Y chromosomes carrying such mutations, and accounting for the survival of these ancestral Y genes on the present-day Y chromosome and the existence of X-Y homologous gene pairs.[10,32] The historical retention of a very few special Y genes is also viewed as a process of dosage compensation, so that X cells have two expressed doses of the X gene (which escapes X inactivation), which is balanced in XY cells by one X dose and one Y dose of functionally similar X-Y partner genes. Nevertheless, the function of the X and Y homologous genes is not always identical, leading to sex-specific effects of each.

The "inevitable forces of genome evolution",[20] just mentioned, recruited diverse X and Y genes, not just those related to reproduction, into roles as agents of sexual differentiation. A fairly diverse set of genes on these chromosomes became sex-biased because of wholesale loss of Y genes and compensatory changes in the X chromosome. Thus, sexual inequality of sex chromosome gene action was not necessarily a result of selection for better reproductive performance. As in the case of X-inactivation, a sex-biasing gene effect could have evolved because it effectively offset another disadvantageous sex-biasing effect. Although the gene content of the X and Y chromosomes was also altered to favor enhanced reproduction of individuals carrying these genes,[20] the X chromosome retains a wide assortment of genes that influence many cellular processes, causing sexual inequality of many functions beyond reproduction.

The X chromosome is left with at least 7 classes of genes or genetic sequences that are *a priori* more or less likely to serve as agents of sexual differentiation (Figure 1).[3,33–36] Although an X gene may have unequal expression if it is regulated by gonadal hormones, here we discuss sex differences originating directly from inequalities in the sex chromosomes themselves. Class (1) comprises *Xist* and genes of the X inactivation center that regulate the process of X inactivation. The XCI machinery regulates the degree of

sex difference in expression of X genes, which is thus central to determining whether the X genes are potential agents of sexual differentiation or not. Any factor influencing XCI thus is likely to change the sexual balance of X gene expression.[19,37,38] Class (2) includes the majority of non-pseudoautosomal X genes, which are subject to XCI. These genes are expressed from one X chromosome in both XX and XY cells, and show little sex difference in expression.[35,36] They are generally thought not to cause sexual differentiation. However, the sexual equivalence of expression of these genes is achieved by sexually unequal regulatory mechanisms in the two sexes, because XX cells require XCI whereas XY cells do not. Any perturbation of the sex-specific balancing mechanisms (Class 1), for example by mutation or disease, could disrupt the equality of expression of these genes, and contribute to sex differences found in disease that are not seen in other contexts. Class (3) and (4) genes escape X inactivation and are expressed from both X chromosomes in XX cells, at a higher level than in XY cells. In humans, about 23% of genes escape inactivation,[35,36] whereas 3–8% escape in mice.[31] Escape from X inactivation is regulated and variable according to cell or tissue type, age, and disease.[31,33,38–42] Class (3) genes have lost their homolog on the Y chromosome, whereas Class (4) genes possess a Y homolog. All X escapees are potentially agents of sexual differentiation, because they are expressed higher in XX than in XY cells, but Class (4) genes may represent the group most likely to cause sexual differentiation, discussed below. Class (5) are genes in the pseudoautosomal region (PAR), shared equally by the X and Y chromosomes. PAR genes have historically been thought to have equal effects in females and males because XX and XY cells both have two copies of PAR genes. However, the X inactivation process appears to reduce PAR gene expression from the inactive X chromosome in XX cells, resulting in greater expression in XY cells.[36] Class (6) genes receive a parental imprint, which can contribute to sexual differentiation,[43] because XY cells express the maternal imprint but XX cells express the imprint of both parents. Class (7) genes or genetic regions have putative sex-biased indirect epigenetic effects on the rest of the genome, because of the presence of a large heterochromatic X chromosome only in XX cells, which is not well understood in mammals.[44] Class (6) and (7) genes are not discussed further here.

Class (4) genes (X escapees that possess a Y homolog) differ in important ways from Class (3) of X escapees without a Y homolog. The X-Y gene pairs are functionally similar genes, former alleles that eventually evolved separately because of lack of recombination of the two chromosomes. X-Y pair genes are widely expressed in many tissues. The X partner genes are involved in critical basic cellular functions, and are expected to be the most dosage-sensitive among X genes.[10,32,33] The survival of the Y partner gene of these gene pairs is remarkable, considering that almost all other Y genes were lost during evolution. The Y chromosome apparently could not afford to lose these Y genes, because the Y gene offsets the haploinsufficiency caused by lack of a second copy of the X gene in XY males. The balance of X and Y gene action also explains the general finding that the X partner gene escapes XCI,[35,36] because XX and XY cells both have two genomic doses of functionally similar genes required to prevent lethality. Indeed, the survival of the Y gene can be seen to constrain the X partner gene into a consistent pattern of escape from XCI. If this compelling scenario were the only consideration, then X-Y gene pairs would be viewed as agents that *prevent* sexual differentiation by balancing each other out in XX and XY

