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Invited Review Article

INVITED REVIEW ARTICLE

X-factors in human disease: impact of gene content and dosage regulation

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Abstract

The gene content of the X and Y chromosomes has dramatically diverged during evolution. The ensuing dosage imbalance within the genome of males and females has led to unique chromosome-wide regulatory mechanisms with significant and sex-specific impacts on X-linked gene expression. X inactivation or silencing of most genes on one X chromosome chosen at random in females profoundly affects the manifestation of X-linked diseases, as males inherit a single maternal allele, while females express maternal and paternal alleles in a mosaic manner. An additional complication is the existence of genes that escape X inactivation and thus are ubiquitously expressed from both alleles in females. The mosaic nature of X-linked gene expression and the potential for escape can vary between individuals, tissues, cell types and stages of life. Our understanding of the specialized nature of X-linked genes and of the multilayer epigenetic regulation that influence their expression throughout the organism has been helped by molecular studies conducted by tissue-specific and single-cell-specific approaches. In turn, the definition of molecular events that control X silencing has helped develop new approaches for the treatment of some X-linked disorders. This review focuses on the peculiarities of the X chromosome genetic content and epigenetic regulation in shaping the manifestation of congenital and acquired X-linked disorders in a sex-specific manner.

Introduction

Sex affects the manifestation, epidemiology and pathophysiology of many common diseases such as cardiovascular diseases, cancer, intellectual disabilities, neurodegenerative disorders, metabolic disorders, immune responses and autoimmune diseases (1). One example, relevant to the current Covid-19 pandemic, is that women mount a more robust immune response to infection than males who are often more severely affected by viral infections (2). Conversely, women are more susceptible to late-onset sporadic Alzheimer's disease than men, although progression may be more rapid in men (3). Sex-stratified differences have been demonstrated in a systematic analysis of all diseases and disease co-occurrences in the Danish population using the ICD-10 and Global Burden of Disease terminologies (4). By compiling sex-specific incidence, risk, temporal aspects of diagnoses and co-occurrence of diagnoses, the authors identified sex-related differences across nearly all major disease types.

Hormones and genetic sex, namely the sex chromosomes, are major biological determinants of sex differences in human disease. In this review, we will show how the sex chromosomes, in particular the X chromosome and its peculiar gene content and modes of regulation, influence the manifestation of diseases in males and females. One important aspect of diseases caused by X-linked mutations relates to the types of genes located on the X chromosome, which have been shaped by evolution. Because of its presence as a single chromosome in males, the

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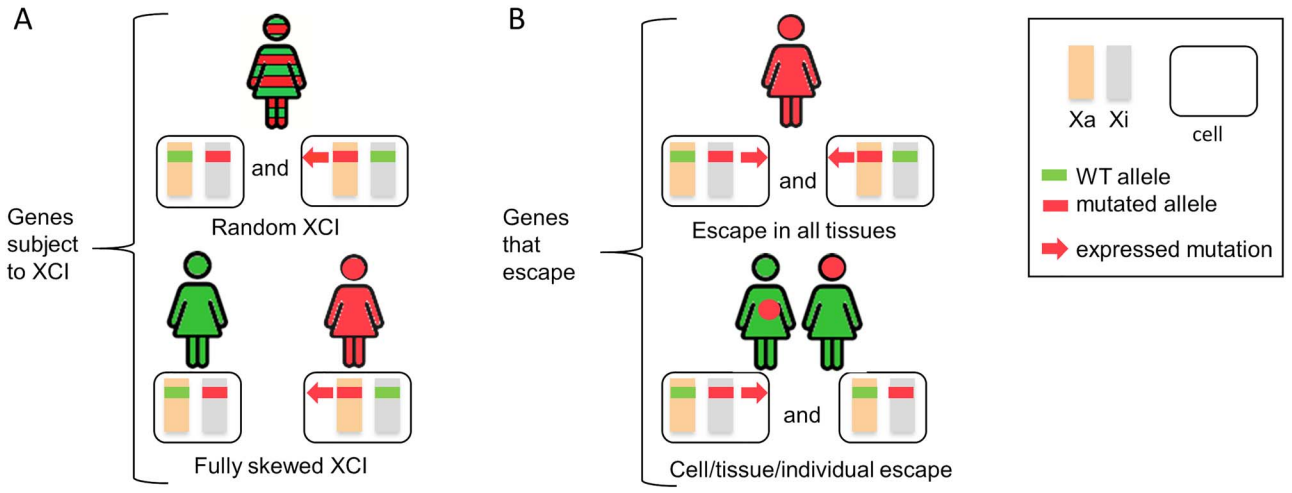

