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Genetics and epigenetics of the X chromosome

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A consequence of Mendelian inheritance of X-linked traits is that women are more than equal to men in the face of X-linked diseases, protected as they are by the presence of two X chromosomes in their genome. This potentially beneficial inequality is diminished by the molecular mechanism known as X-chromosome inactivation (XCI), which triggers the transcriptional silencing of one of the X chromosomes in each female cell. The determination of which X to inactivate, a process that occurs during early embryogenesis, is random and clonally inherited. As a result, females are mosaic for the expression of X-linked genes. XCI is a highly regulated process involving large noncoding RNAs, chromatin remodeling, and nuclear reorganization of the X chromosome. It is a paradigm for epigenetic regulation and is frequently used as a biomarker for monitoring long-range gene reprogramming during cell differentiation and dedifferentiation. Our review analyses how XCI affects the expression of X-linked mutations, describes some of the most recent discoveries on the molecular mechanisms triggering XCI, and explores the therapeutic potentialities of the XCI process per se.

Keywords: X-inactivation; chromatin; epigenetic reprogramming; cellular mosaicism

Introduction

If Gregor Mendel had used as experimental model calico cats instead of peas, he would certainly have noticed that the tortoiseshell coat color was only seen in females and come to the conclusion that this trait is sex linked. But he would surely have struggled trying to apply his famous rules of heredity to the transmission of this mosaic patterning and, more generally, to the transmission of X-linked traits in mammals. This is because the X chromosome is submitted to a unique regulation system that not only relies on formal genetics but also—and maybe above all—on classical epigenetics.

In humans, sexual dimorphism is associated with the presence of two X chromosomes in females, and an X and a Y chromosome in males. The X chromosome is 155 Mb long and carries some 1,250 known genes (http://www.ensembl.org/Homo_sapiens/). The Y chromosome is some three times smaller and has the lowest known gene density of any human chromosome (Fig. 1A). In contrast with the X chromosome, the Y chromosome has changed rapidly in mammalian evolution (for review on sex chromosome evolution, see Refs. 1 and 2). Its unique structure, the paucity of genes on it, and the high content of repetitive elements all testify to a rapid loss of active genes and accumulation of repetitive sequence on the Y chromosome. This loss is thought to have been driven by the acquisition of a novel male-determining gene (SRY), followed by acquisition of male advantage genes (i.e., spermatogenesis genes) nearby; recombination was suppressed to keep together the male-specific package of genes. The absence of recombination with the X chromosome promoted accumulation of mutations and deletions because of drift and inefficient selection.[1] This results in the unusual situation whereby the homology regions shared by the X and Y chromosomes are reduced to two extremely small regions, called the pseudo-autosomal regions 1 and 2 (PAR1 and PAR2), located at each end of the X and Y chromosome (Fig. 1B). The quasi-hemizygote state of the X chromosome resulting from this situation in males renders them more susceptible to X-linked mutations than females who carry two copies of