cells. In fact, evidence suggests that this class of X genes are particularly involved in sexual differentiation. The balance of action of X and Y partners is probably critical only in some tissues or at specific developmental stages, and in other tissues the two genes appear to be out of balance. Sex differences in selection pressures seem to have caused some divergence of X-Y gene function, while still retaining overlap of functions (for example for the pair *Kdm6a* and *Uty*, discussed below).[45,46] Moreover, the summed level of expression of the X and Y homologs varies across tissue types and is often not the same in XX and XY cells; in some cases the Y homolog has evolved enhanced tissue-specific expression and a male-specific role.[47] Class (4) genes are predicted to be a group of genes highly enriched for agents of sexual differentiations (for example, *Kdm6a* and *Kdm5c* discussed below) because of their higher dosage sensitivity, their pleiotropic effects, their consistent pattern of XCI escape, their higher expression in XX vs. XY cells, and their unbalanced expression of the X and Y homologs. However, establishing the role of the Class (4) X gene as an agent of sexual differentiation requires manipulations of X gene dose to assess the functional effects of the XX vs. XY difference in level of expression, and assessing whether the Y homologous gene offsets the sexual inequality of X gene action. Class (3) genes, XCI escapees for which the Y partner gene has been lost, may include genes for which a sex-biasing role is possible because of the XX>XY pattern of expression. However, Class (3) genes are predicted to have the lowest dosage sensitivity among non-PAR X genes, based on evolutionary and comparative analysis,[33] so in some cases the sex difference in expression may cause little functional difference in XX and XY cells. Below we discuss evidence based on direct manipulation of X gene dose.

During evolutionary history, the X and Y chromosome received new genetic regions which began to diverge from each other at different stages. The most recently added X genes are more likely to escape XCI than older regions, and to be expressed higher in XX than XY cells,[48] suggesting that subjecting an X gene to inactivation may not depend solely on its function, but also on the history of its DNA neighborhood.[20,35,49] Because escape from inactivation influences whether a gene can cause a phenotypic sex difference, the role of X genes as agents of sexual differentiation may be influenced by historical accident.

Case studies of X agents of sexual differentiation

X escapees, and especially X-Y gene pairs, are implicated as potential causal agents of sexual differentiation, based on association of gene mutations with human disease[21,45] and analysis of evolutionary patterns of regulation of classes of X genes.[10,33] The sex-biasing effects of specific X genes can be tested rigorously in whole animal mouse models, in which sex-biasing effects of different doses of the X gene are measured in mice engineered to have different copy numbers of the X gene. These studies offer evidence that several X genes are agents of sexual differentiation.

Kdm5c dose contributes to sex differences in body weight and metabolism.

Kdm5c is a histone demethylase that removes methyl groups from methylated H3K27, but also has diverse mechanisms of action not requiring demethylase activity.[45,50] *Kdm5c* is an X escapee with a Y homolog, *Kdm5d*. *Kdm5c* is reliably expressed higher in XX

than XY cells in mice and humans.[14,36] Recent studies implicate *Kdm5c* in regulation of metabolism of mice. In healthy FCG mice,[25,51] gonadal males weigh more than gonadal females, irrespective of their sex chromosome complement (XX or XY), suggesting that levels of circulating gonadal hormones are the dominant source of sex differences in body weight in adults. That conclusion is confirmed because the sex difference largely disappears after gonadal hormones are removed by gonadectomy of adults. However, thereafter the XX mice gain weight more than XY mice, and accumulate much more fat. The XX>XY difference in body weight and fat is also found in gonad-intact mice, and is exacerbated when the mice eat a high fat diet. XX mice eat more than XY mice during the light phase of the circadian cycle. Independent of diet and presence/absence of the gonads, high-density lipoprotein levels in plasma are higher in XX than XY mice with the same type of gonad.[52] The hormonal and sex chromosome control of body weight is a case of counterbalancing effects because mice with male gonads weigh more, but those with male sex chromosomes (XY) weigh less. Thus, the effects of gonadal hormones reduce the effect of sex chromosomes, and vice versa. Use of the XY* mouse model shows that it is the number of X chromosomes that causes the sex chromosome effects, and presence or absence of the Y chromosome has little apparent effect. The effects of one vs. two doses of the X chromosome are mimicked by the effects of one vs. two doses of *Kdm5c*.[25] Gonadal female XX mice with two doses of *Kdm5c* (+/+) differ from those with one dose (+/−) in their greater body weight, greater proportion of body fat, and more daytime eating. Although the dose-dependent effects of *Kdm5c* occur in mice eating a low-fat or a high-fat diet, the sex differences caused by sex chromosomes are more prominent in the mice on a high-fat diet, suggesting that the sexual balance of hormonal and sex chromosome effects depends on the type of diet. *Kdm5c* may act predominantly in preadipocytes to influence their development and metabolic effect, because knockdown of *Kdm5c* alters chromatin accessibility and reduces cell proliferation and accumulation of lipids in a preadipocytic cell line. Although the possible counterbalancing effects of the Y partner gene, *Kdm5d*, have not been reported, *Kdm5d* may not have the same effect as *Kdm5c* because the presence of a Y chromosome does not compensate for the lack of a second X chromosome in the XY*model.[51] Thus, this example is a case in which the dose of an X chromosome gene, a member of an X-Y gene pair, contributes to sex differences in normal physiology and susceptibility to metabolic disease.