Figure 1. Influence of XCI on phenotypes in X-linked disorders. (**A**) Phenotypic effects of mutated genes subject to XCI. Following random XCI (top), a mixture of cells expressing either the wild-type allele (green) or the mutated allele (red) is present in female patients (red and green stripes). In case of fully skewed XCI (bottom), female patients will solely express either the wild-type allele (green) and show no disease (green), or the mutant allele (red) and show a disease phenotype (red). (**B**) Phenotypic effects of mutated genes that escape XCI. Patients with a mutation in a constitutive escape gene (top) will display a disease phenotype in all tissues regardless of random or skewed XCI. Patients with a mutation in a variable escape gene (bottom) will have a disease phenotype in tissues in which the gene escapes (red), even if skewed XCI favors the expression of the normal allele (green) in other tissues (green). This pattern may also differ between individuals. Orange and grey rectangles represent active and inactive X chromosomes. Green and red represent normal and pathogenic genotypes (bars) and phenotypes (symbolic female). Red arrow indicates the expression of the mutant allele.

X chromosome is a choice location for genes favorable to males. Thus, families of testis-specific genes implicated in male fertility have accumulated on the X chromosome (5–7). Genes expressed in the brain especially in the cortex and the hypothalamus are also enriched on the X chromosome, a phenomenon probably driven by sexual selection (8–11). This is supported by a 3.5-fold higher incidence of X-linked versus autosomal mutations that cause intellectual disability (12). Other types of genes enriched on the X chromosome are genes involved in muscle function and in immune response. Hence, diseases caused by X-linked mutations often affect these tissues/cell types. Following a discussion of the role of X inactivation and escape from X inactivation on the manifestation and potential alleviation of X-linked mutations, we will address the role of X-linked genes in specific disorders including X aneuploidy, immune disorders and cancer, followed by a discussion of potential therapeutic approaches.

Influence of X Chromosome Inactivation on the Manifestation of Congenital Diseases

The human X chromosome carries more than 1100 genes and pathogenic variants cause at least 546 known X-linked diseases (13). Males are usually affected by X-linked pathogenic variants as they lack a second compensatory allele, while females are often asymptomatic carriers of the defect even though only one allele, maternal or paternal, remains expressed in their cells after X chromosome inactivation (XCI) (Fig. 1A) (14). Prior to XCI in pre-implantation human female embryos, single-cell studies have detected expression from both X chromosomes, with evidence of possible dampening of gene expression from each X allele to compensate for the high X versus autosome expression, but this possibility remains controversial (15–17). Following implantation, XCI is initiated by the upregulation of the long non-coding RNA (lncRNA) *XIST* on the future inactive X chromosome chosen at random. This triggers the recruitment of a number of proteins to silence X-linked genes by implementing layers of repressive epigenetic modifications such as histone modifications, chromatin condensation and methylation at CpG islands (16).

The manifestation of X-linked mutations depends on the developmental stage considered. Prior to XCI, the presence of two expressed alleles in females may provide a fitness advantage in cases of a heterozygous recessive X-linked mutation (but not a dominant one). However, once XCI has taken place, only a single expressed allele persists in each somatic cell of both sexes. While X-linked-gene hemizygosity in males (except for genes in the pseudoautosomal regions PAR1 and PAR2) results in full expression of deleterious variants, heterozygous females are natural mosaic for cells with either parental X chromosome being active, which confers protection from pathogenic X-linked variants in many conditions (Fig. 1A) (13,18). One of the consequences of random XCI is that cell-to-cell variability for each variant haplotype is doubled in females compared with males, which may enhance the breath of responses to stimuli or injuries, providing further advantage to females in both health and disease (19). Female tissues are a patchwork of cells that express either paternal or maternal X alleles, with patches variable in size and among cell types and tissues due to the timing of silencing during embryogenesis, which occurs when a few hundred cells are present. A surprisingly wide variation in the proportion of cells with maternal or paternal allele expression has been found among tissues of female mice by imaging each allele of a reporter gene labeled with a red or green fluorescent marker (20). A study of healthy human monozygotic female twins has also revealed remarkable differences in X inactivation skewing within pairs, especially in fat and skin tissues (21). However, analyses of gene expression in bulk tissues obscure the variable distribution of cells expressing either paternal or maternal alleles. Single-cell studies in multiple human tissue types, cell types and developmental stages will help determine the extent of XCI variability in women, which could explain variable phenotypes not only in disease but also in healthy individuals.