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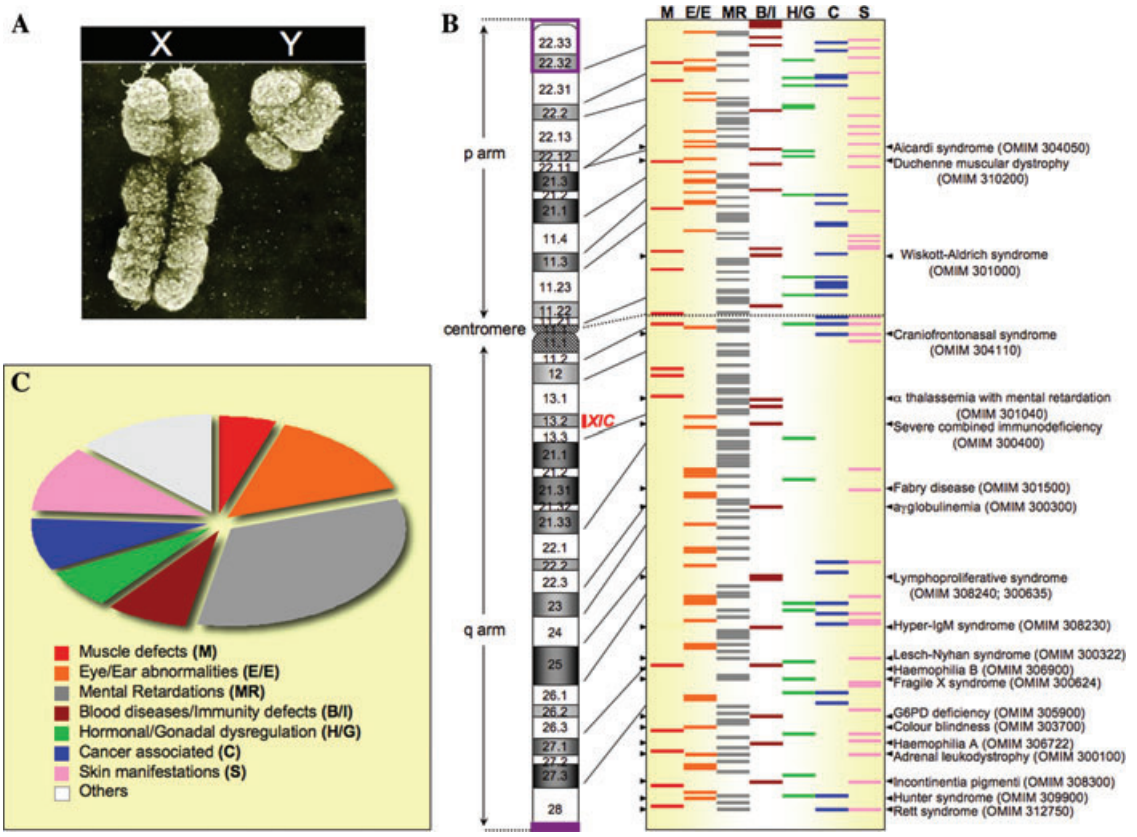

Figure 1. Human X-chromosome map and distribution of X-linked diseases/syndromes. (A) Scanning electron micrograph of an X and Y chromosome (image courtesy of Indigo Instruments, Canada). (B) Ideogram of the X chromosome showing the position of 275 known X-linked diseases or syndromes and the associated clinical features (M, muscle defects; E/E, eye/ear abnormalities; MR, mental retardation; B/I, blood disease/immunity defects; H/G, hormonal/gonadal dysregulation; C, cancer; S, skin manifestations) (see also Table S1). The diseases cited in this review are shown on the right of the table. The location of the X-inactivation center (*XIC*) controlling XCI initiation and of the pseudo-autosomal regions (PARs) are shown in red and violet, respectively. (C) Graph showing the distribution of X-linked diseases/syndromes within the seven categories of clinical features described in (B).

each gene. This genetic vulnerability is reflected in the preferential occurrence of X-linked diseases in males, and probably contributes, at least in part, to the increased male mortality rates observed at every stage of life.[3]

Sexual dimorphism also introduces two different types of disequilibrium into the dosage of X-linked genes. First, in males, the copy number of X-linked genes is reduced by half as compared to autosomes. In mice, this is compensated for by an upregulation of transcription (1.4- to 1.6-fold) of all X-linked genes during early embryogenesis in both the male and female.[4] This upregulation reaches twofold during the later stages of differentiation, thereby equalizing gene expression levels between the single X and the diploid autosomes in male cells. The molecular mechanisms responsible for this upregulation have not as yet been characterized. However, the comparison of triploid cells bearing a single active X (Xa) with triploid cells carrying two Xa's has shown that expression levels of only a subset of X-linked genes, whose expression dosage in relation to autosomal expression may be critical, are adjusted.[5]

The second disequilibrium results from the imbalance in the number of sex chromosomes between males and females. Various strategies have been adopted by different species to achieve this second type of dosage compensation. In fruit flies, the single X chromosome doubles its transcriptional activity compared to either of the two female X chromosomes, whereas in nematodes, hermaphrodites XX animals halve the level of X-linked transcription.