Although *Kdm5c* regulates numerous phenotypes, it does not always cause sex differences. Null mutations of *Kdm5c* in female mice, or of both *Kdm5c* and *Kdm5d* in males, reduce embryonic size, and cause cardiomyopathy and neonatal death. The cardiac deficits were not observed in mice with one functional copy of *Kdm5c* in females, or a copy of *Kdm5d* in males.[53] Thus, *Kdm5c* and *Kdm5d* have redundant effects on cardiac development, and seem not to produce sex differences in these phenotypes. Similarly, the X-Y gene pair *Ddx3x* and *Ddx3y* have similar effects on murine development.[54]

Kdm6a *dose contributes to sex differences in mouse models of bladder cancer, Alzheimer's Disease, and autoimmunity*.

Kdm6a is an X escapee, also known as *Utx*, that encodes a histone demethylase acting on H3K27me3.[55] It's Y partner gene, *Uty*, has lost histone demethylase activity.[46] *Kdm6a*

consistently escapes XCI across tissues and species, and is expressed higher in XX than XY cells.[36] Knockout of *Kdm6a* is lethal to XX mouse embryos at midgestation,[46] in part because of effects on cardiovascular development and neural tube defects. *Kdm6a* has been shown to have important roles in embryonic stem cell differentiation, and development of numerous specific tissues in the immune system, heart, and mammary glands. The effects of homozygous knockout of *Kdm6a* are partially prevented by the presence of *Uty* in male embryos, indicating that some effects of *Kdm6a* do not require histone demethylase activity.[55] For example, XY mice lacking *Kdm6a* have reduced midgestation mortality, and can live to adulthood (albeit with reduced body size) and reproduce.[46] XX mice with one copy of *Kdm6a* are viable, but may not reproduce well. *Kdm6a* interacts with numerous epigenetic modifiers such as histone methyltransferases acting on H3K4, and histone acetyltransferases.[55]

Kdm6a is thought to be a tumor suppressor in various cancers,[21] for example in a mouse model of bladder cancer, a disease that occurs 3–5 times more often in men than women. FCG mice were injected with the bladder-specific carcinogen *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine.[56] Mice with testes showed greater mortality than mice with ovaries, illustrating a gonadal hormone effect. But XY mice showed greater mortality than XX mice, irrespective of their type of gonad, establishing a sex chromosome effect that acts synergistically with the hormonal effects. In bladder cancer cell lines, direct manipulations of *Kdm6a* show that it reduces cell proliferation by activating genes downstream of the tumor-suppressor *p53*, via different mechanisms that were dependent on or independent of *Kdm6a*'s histone demethylase activity. In mice with a conditional knockout (cKO) of *Kdm6a* in the urothelium, XX females with a null mutation of *Kdm6a* died with bladder cancer, more than females expressing *Kdm6a*, establishing *Kdm6a* as a tumor-suppressor *in vivo* (Figure 2A). In XY males, cKO of *Kdm6a* did not suppress tumors or change survival of the animals, suggesting that males possess some factor that protects from the effects of loss of the single copy of *Kdm6a* in XY cells. One potential explanation is that *Uty* also possesses some tumor-suppressor activity in this model.

Kdm6a is also identified as an agent of sexual differentiation causing sex differences in a mouse model of Alzheimer's Disease, because of a greater protective effect in XX than XY animals.[57] Men with Alzheimer's disease die earlier than women, and show worse cognition, more cognitive decline, and increased measures of neurodegeneration. In FCG mice carrying the human amyloid precursor protein gene (hAPP), gonadectomized to remove effects of gonadal hormones in adulthood, survival of mice is worse in XY than XX mice, a difference that shows some interaction with gonadal type because it is particularly evident in gonadal females (Figure 2C). Stated differently, there is a hormone effect (difference between mice with testes vs. ovaries) only in XY mice. Moreover, XY mice show worse performance than XX mice in tests of learning and memory. In XY* model mice carrying the hAPP gene, mice with one X chromosome (either XO or XY) have reduced longevity compared to those with two X chromosomes (XX or XXY), and also show significant forgetting on memory tests. *Kdm6a* was investigated as a candidate X gene causing these effects. *In vitro*, treatment of neurons with the neurotoxin Aβ42 causes greater cell death in XY than XX neurons. Knockdown of *Kdm6a* in XX neurons, to the level of XY neurons, significantly increases neurotoxicity (Figure 2D). Conversely, increases