Although random XCI results in two distinct cell populations, not all women have an overall 50:50 ratio of cells with one

Table 1. Examples of inherited X-linked diseases affected by XCI patterns

X-linked disease	Gene	XCI status in carriers	Mechanism	Phenotype	References
Duchenne muscular dystrophy	*DMD*	Random XCI	Sufficient number of cells expressing cell autonomous protein	N	28
Duchenne muscular dystrophy	*DMD*	Skewed XCI toward mutated allele	X:autosome translocation causes skewing	A	29
Hunter syndrome	*IDS*	Random XCI	Sufficient amount of secreted protein	N	30,31
Fabry disease	*GLA*	Random XCI	Normal protein product not taken by mutant cells	A	32, 33
Lesh-Nyhan	*HPRT*	Random XCI in fibroblasts/skewed XCI toward normal in blood	Gap junctions between fibroblasts/cell selection in blood	N	34
Adrenoleukodystrophy	*ABCD1*	Skewed XCI toward mutated allele	Growth advantage of cells expressing mutation	A	26
Craniofrontonasal syndrome	*EFNB1*	Random XCI	No substitution for EFNB1 deficiency	A	35
Rett syndrome	*MECP2*	Variable XCI skewing	Critical protein; mutation lethal in males	A	36
ICF syndrome	*DNMT3B*	Aberrant XCI	Hypomethylation of various sequences	A	39
XLID due to escape genes	e.g. *KDM5C, KDM6A*	Escape XCI; partial XCI skewing	Haploinsufficiency	A	61–65
X aneuploidy	Escape genes; e.g. *KDM6A*	Random XCI	Dosage imbalance; genome-wide expression and DNA methylation effects	A	74
SLE	*TLR7, TLR8, IRAK1, CXORF21*	Eroded XCI	Higher gene expression in B- and T-cells	A	84–86

or the other X chromosome active. About 10% women display strong XCI skewing, such that greater than 95% of their cells express the same parental allele (Fig. 1A) (22). The proportion of each cell population has profound effects on the manifestation of X-linked diseases in heterozygous females. Monozygotic twin studies reveal that XCI skewing can change with age, a phenomenon often observed in blood and attributed to clonal hematopoiesis (21). Methods to read out skewing based on testing DNA methylation at a single locus, e.g. at the *AR* gene, have been supplemented by whole X chromosome allele-specific gene expression read-out by exome sequencing (23). Replication analyses can also be used to identify the inactive X chromosome based on its late replication pattern, especially in cases of structural X anomalies (24). Nonrandom XCI can be a chance event during embryogenesis or can be due to a rare mutation in the promoter of the *XIST* gene, which is essential for the onset of XCI (25). More often, skewing favors the normal allele due to cell selection, in which case a female carrier would usually not display clinical abnormalities (Fig. 1A; Table 1). The rate of cell selection in female tissues varies, which would affect the expression of the disease variant. While such selection mostly favors normal alleles, a rare example of the opposite effect is seen in adrenoleukodystrophy where cells with an *ABCD1* mutation have a growth advantage (Table 1) (26). In some cases, complete XCI skewing can result from an undetectable lethal mutation on the so-called 'normal' X chromosome, which becomes preferentially silenced, potentially allowing for full expression of a mutation on the other X chromosome. Complete skewing can also be due to confined placental mosaicism in uniparental disomy (27).

In some conditions, interactions between mosaic cell populations in female tissues can foster a metabolic cooperation and normal cells may provide essential gene products to correct the defect in mutant cells. In cases of mutations in cell autonomous products, females might not display symptoms if random XCI results in a sufficient number of cells that express the protein, for example in Duchenne muscular dystrophy carriers (Table 1) (28). However, rare cases of carrier women with full expression of the disease occur in individuals with an X; autosome translocation or other structural rearrangement that breaks the *DMD* gene and causes complete skewing of inactivation of the normal allele (Table 1) (29). In conditions due to mutations in secreted proteins, there may be sufficient product even without skewing of XCI. For example, females with Hunter syndrome, an X-linked lysosomal disease caused by deficiency of iduronic sulfatase (IDS), rarely have any clinical symptoms (Table 1) (30,31). In contrast, females with Fabry disease, another lysosomal disease caused by a deficiency in *a*-galactosidase (*GLA*), may have some clinical symptoms also seen in affected males, which can be explained by inability of the lysosome to take up the normal product (Table 1) (32,33). Tissue-specific differences in XCI skewing have also been reported. For example, in asymptomatic women carriers of an *HPRT* mutation XCI skewing does not occur in fibroblasts where gap junctions allow protein transit between cells but occurs in blood cells that lack junctions (Table 1) (34). Paradoxically, women carriers of an X-linked variant can occasionally manifest more severe symptoms than men. For example, men with a mutation in ephrin-B1 (*EFNB1*), which causes craniofrontonasal syndrome, are spared severe phenotypes because of redundancy in the essential functions of EFNB1, while women are severely affected because their mosaic state does not permit substitution for EFNB1 deficiency (Table 1) (35). In conditions where the disease is lethal in males, only females are born, for example,

in Rett syndrome caused by a loss of function mutation in *MECP2* (Table 1) (36). We refer the reader to a recently published comprehensive review of the effects of X-linked mutations on women health for additional examples (13).