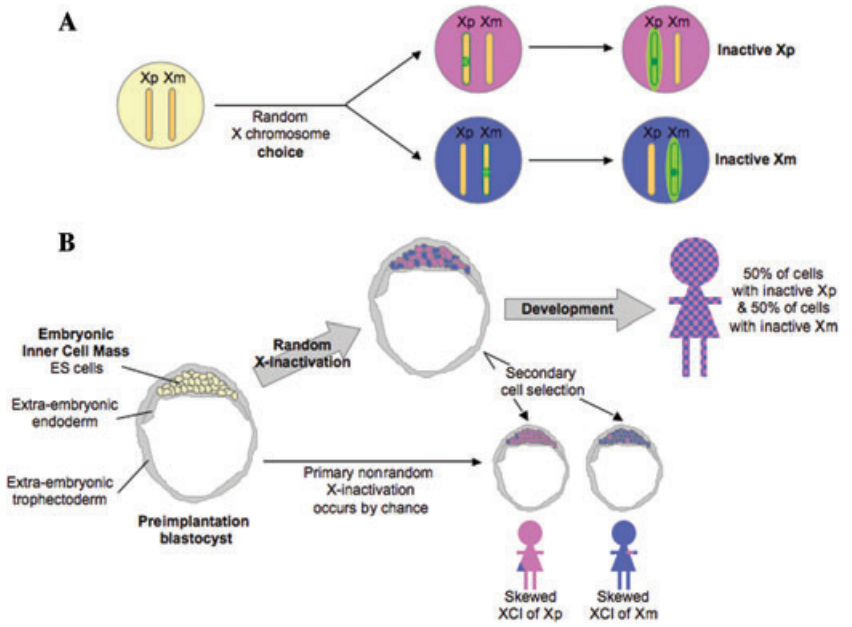

Figure 2. X-inactivation skewing. (A) Schema of X-chromosome random choice. Before XCI, both Xs (the maternal: Xm and the paternal: Xp) are active (yellow cells). *XIST* RNA is randomly selected to be expressed from either the maternal X (bottom row, blue cells) or the paternal X (top row, pink cells). *XIST* coats the chromosome from which it is produced (green halo surrounding the Xi) and triggers its transcriptional silencing and condensation. (B) Models of skewed X-inactivation. During embryogenesis, XCI is initiated in the inner cell mass of the female blastocyst carrying two active Xs (yellow cells). In most cases, random XCI results in a patchwork of cells with an inactive Xm (blue cells) or an inactive Xp (pink cells). With further development and cell divisions this random distribution is maintained, ultimately giving rise to women who are mosaic for the expression of X-linked genes: 50% of cells express the paternal copy (blue cells) and 50%, the maternal copy (pink cells). In primary nonrandom XCI, some factor or modification alters this randomness, so that either the Xp or the Xm is preferentially inactivated. In secondary cell selection, random XCI occurs normally but a mutation of a gene on one or the other X conferring a growth advantage or, conversely, inducing cell lethality favors the selection of cells carrying either an inactive Xp or an inactive Xm.

In mammals, one of the two X chromosomes, determined randomly, is silenced during early female embryogenesis. This random X-chromosome inactivation (XCI) results in cellular hemizygosity of X-linked gene expression and in balanced mosaicism at the level of the organism, with about half of the cells expressing the maternal X and the other half the paternal X (Fig. 2). The female calico cat illustrates very well this phenomenon: cells in which the X chromosome carrying the orange coat color (X^O) allele is inactivated express the alternative black ($\mathbf{X}^B$) allele whereas cells in which the black (X^B) allele is inactivated express the orange (X^O) allele, hence the orange/black tortoiseshell coat. In addition, some placental mammals (i.e., mice, cows) also show an imprinted form of inactivation of the paternal X in extra-embryonic tissues.