of *Kdm6a* expression from a lentivirus in XY cells attenuates neurotoxicity (Figure 2E). *In vivo*, lentiviral overexpression of *Kdm6a* in the hippocampal dentate gyrus of XY males causes an increase in learning and memory performance. Thus, *Kdm6a* is established as an agent of sexual differentiation in this disease model, with two copies of the gene causing more protection than one copy of the gene. The effects of *Uty*, the Y partner gene of *Kdm6a*, have not been reported in this model.

Kdm6a also contributes to sex differences in autoimmune disease in mice. Most autoimmune diseases occur in women more than men. Multiple sclerosis (MS) is a putative T-cell mediated disease with a 3-fold higher risk in females, although disease progression appears worse in males. The same female bias in incidence is found in Experimental Autoimmune Encephalomyelitis (EAE), an MS-like disease induced by injecting mice with myelin protein autoantigens and an adjuvant. Manipulations of levels of gonadal hormone show protective effects of androgens, explaining the sex difference in part. Estriol, an estrogen elevated during pregnancy, is also protective, mimicking the improvement in MS symptoms that occur during pregnancy in humans.[58] In gonadectomized FCG mice, XX mice experience the disease more than XY mice, and show greater neurodegeneration.[59] *Kdm6a* was selected for study because it was the most sexually dimorphic X escapee with higher expression in XX than XY CD4+ T cells. Conditional loss of *Kdm6a* only in CD4+ T cells led to strong protection from EAE (Figure 2B), and reduction in neuroinflammation.[60] Analysis of the transcriptome showed that the loss of *Kdm6a* upregulates T helper cell-related gene pathways, and downregulates neuroinflammation genes.

Kdm5c is also implicated in causing sex difference in EAE in mice.[61] Adoptive transfer of Th17 CD4+ T cells to recipient mice causes EAE that is worse when the donor cells are male rather than female. The sex differences are attributed to sex chromosome complement, because the effect was seen when comparing XY vs. XX cells from FCG donor mice that each had either testes or ovaries. Overexpression of *Kdm5c* reduced disease caused by Th17 cells, and pharmacological inhibition of *Kdm5c* increased disease, suggesting that sex differences in the dose of T cell *Kdm5c* affects EAE. Further studies are needed to resolve the effects of *Kdm5c* and *Kdm6a* in different models of EAE in mice,[60,61] and to determine which results relate to the observed sex differences in MS in humans, in which females are at much greater risk of disease, but males may experience a worse course of disease.

Ogt causes sex differences in placental function.

In a model of neurodevelopmental disorders, stress of pregnant mice during the first week of gestation causes offspring to show later patterns of neural and metabolic dysregulation, including hyper-responsiveness to stress.[62,63] These effects are shown more by male offspring than females. The X-linked gene *Ogt* (O-linked N-acetylglucosamine transferase, with no Y homolog) is implicated as mediating some effects of prenatal stress. *Ogt* uses cellular nutrients to glycosylate thousands of proteins. *Ogt* escapes XCI and is expressed at higher levels in female than male placenta. Trophoblast-specific reduction of *Ogt* expression in females causes a major shift, towards a more masculine pattern, in gene expression in both the trophoblast and embryonic hypothalamus. Reduction of *Ogt* expression in the trophoblast also recapitulates some of the effects of prenatal stress in males, and knockout

of trophoblast *Ogt* in males alters gene expression in the hypothalamic regions that regulate the response to stress. The effects of *Ogt* appear to be mediated in part by its regulation of H3K27me3 levels. These studies implicate *Ogt* as an X agent of sexual differentiation causing sex differences in placental function with long-term effects on neural development and metabolism, with higher expression protecting females from environmental disruptions during early embryonic development.[62,63]