Women may also manifest diseases caused by mutations in genes encoding essential components of the XCI machinery itself, a complex process with multiple layers of control (37,38). As mentioned above, a rare mutation in the promoter of the *XIST* gene results in skewing of inactivation (25). Mutations that affect XCI components often alter many other regulatory mechanisms that employ the same epigenetic processes throughout the genome. An example is afforded by mutations in the DNA methylase *DNMT3B*, which cause ICF (Immunodeficiency, centromeric region instability, facial anomalies) syndrome (Table 1) (39). This syndrome is characterized by T-cell but not B-cell deficiency and hypomethylation of centromeric repeats, which leads to chromosomal rearrangements throughout the genome. Hypomethylation of some X-linked genes occurs in a subset of ICF female patients, but there is little gene reactivation owing to the multiple layers controlling XCI (39). Few disorders caused by mutations in components of the machinery that specifically control XCI onset and stability have been discovered. One can speculate that such disorders might cause female lethality or complete XCI skewing since silencing of one X chromosome is essential for female survival. Such sex-specific lethality has been reported in mice with a mutation in *Smchd1*, a gene essential for the onset of XCI and for compaction of the human inactive X chromosome (40,41). In fact, it has been reasoned that the distorted sex ratio at birth (more males than females are born) could be contributed at least in part by female-specific susceptibility to failure of properly silencing one X chromosome (42). Interestingly, stem cell differentiation during embryonic development depends on successful completion of XCI for release of pluripotent factors, as shown by single-cell analyses in which X-linked genes implicated in the MAPK signaling pathway were identified as critical for this process (43–45). Mutations in X factors important for maintenance of XCI have been reported in autoimmune disorders and cancer (see below).

Escape from XCI and Role in Diseases

In human, ~20–30% of X-linked genes are expressed to some extent from the silent X chromosome (46–49). Except for PAR1 genes, which are mostly male-biased, escape genes often have higher expression in women, potentially leading to sexually dimorphic traits in susceptibility to disease (50). However, expression from the allele on the inactive X chromosome is usually lower than that from the active allele, probably due to their embedding within silenced chromatin (49). Genes that escape XCI are often clustered in domains that lack repressive histone marks on both alleles, and they are usually hypomethylated at their CpG island, which has helped their identification (51,52). In addition, the body of some escape genes is marked by non C-G methylation in neuronal cells (53). Escape genes have diverse functions, with those that retain a Y-linked paralog often highly dosage-sensitive and exhibiting critical functions related to the regulation of a number of other genes (54,55). There is a great deal of variability in the extent of escape from XCI among tissues/cell types and individuals, which has led to the definition of constitutive (always escape) and facultative (variable escape) escapees (56,57). Despite potential difficulties with low coverage, single-cell studies of gene expression by RNA-seq have helped identify escape genes based on SNPs. So far, such studies largely validated the previously reported annotations of human escape genes in fibroblast and lymphoblast transcriptomes, but this approach promises to extend our knowledge to many other cell types within human tissues for a better understanding of their role in disease (58). The profiles of escape from XCI vary among mammalian species, which makes it difficult to study them in animal models, except for genes that consistently escape XCI in both human and the model species (44). As an example, recent studies in mouse models have implicated *Kdm6a*, a gene that escapes XCI in human and mouse, in both Alzheimer disease and immune responses, which makes *KDM6A* an attractive candidate for a role in the corresponding human conditions (59,60).

Mutations in escape genes located in the pseudoautosomal regions of the sex chromosomes generally behave like mutations in autosomal genes. However, mutations in escape genes located outside the pseudoautosomal regions usually cause abnormal phenotypes in both sexes (Fig. 1B). In carrier females, the severity of phenotypes depends on whether the mutation is dominant, on the expression level from the inactive X and on the level of XCI skewing. Because many escapees are highly dosage sensitive, variants often cause a deficiency, only partially ameliorated by expression from the inactive X allele in women. Skewing of XCI in favor of the normal allele can alleviate the effects of a pathogenic variant and partially protect women from abnormal phenotypes. These effects are exemplified by mutations in the escape genes *KDM6A* and *KDM5C*, which cause multiple abnormalities including X-linked intellectual disability (XLID) in both sexes, but with more severity in males than females (Table 1) (61–64). Mutations in escape genes are an especially common cause of XLID (12,65). As discussed below, autoimmune diseases, which are much more common in women, are likely caused by abnormal expression of escape genes. Furthermore, abnormal escape gene dosage due to X aneuploidy contributes to a milieu of deleterious phenotypes including infertility, intellectual disability, immune diseases and cancer (66).