One of the most striking features of XCI is that it requires that two homologous chromosomes within the same nucleus be treated differently. Historically, the XCI process has been divided into three phases: initiation, spreading, and maintenance of the inactive state (Fig. 3, for review see Ref. 6). Initiation involves a *trans*-sensing of the two X chromosomes, resulting in the counting of the number of Xs in the cell to keep only one Xa chromosome per set of autosomes and of which X to inactivate and which X to maintain active. The *cis*-spreading XCI signal is achieved through sequential epigenetic modifications of the future inactive X (Xi), including the accumulation of the large noncoding *Xist* (*X-inactive specific transcript*) RNA, the loss of active and the acquisition of silent chromatin marks through the recruitment of, among others, polycomb repressor complexes (PRCs), the shift to late replication timing, the enrichment in a specific histone variant, and CpG island methylation of promoter regions (Fig. 3). These multiple

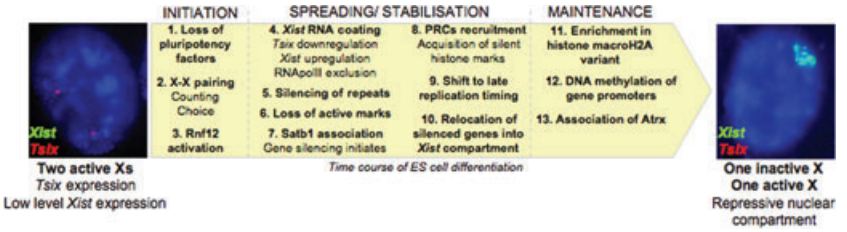

Figure 3. Sequential modifications of the inactive X chromosome. Main steps leading to XCI during the time course of mouse ES cell differentiation. Before XCI, both X chromosomes of female ES cells are active and characterized by low-level *Xist* expression and high-level *Tsix* expression, as shown by RNA-FISH using *Xist* (green) and *Tsix* (red) specific probes on DAPI-stained nuclei (left picture). *Tsix* transcripts are visualized as pinpoint signals at the *Xist/Tsix* loci of both active X chromosomes. *Xist* expression is almost undetectable. At the onset of differentiation, when counting and determination of which X to inactivate are thought to occur, the loss of pluripotency factors (Nanog, Oct4, Sox2,[45] Rex1, Klf4, and c-Myc),[46] the upregulation of Rnf12[54] associated with X–X *trans*-interactions[51,89] result in *Tsix* downregulation, *Xist* upregulation, and *Xist* RNA coating of the future Xi, as illustrated on the RNA-FISH picture on the right. The precise kinetics of the three events in the XCI initiation phase remains to be established. Almost simultaneously, RNA pol II enzymes are excluded from this repressive compartment, active histone marks are lost, and polycomb complexes are recruited[90,69,91] to set up silent histone marks.[74,92] Concomitantly, silencing of X-linked repeats followed by gene silencing (mediated by Satb1[73]) occurs and the Xi shifts to late replication phase. Later on, silenced genes are relocated to the *Xist* repressive nuclear domain,[71] the Xi becomes enriched in the macroH2A histone variant[93] and in the ATRX protein,[94] whereas promoter regions of X-linked genes are methylated. For a recent review of the modifications of the Xi see Ref. 33.

modifications ensure the stabilization and maintenance of the inactive state throughout subsequent mitotic divisions.

The first part of this review describes the consequences of XCI on the expression of heterozygote X-linked mutations and how observed X-linked phenotypes result from Mendelian inheritance of X-linked traits, whose expression are then modified by the XCI process. The second part of this review summarizes our current knowledge of the molecular mechanism of XCI, with special emphasis on the most recent advances in our understanding of the initiation and spreading phases of XCI. The last section of this review reports how XCI can be used as a diagnostic tool to detect X-linked mutations, and describes some of the therapeutic strategies that have been envisaged as treatment for specific X-linked diseases. We also speculate on how the molecular properties of XCI might be "hijacked" to ectopically silence abnormal gene expression.