Xist and XCI machinery as agents of sexual differentiation

The evolution of the sex difference in the number of X chromosomes locked virtually all X genes into a web of potential sexual differentiation, not because the genes were especially involved in sexually dimorphic functions, but simply because the genes were located on the chromosomal partner of the degenerating Y chromosome. Loss of nearly all Y genes meant that their X homologous genes initially passed through an evolutionary phase in which the dose of the X gene was sexually unequal, higher in XX than XY cells, before any compensatory mechanism could offset the imbalance. Depending on gene function, such inequality was either selected for or against, or persisted because it had little functional effect. Selection against sexual inequality of expression favored mechanisms to compensate for and reduce the sexual inequality, such as XCI, or retention of the Y homolog. Although XCI makes expression of most X genes functionally equivalent in the two sexes, this equality requires an inherent sexual inequality in regulation of X gene expression, because most X genes in females are regulated by XCI, but none is similarly regulated in males. Although almost any discussion of the function of XCI requires emphasis of its sex-specificity, the sex-biasing effects of XCI have not been adequately incorporated into discussions of the proximate mechanisms of sexual differentiation. XCI, and limits on XCI, lie at the heart of sexual differentiation of most X gene effects on emergent phenotypes. Regulation of XCI determines which X genes have sexually unequal levels of expression, and results in mosaic expression of different parental imprints that may contribute to phenotypic sex differences. Because XCI is regulated differently for different types of X genes,[33,39] in different tissues,[31,38,40] at different life stages, as a function of disease,[41] and even within a single cell type in a tissue,[42] the machinery of XCI is a major point of control of sexual equality and inequality. Dynamic regulation of XCI in different cell types of the immune system, for example, caused by immune activation, is suggested to influence expression of X genes in females and contribute to sex differences in autoimmune diseases.[41] In adult female human B cells, *Xist* is required for continual maintenance of inactivation of X genes with hypomethylated promoters. Loss of XIST function in XX (but not XY) B cells causes these genes to escape XCI, promoting differentiation of naïve B cells into atypical B cells characteristic of aging and infectious and autoimmune diseases.[38]

To initiate random XCI in the embryo proper, *Xist* is expressed from one X chromosome and recruits numerous interacting partners that repress transcription of X genes in *cis*. XCI is existentially important for females, but not for males. Preventing XCI in the embryonic trophoblast (extraembryonic tissues) is lethal in XX female embryos.[64] Deleting *Xist* in most or all tissues of the epiblast (embryo proper), prior to onset of X inactivation, also results in death of all XX females but no XY males, although a minority of XX females remarkably live up to several weeks after birth.[65] Once XCI is established, deletion of

Xist undermines the maintenance of XCI and causes upregulation of X gene expression in numerous tissues of XX individauls.[66] The functional effects of *Xist* deletion depend on cell type. Knockout of *Xist* in hematopoietic cells results in development of lethal hematologic cancers in 100% of XX females and 0% of XY males.[19] Thus, *Xist* is a tumor suppressor in females but not males, and successful survival of females is anchored by a female-specific mechanism. Deletion of *Xist* in the gastrointestinal epithelium makes XX females susceptible to the tumorigenic effects of a carcinogen coupled with an inflammatory agent.[66] However, deletion of *Xist* in epithelial cells, B cells, or brain has little apparent effect in laboratory mice.[66,67] Although deletion of *Xist,* in one tissue at a time, has surprisingly little effect on viability of mice and on tissue functions studied, the range of potential phenotypes measured to date as a function of *Xist* expression is limited. Moreover, these studies are in laboratory mice protected from many environmental insults. *Xist* is an agent that has XX female-specific effects that prevent hematologic and gut cancer, and establishment of XCI is required for viability of females not males. Clearly *Xist* is an agent of sexual differentiation, a concept that can rationalize further studies of *Xist*'s role in contributing to sex differences in diverse phenotypes and under different environmental conditions.

In addition to the canonical role of *Xist* in triggering and maintaining XCI, new mechanisms of *Xist* action have recently been proposed. The *Xist* lncRNA binds numerous miRNAs as a sponge, reducing action of the miRNAs and putatively influencing progression of various cancers, pulmonary fibrosis, inflammation, neuropathic pain, stroke and cardiomyocyte hypertrophy.[68] As one example, in a mouse model of stroke, *Xist* expression is increased in males over a period of a week following the ischemic event. Silencing *Xist* increases the size of the neural infarct and increases neurological deficits, and evidence suggests that *Xist* regulates expression of angiogenic factors by targeting miRNa-92a.[69] Female mice were not tested. In such investigations, it will be important to consider the sex-biasing effects of *Xist* as a rationale for answering whether acute changes in *Xist* expression alters the inactivation of the X chromosome and results in any general or sex-specific change in expression of X genes.

Studies of sex-biased neural tube closure defects illustrate the sex-biasing role for *Xist* and X inactivation center genes. Null mutations of p53 in mice lead to neural tube closure defects and embryonic lethality that predominantly affect females because of the sex difference in number of X chromosomes.[70] Loss of p53 reduces *Xist* expression and binding to the inactive X chromosomes, reducing foci of H3K27me3 associated with XCI, and upregulates expression of X genes that are typically inactivated. P53 directly binds to the X inactivation center, causing these changes and resulting in female-specific neural tube closure defects.[37]

Contrasting hormonal agents and X chromosome agents of sexual differentiation: functional vs. locational coherence