X Chromosome Aneuploidy

Sex chromosome aneuploidies, characterized by the loss or gain of one or more sex chromosomes, disturb the balance of gene products and result in recognizable syndromes such as Turner syndrome (45,X) and Klinefelter syndrome (47,XXY) (Fig. 2A). Turner syndrome is a near-lethal condition during embryogenesis, with individuals who survive presenting an array of abnormal phenotypes including infertility, short stature and heart anomalies (67,68). Klinefelter syndrome is a common cause of male infertility but also presents with autoimmune diseases and cognitive disturbances (69–71). Other X aneuploidy syndromes include triple X syndrome (47,XXX) associated with mild intellectual disability, while a greater number of sex chromosomes, e.g. 49,XXXXX, significantly increases morbidity (72,73). Yet, in all these conditions, only a single X chromosome remains active and all others are silenced regardless of sex. Thus, genes expressed prior to XCI in early development and genes that escape XCI are candidates to explain abnormal phenotypes. Sex-chromosome dosage (SCD) effects on genome-wide expression within a large cohort of individuals with diverse karyotypes (X, XX, XXX, XXXX, XY, XXY, XYY, XXYY and XXXXY) revealed a clear effect of chromosome ploidy on expression of escape genes (Fig. 2B) (74). Interestingly, some X-linked genes were upregulated on the single X chromosome in 45,X compared with 46,XX or 47,XXX individuals, suggesting a partial compensatory mechanism in Turner syndrome. In addition, an increasing number of sex chromosomes did not increase gene expression

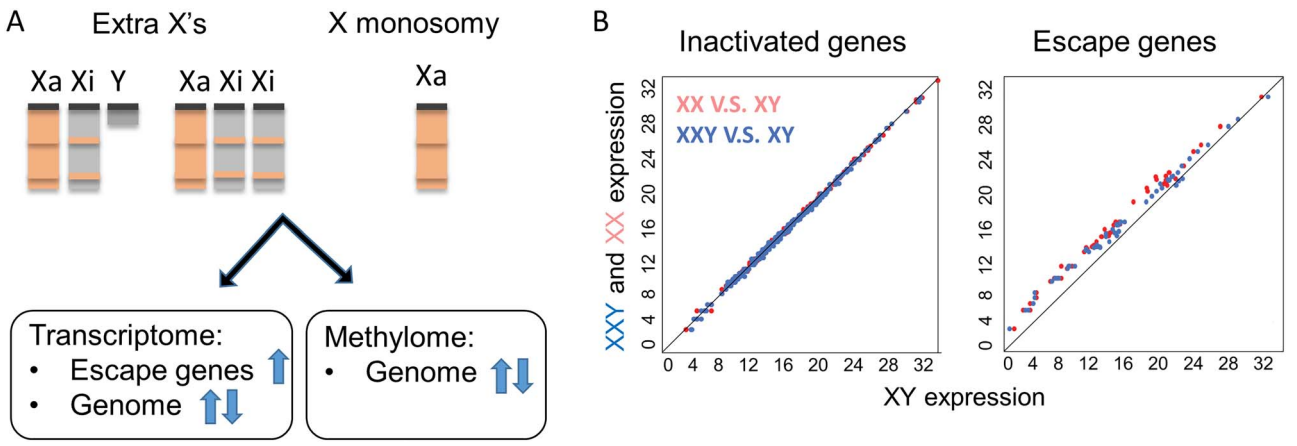

Figure 2. Sex chromosome aneuploidies disturb both the transcriptome and methylome in cells. (**A**) Extra X chromosome copies in Klinefelter syndrome (XXY) or Triple X syndrome (XXX), and the unique X chromosome in Turner syndrome (45,X) all disturb escape gene transcription, genome-wide gene transcription and DNA methylation. (**B**) Scatter plots of X-linked gene expression in female (XX, red) or Klinefelter (XXY, blue) versus male (XY). The expression of escape genes (right) is higher in Klinefelter males and normal females compared with normal males, while the expression of genes subject to XCI (left) remains similar between samples. Data reanalyzed from (130).

linearly, indicating extensive trans-acting inverse effects of SCD on autosomal expression. Other factors may stem from the presence of multiple heterochromatic structures in the nucleus of aneuploid cells, which could disrupt epigenetic marks elsewhere in the genome and affect gene expression and homeostasis. In fact, the 3D structure of the active X chromosome in cells with sex chromosome aneuploidy is disrupted (75). Although dosage imbalance arising from escape genes is believed to contribute significantly to aneuploidy phenotypes, so far few specific genes have been implicated. One potential candidate is *KDM6A*, which regulates reproduction-related pathways in females and thus may be involved in ovarian dysfunction in Turner syndrome (76,77).