Consequences of Mendelian inheritance and X chromosome inactivation on X-linked haplotype expression

Ontology of X-linked genes and mutations

A closer examination of human X-linked diseases reveals that the X chromosome is especially enriched in genes whose mutation leads to mental retardation (for review on X-linked mental retardations, see Ref. 7) and in genes related to sexual reproduction functions (Fig. 1C). This unique distribution supports the so-called faster-X hypothesis, which suggests that sex chromosomes evolve more rapidly than autosomes due to the hemizygote status of X-linked genes in males, which imposes a high selection pressure leading to an enrichment in a specific category of genes (for review on sex chromosome evolution, see Ref. 1). Interestingly, the excess of genes involved in brain functions correlates to the fact that X-linked genes are highly expressed in brain in comparison with autosomal genes,[6] an effect that appears especially pronounced in humans, in contrast to rodents.[8] This has led to the hypothesis that, over the last few million years of hominid evolution, females have selected smart males to mate with—perhaps because smarter males are better breadwinners?—thereby explaining the extensive brain development in humans. Differences in X-linked gene expression in brain tissues between the sexes may also explain supposed differences in the brain capacity of men and women, although the very existence of such differences is the subject of a long-standing debate. Indeed, qualitative and quantitative analyses of X-linked gene expression in brain tissues have failed to detect any major differences between female and male.[8]

The "faster-X hypothesis" also explains the X-chromosome enrichment for genes expressed in

testis; the X chromosome is enriched for genes expressed in spermatogonia[9] but not for genes expressed in later stages of spermatogenesis, likely because of silencing at meiosis (MSCI).[10] These genes are thought to have accumulated on the X because recessive mutations expressed in males, due to hemizygosity of the X, could give rise to novel functions that enhance male sexual reproduction (i.e., bigger, faster, more sperm). Even if the mutations are deleterious to females (as "sexually antagonistic" genes often are) the effect will not be felt until the allele is sufficiently frequent to produce female homozygotes. By this stage there is strong selection to restrict expression of the gene to the testis, to mitigate disadvantage to females.[2] Testis-specific genes that have been selected-for include, among others, microRNAs[11] and arrays of so-called testis-cancer antigen genes required for fertility that lie in large palindromic loops.[12]

Females are mosaic for the expression of X-linked genes

Although human autosomal monosomies are incompatible with life, the quasi-monosomic status of the X chromosome in males is without phenotypic consequence. X-chromosome aneuploidies, including Turner (XO females) and Klinefelter's syndrome (XXY males), which are among the most common viable chromosomal abnormalities, lead to syndromes having a relatively moderate clinical impact. This is due to the counting property of the XCI process, which triggers the transcriptional silencing of all but one X chromosome per diploid set of autosomes. The counting prevents XCI from occurring in XO female and inactivates one of the extra X in XXY males. Turner symptoms result, at least in part, from the absence of a second copy of the PAR regions.

The balanced female mosaicism for the expression of X-linked genes—50% of cells express the maternal haplotype and the other 50% the paternal haplotype—is a reflection of the randomness of which X undergoes inactivation in epiblast cells of the early female embryo. Critically, information determining which X is activated is then transmitted throughout subsequent mitotic cell divisions (Fig. 2). Some of the most visual examples of female mosaicism are provided by cutaneous manifestations[13] such as incontinentia pigmenti,[14] which is caused by mutations in the X-linked gene NEMO and is lethal in males. Heterozygous females show skin hyperpigmentation that develops as characteristic whirls and streaks on the trunk. The extent and shape of these pigmentation patterns is extremely variable between individuals. They reflect the number and the migration path of the precursor cells that have initially inactivated the wild-type and the mutated X chromosomes.