The discussion above indicates that several X genes are agents of sexual differentiation, including *Xist*, *Kdm5c, Kdm6a, Xist*, and *Ogt*. Other X genes will certainly be added to

this list. We can begin to ask if these genes have common properties that make them more likely to serve as agents of sexual differentiation. Among the properties shared by these genes are: (1) each is expressed at a higher level in females than males because they control or escape X inactivation; (2) each is important or essential for cell functions; (3) each is expressed widely in many cells types; (4) each has pleiotropic effects; and (5) each is dosage sensitive, meaning that it exerts varying phenotypic effects depending its level of expression, as documented above and predicted by comparative analysis of gene regulation.[10,33] These different attributes are related. It makes sense that genes playing widespread, critical roles in cell function are most likely to have dose-dependent effects. The pleiotropic nature of these genes, however, presents a complex regulatory situation making it difficult to optimally tune the sex difference to each of the gene's effects in diverse cell types and under environmental or disease conditions.[71] Thus, sexual inequality in an X gene's expression in one tissue may be a side effect of adaptive sex differences in other tissues, and may have the appearance of being accidental instead of being the result of positive selection (Figure 3).

The X agents of sexual differentiation differ as a group from the hormonal agents of sexual differentiation. Unlike the autosomal genes regulated by gonadal hormones, X genes are likely all to have passed through a period of evolution in which they were forced to be expressed at different levels in the two sexes. In cases in which this sexual bias in expression and phenotypic effect has been eliminated by evolutionary events, the X genes remain primed for sexual bias because the process of eliminating inequality of expression leaves X genes under different regulatory control in XX and XY cells. The compensatory regulation of X genes itself represents a new set of sexually differentiated forces that accounts for some sex differences in tissue functions and response to disease. Despite selection pressures that have enriched the X chromosome for genes related to reproduction,[20] the X genes are highly diverse. They are therefore not functionally coherent as a group, but coherent based on their genomic location that determined their peculiar sex-specific regulation. This pattern of constraints on X agents of sexual differentiation contrasts with the patterns for genes that evolved sensitivity to gonadal hormones. The latter group, not located on any one chromosome, lacks the underlying sexual bias stemming from inequality of chromosome number. Rather, hormone-sensitive genes are more functionally coherent, because they are generally related to reproduction.

Despite the difference in attributes of these two classes of agents, they share some important properties. Like sex chromosome agents, gonadal hormones have widespread and pleiotropic effects, complicating selection pressures on their roles in producing phenotypic sex differences. A cell type might well evolve sensitivity to gonadal hormones, producing a sex difference that is not entirely advantageous in all environmental or disease conditions, leading to evolution of compensatory effects that reduce the sexual difference in a sex- or condition-dependent manner.[2] Moreover, all X agents of sexual differentiation face sex-specific selection pressures related to reproduction, because XX cells generally function in individuals with ovaries, but XY cells function in individuals with testes. X chromosome agents of sexual differentiation could be the X genes that are advantageously expressed unequally because of their differential positive effect on male or female reproduction, even if that pattern has not yet emerged as a common theme in this group. Sex chromosomal and hormonal mechanisms of sexual differentiation operate in the same tissues and probably in

the same cell types, but the offsetting and synergistic actions of these agents are generally unknown.

Conclusion

Two major modes of sexual differentiation are controlled by gonadal hormones, and by sex chromosome genes, both of which are inherently expressed differently in females and males. The tight linkage of gonadal hormones to reproductive development and function makes this mechanism highly relevant to reproduction itself. The sex chromosome genes became sexually dimorphic originally because of genomic forces that were not directly driven by reproduction, and produced sexual imbalance in a heterogeneous set of genes that happened to be on the same chromosome that was unequally represented in the two sexes. Both mechanisms involved complex regulation that likely involved both positive selection of adaptive sexual differences, and sex-specific mechanisms to offset some disadvantageous sex differences that were unavoidable because of pleiotropy. Further research is required to understand the intersection of these regulatory mechanisms and how they produce sex differences in disease.

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Key Points Box

Phenotypic sex differences arise because of the unequal expression of sex chromosome genes, including downstream unequal effects of gonadal hormones.

Recent evidence implicates specific X chromosome genes as agents causing sex differences in a wide variety of tissues, relevant to many diseases.

Two major groups of agents of sexual differentiation, sex chromosome genes and gonadal hormones, may differ in their relevance to reproduction because of their different evolutionary history and chromosomal linkage.

Sex-biasing effects of sex chromosome genes and gonadal hormones may be favored because they produce a *de novo* adaptive effect or offset another disadvantageous sex difference.

Because gonadal hormonal and sex chromosomal agents of sexual differentiation both have pleiotropic effects, they are likely to produce diverse sex differences that are not all equally advantageous.

Sex differences in disease may occur even in tissues that function equally in healthy individuals, if the sexual equality is based on different compensatory mechanisms in the two sexes.