X chromosome aneuploidy leads to widespread epigenetic changes, including differential DNA methylation in target genes implicated in pathways associated with clinical features. Interestingly, DNA methylation levels on the X chromosomes of 47,XXY patients are distinct from both those of 46,XX and 46,XY controls (Fig. 2A) (78). Furthermore, differences are also evident between 47,XXY (hypermethylated X) and 45,X patients (hypomethylated X) (79). As with expression analyses, DNA methylation changes are not limited to the X chromosomes (80,81). Interestingly, loss of an X chromosome has a distinctive effect on autosomal genes compared to gain of an additional X chromosome. Approximately 80% of the CpG island methylation changes in 45,X individuals represent hypomethylation of autosomal loci, whereas nearly equal numbers of hypo- and hypermethylated CpG sites are observed in autosomes from 47,XXY individuals (82). Importantly, pathway analysis with differentially expressed genes and differentially methylated genes are found to be complementary, rather than overlapping. This finding suggests that DNA methylation as an epigenetic hallmark is not necessary illustrated by the transcriptome. Conversely, reversal of DNA methylation may not lead to a normal transcriptome, which has implications for future therapeutic target screenings (76).

XCI and Immune Diseases

As discussed above, XCI skewing and escape from XCI often vary among individuals, tissues and cell types, which results in phenotypic diversity (21,50,83). Intriguingly, B- and T-cells from women show a less completely inactivated X chromosome, with more escape genes, some immune-related (Fig. 3) (84–86). This may account for a more robust response mounted by women in response to infections, e.g. to COVID-19 (2). The flip side of this sex-specific advantage is that some autoimmune conditions are prevalent in women in whom aberrant escape from XCI has been observed. For instance, multiple sclerosis (MS), a disease in which the immune system attacks the insulating myelin around nerve fibers, is about three times more common in women than men (87). In a recent study, Itoh and colleagues employed an experimental autoimmune encephalomyelitis mouse model of MS to identify the escape gene *Kdm6a* as the main candidate in MS susceptibility (60).

TLR7, a member of the TLR family important for pathogen recognition and activation of innate immunity, escapes XCI and thus has higher expression in interferon-α producing cell types including B-lymphocytes, monocytes and plasmacytoid dendritic cells of XX and XXY versus XY individuals (88,89). The escape status of *TLR7*, and of other genes important in immunity including *RPS6KA3*, *CYBB*, *BTK* and *IL13RA1*, was confirmed in human plasmacytoid dendritic cells, stressing the contribution of multiple X-factors to sex differences in immune responses (90). *TLR7* is also more abundant in female than male microglia, which could influence sex differences in brain function (91). A related gene, *TLR8*, escapes XCI in macrophages, a cell type activated in the inflammatory response to viral infection through the production of granulocyte-macrophage colony-stimulating factor (CSF2) (92). A recent study of human CD11c+ atypical memory B-cells, which can expand during aging and cause aberrant responses to infectious diseases as well as female-biased autoimmunity, identified *XIST* RNA-independent and *XIST* RNA-dependent X-linked genes critical for maintenance of XCI stability (93). One of the genes whose silencing depends on continuous *XIST* expression is *TLR7*, implicating *XIST* RNA as a critical factor for maintenance of silencing of specific genes. Both *TLR7* and *TLR8* are highly expressed in systemic lupus erythematosus (SLE), an autoimmune disorder prevalent in women in whose T-cells *XIST* RNA localization and the *XIST* RNA interactome are perturbed (Fig. 3). Other escape genes implicated in SLE include *IRAK1*, and

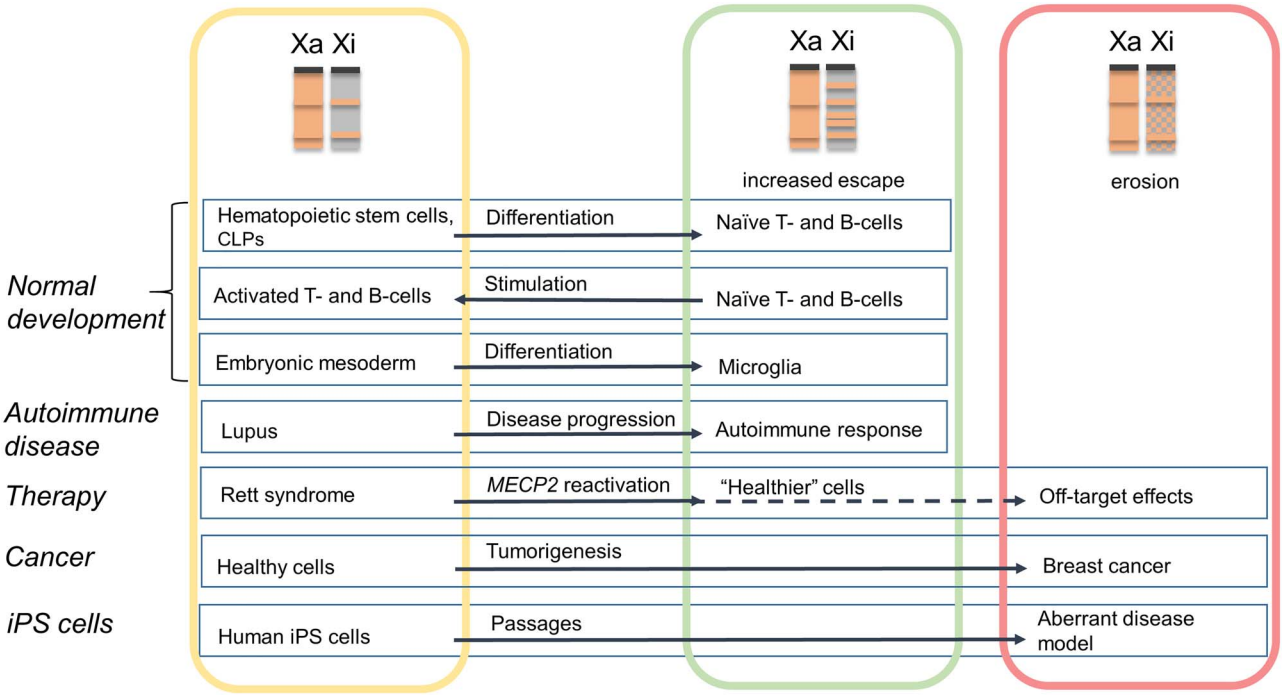

Figure 3. XCI changes in normal development, disease, therapeutic treatment and cell engineering. Cells in the yellow rectangle have normal XCI, cells in the green rectangle have partial X reactivation with an increase in the number of escape genes, and cells in the red rectangle have eroded XCI. CLPs are common lymphoid precursors. The dotted line indicates the potential for erosion due to off-target effects following therapy. Note that X reactivation in cancer cells could be beneficial due to enhanced neoantigens and better response to immunotherapy. Normal reactivation and potential dampening that occur in precursors of germ cells are not depicted.

CXORF21, both potential targets for therapy (94,95). Interestingly, XXY males with Klinefelter syndrome are also susceptible to SLE (96).

X Chromosome and Cancer

Abnormal X chromosome copy number and aberrant patterns of XCI can occur in cancer (97,98). Imbalanced dosage of X-linked genes caused by copy number alterations of the whole X chromosome or of regions wherein and by erosion of XCI, has been implicated in oncogenesis. In the early 1950s, Barr and Moore discovered that nuclei of certain breast tumors lacked a Barr body (99). It was later shown that XCI is unstable in some breast tumors, a phenomenon associated with dispersed *XIST* RNA in the cancer cells (Fig. 3) (100). Translocations involving regions of the X chromosome could also alter gene expression profiles and lead to cancer. For example, relocation of regions of the inactive X chromosome to an autosome could result in reactivation of previously silent X-linked oncogenes. Conversely, loss of expression of an autosomal tumor suppressor can result from translocation to the inactive X chromosome (97).

XIST RNA is a key regulator of dosage compensation and is critical in maintaining XCI. Direct causal relationship between *Xist* RNA and cancer was confirmed in a mouse model in which *Xist* deletion in hematopoietic cells induced myeloproliferative neoplasm and myelodysplastic syndrome (101). In human, loss of *XIST* expression in iPS cells is significantly associated with upregulation of X-linked oncogenes (102). Recently, *XIST* has been reported to be dysregulated in gastric cancer (103), hepatocellular carcinoma (104), nasopharyngeal carcinoma (105), breast cancer (106) and ovarian cancer (107). *XIST* appears downregulated in recurrent ovarian tumors, but this is often due to the loss of the inactive X chromosome because of genomic instability in the tumor cells (108). Indeed, loss of either the inactive X chromosome in females or the Y chromosome in males is common in older individuals and may play a role in cancer susceptibility (109–112). Paradoxically, high *XIST* expression may also be deleterious in patients with a solid tumor, possibly due to sponging of miRNAs leading to upregulation of oncogenes by *XIST* RNA (113). Another X-linked lncRNA, *FIRRE*, implicated in XCI is also highly expressed in cancer cells, but its specific role remains to be discovered (114,115).