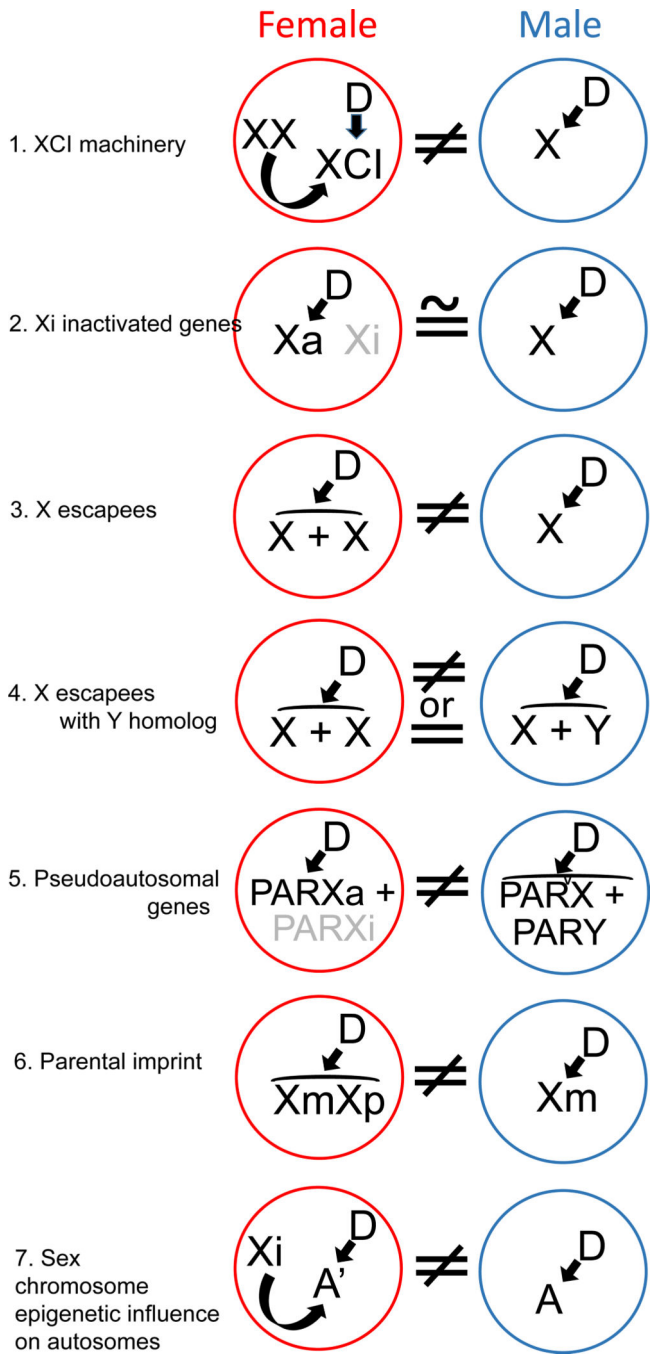

Figure 1.

Classes of X gene that contribute more or less to sexual differentiation. The figures contrast the effects of disease (D) on specific mechanisms operating differently in female and male cells, depending on which type of X genes contribute to a specific phenotype. Class 1 genes are involved in X chromosome inactivation (XCI), which only occurs in XX cells. Therefore a disease effect on XCI machinery can create a sexual imbalance in effect of the disease. Class 2 genes are not expressed from the inactivated X chromosome (Xi) in XX cells, making expression of the X genes similar in XX and XY cells. Effects of disease

on phenotypes affected by this class are likely to be similar in the two sexes, unless there is skewing of X inactivation. Class 3 genes escape X inactivation and are expressed from all X chromosomes and in a XX>XY pattern. Disease effects on phenotype affected by these genes can be unequal in the two sexes. Class 4 genes also escape X inactivation, but have a homologous Y gene that may or may not offset the sexual imbalance of the X gene effect on phenotype. Class 5 genes in the pseudoautosomal region (PAR) are expressed less from Xi (PARXi) than from the active X chromosome (PARXa), and therefore show lower expression in XX than XY cells. Class 6 X genes receive a parental imprint which occurs unequally in XX and XY cells because the paternal imprint (Xp) occurs in XX cells only, whereas the maternal imprint (Xm) occurs in all cells. Class 7 genes or non-genic regions of the X chromosome include indirect effects on autosomes (A') caused by the difference in number of X chromosomes, for example the possible epigenetic effects of the large heterochromatic Xi that exists only in XX cells.

Figure 2.