Acquired somatic mutations in X-linked genes are a frequent cause of cancer. Comparison of 402 whole genomes from a diverse set of childhood and adult tumors revealed hypermutation of the X chromosome (116). Somatic mutations in genes located in repressed chromatin domains including the inactive X chromosome are more frequent than in active domains, which is linked to the 3D chromatin structure (117). The main types of genetic alterations that lead to cancer—tumor-suppressor inactivation and oncogene activation—produce different results when they target X-linked versus autosomal genes. In females, a mutation propagated in a clonal manner would remain silent if it occurs on the silent allele of an X-linked gene. However, males would express the mutation. Accordingly, many cancers with a clear sex difference affect males more than females (118). To note, because XCI is usually stable once established, clonal expansion of a somatic cell in cancer results in a cell population with completely skewed XCI in women. Nonrandom XCI skewing can result in a selective growth advantage of cells with expression of the normal or the pathogenic allele. When selection severely disfavors cells that express the variant, heterozygous females rapidly lose mutant cells and render no symptoms. In contrast, if variant cells have a selective growth advantage,

females manifest symptoms of the disease. For example, *Foxp3* heterozygous female mice develop breast cancer at an enhanced rate as they age, due to the growth advantage of the mutant cells (119).

For oncogenes that escape XCI, acquired mutations may result in a similar disease susceptibility in men and women. However, for tumor suppressor genes that escape XCI, women would be protected by their second copy, resulting in a lower prevalence compared with men. Indeed, in a large study of about 4100 specimens across 21 tumor types, six escape genes called EXITS (Escape XCI Tumor Suppressor), including *ATRX*, *CNKSR2*, *DDX3X*, *KDM5C*, *KDM6A* and *MAGEC3*, were identified as genes which loss-of-function mutations affected men more frequently than women (120). In some cases, increased expression of an escape gene activated by a carcinogen can increase the risk of cancer in women. For example, *GRPR*, an escape gene expressed in lung epithelial cells, is associated with an increased risk of lung cancer due to tobacco exposure in women (121).

Therapeutic Induction of X Chromosome Reactivation

As stated above, there is a 3.5-fold increase of XLID disorders, compared with those due to mutations in autosomal genes. Here, we refer the reader to recent reviews on the subject (11,12), and we focus on new research that aims to cure deleterious neurological effects of X-linked disorders by reactivation of the normal allele in female carriers. Reactivation of X-linked genes occurs naturally in germ cells (122,123). However, silencing is highly stable in somatic cells; hence, reactivation is difficult. Rett syndrome caused by a mutation in *MECP2* has been the focus of intense searches for compounds that would reactivate the normal allele using inhibitors of *XIST* expression in cell-based systems (Fig. 3) (124,125). Reactivation has also been achieved *in vivo* in a mouse model of Rett syndrome, in which small molecule inhibitors of 'XCI factors' reactivated *Xist* and corrected the neurological phenotype. Fortunately, while *Mecp2* was reactivated, there did not appear to be widespread off-target effects on overall X-linked gene expression, suggesting compensatory mechanisms to maintain a normal balance of expression between X and autosomes (126). A similar strategy could potentially be applied to other X-linked disorders that affect female carriers, including neurological disorders caused by mutations in *CDKL5*, *HDAC8* and *KIAA2022*. A new strategy was recently used to reactivate *CDKL5* by editing DNA methylation using the demethylase TET1 and dCas9 with guides targeted to the CpG island of the gene (127). Such precise strategy has the advantage of limiting off-target effects on the genome. Female carriers affected by mutations in escape genes, e.g. *DDX3X*, *KDM5C*, *KDM6A*, *USP9X* or *SMC1A*, may also benefit from X reactivation to increase expression from the normal allele when it is on the inactive X chromosome. Finally, reactivation of the hypermutated inactive X chromosome in female cancer cells by inhibition of repressive chromatin marks has been proposed as a way to enhance the response to immunotherapy by increasing neoantigens (117).

Conclusions

The content and peculiar regulation of X-linked genes, which were shaped by evolution, profoundly influence the expression of diseases caused by mutations or copy number changes (Table 1). The advent of new methods to examine the onset and stability of X chromosome silencing in females has greatly improved our understanding of X-linked disorders. Future studies conducted in single individual cells promise to add much more information on the modalities of X chromosome regulation, which will help find better approaches to design an effective therapy of congenital and acquired disorders. Such studies can be supplemented by analyses of human induced pluripotent stem (iPS) cell lines to examine sex-specific effects of X-linked mutations in cell types relevant to a disorder. To note, XCI can be eroded in female iPS cells and it is usually skewed toward one allele due to cell cloning, necessitating the analysis of multiple clones together with the verification of the integrity of the inactive X chromosome (Fig. 3) (128,129). Nonetheless, analyses of organoids derived from stem cells obtained from patients with X-linked mutations will help understand their impact during development and in specific tissue/cell types.

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