Examples of diverse sex differences caused in part by *Kdm6a* in mice. A, Sex differences in bladder cancer. In Four Core Genotypes mice injected with a bladder-specific carcinogen, both gonadal hormones and sex chromosome complement contribute to the greater lifespan of females relative to males (data not shown). In A, conditional homozygous knockout (cKO) of *Kdm6a* in the urothelium reduced lifespan of females, but not of males, suggesting that *Kdm6a* dose contributes to the sex difference, but that gonadal sex or XY genotype modifies the *Kdm6a* effect. Data and figure from[56] B, In Experimental Autoimmune Encephalomyelitis, a model of multiple sclerosis, mice are injected with a myelin autoantigen to induce an MS-like disease, on day 1. Disease progression is measured by clinical score varying from 0 (completely healthy) to 5 (moribund). Progression is worse in mice with two X chromosomes than one (not shown). Conditional KO of *Kdm6a* in CD4+ T cells in XX females protects from EAE, relative to wild type (WT) females, suggesting that the normal F>M *Kdm6a* dose contributes to the sex difference in EAE because of disease-promoting effects of a higher *Kdm6a* dose in XX T cells. Data and figure from [60] C, In a model of Alzheimer's Disease, mice carry the human amyloid precursor protein (hAPP). In Four Core Genotypes mice, XX-hAPP mice live longer than XY-hAPP mice. D, Cortical neurons were cultured and administered Aβ neurotoxin, after knockdown of *Kdm6a* expression in XX neurons, to the level of XY neurons. Short hairpin interfering RNAs reducing Kdm6a expression (shKdm6a) increased neurotoxicity, compared to scrambled shRNA (SCR). In E, lentiviral overexpression of *Kdm6a* in XY neurons, to the level of XX neurons, reduced Aβ neurotoxicity. The combined evidence in D and E suggests that the higher dose of *Kdm6a* in XX neurons is neuroprotective in this model of Alzheimer's disease.[57] Data and figures C,D, and E from [57]

NOTE: For Figure 2A, please refer to Figure 3A of Kaneko, S. & Li, X. X chromosome protects against bladder cancer in females via a KDM6A-dependent epigenetic mechanism. *Sci Adv* **4**, eaar5598, doi:10.1126/sciadv.aar5598 (2018).

For Figure 2B, please refer to Figure 2C of Itoh, Y. *et al*. The X-linked histone demethylase Kdm6a in CD4+ T lymphocytes modulates autoimmunity. *J Clin Invest* **130**, 3852-3863, doi:10.1172/JCI126250 (2019).

For Figures 2C, 2D and 2E, please refer to Figures 3D, 7F, and 7H of Davis, E. J. *et al*. The second X chromosome confers resilience against Alzheimer's disease-related deficits in male and female mice. *Science Translational Medicine* **12**, eaaz5677, doi:10.1126/scitranslmed.aaz5677 (2020).

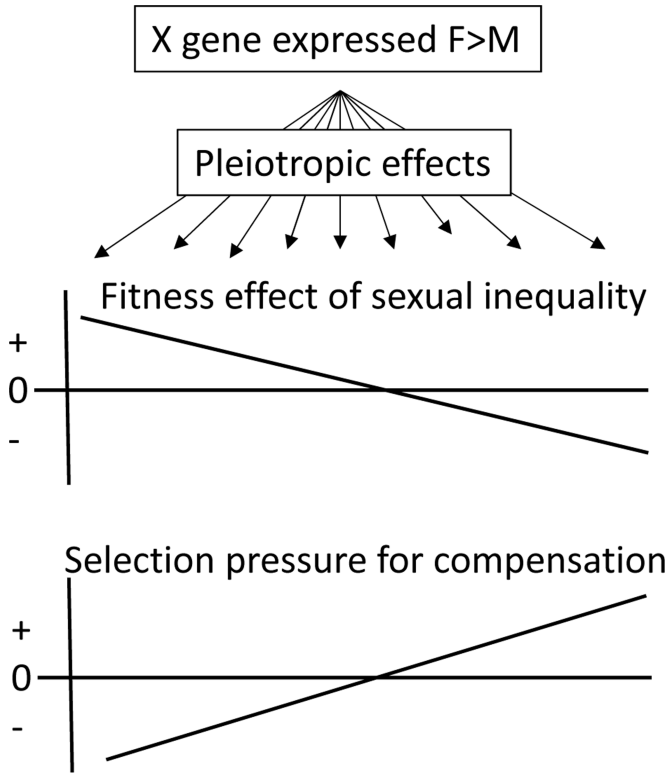

Figure 3.

Side effects of pleiotropic agents of sexual differentiation. X genes evolved higher expression in XX than XY cells. When those genes had pleiotropic effects, the gene caused diverse sex differences that varied in their fitness value, setting up selection pressures for or against the sex differences in expression. Disadvantageous sex differences created pressure in favor of mechanisms offsetting the sex difference in gene action in specific tissues or under specific environmental or disease conditions. The compensatory mechanisms could involve X inactivation, hormones expressed differently in the two sexes, a Y homologous gene with similar phenotypic effects, X genes with opposite effects, negative feedback pathways, etc. If selection was inefficient, then a subset of sex differences caused by a pleiotropic gene likely remained when they have little advantage, as side effects of selection pressures on other effects of the gene that established the overall sex difference in expression of the X gene.