Consequences of cellular mosaicism

Cellular mosaicism in X-linked gene expression generally provides a biological advantage for females.[15] In contrast to autosomal recessive mutations where wild-type and mutated copies are co-expressed within the same cell (except for monoallelically expressed genes), cell populations mosaic for the expression of an X-linked mutation can compensate for each other in certain cases to abrogate the mutation effect on disease etiology. Females heterozygous for X-linked mutations thus generally behave as *asymptomatic carriers*. Two types of compensation are observed: the most common is cell elimination, but metabolic cooperation is also found. An example, of cell elimination is at work in Lesch-Nyhan syndrome, where blood cells expressing the mutated allele show a growth disadvantage and progressively disappear from the population of blood cells.[16] In rare cases, such as adrenal leukodystrophy, the mutation confers a proliferative advantage on the cell, leading to the mutant cell population progressively taking over from the normal cell population and, in women, to an increasing disease severity with age.[17]

Cell populations can also be involved in metabolic cooperation, which involves the eventual exchange of molecules between cells.[3] In the case of Fabry's disease, normal cells secrete a critical lysosomal enzyme that can be taken up by adjacent abnormal cells by endocytosis, reducing the eventual clinical severity of the mutation. In other rare cases, cell–cell interactions can lead to a negative outcome, as occurs in craniofrontonasal syndrome. Males with a mutation in the ephrin-B1 gene, responsible for the disease, suffer only from mild hypertelorism, whereas females may suffer from both facial dysmorphism and premature fusion of some bones. This extreme phenotype is due to the intermingling of ephrin-B1^{+} and ephrin-B1^{-} cells, which results in the formation of an artificial boundary

that prevents gap junction proteins from moving from one cell to the other and interferes with downstream signaling pathways (metabolic interference).[18]

Skewing of X-inactivation

Cell selection, as in adrenal leukodystrophy and Lesch–Nyhan syndrome, results in unequal proportions of cells with a paternal Xi and those with a maternal Xi in specific tissues. This phenomenon, called X-inactivation skewing, is rather common, with some 10% of women showing a deviation from equal inactivation of each parental allele. In extreme cases, up to 90% of cells show expression of the same allele.[3] This XCI bias may occur either by primary nonrandom inactivation during early embryogenesis or by secondary cell selection later in the blastocyst (Fig. 2B). At the onset of XCI, when the embryo consists of only a few cells, it is possible that substantially more cells choose to inactivate the maternal or paternal X simply by chance. A compelling illustration of this is provided by autoimmune diseases such as scleroderma, autoimmune thyroid disease, and systemic sclerosis, where females are two to eight times more likely to develop symptoms than males (for review, see Ref. 19). In these diseases, skewed XCI is almost invariably observed in thymic dendritic cells (TDCs) involved in the scanning and elimination of autoimmune T cells (tolerization process). This skewing may depend on the restricted number of cells present in the late blastocyst when the TDC lineage is initiated. Stochastic XCI in the precursors will give rise, in rare cases, to a minor TDC population expressing a given X chromosome (either the Xm or the Xp) and a major population expressing the other X. Hypothesizing that putative genes involved in the immune T cell scanning process are X linked and that alleles of these genes may show different affinity properties, a disequilibrium in the number of TDCs expressing one or other of such X-linked alleles would impact the T cell scanning process with T cells that are autoimmune to the minority TDC population more likely to escape the tolerance process and trigger autoimmune reactions. Future studies will identify the X chromosome regions containing candidate genes for autoimmune susceptibility.

Studies of phenotypic variation among female monozygotic twins who are carriers for an X-linked disease suggest that such primary XCI skewing may be responsible for the oft-remarked appearance of X-linked diseases in only one of two twins: with the clinically affected sister showing preferential nonrandom XCI of the chromosome carrying the wild-type allele, whereas the unaffected twin has either predominant XCI of the mutated chromosome or random XCI. Reported examples of X-linked diseases with phenotypic discordance between monozygotic twins correlated with skewed XCI in the affected sister include fragile-X syndrome, color blindness, Duchenne muscular dystrophy, Hunter's syndrome, hemophilia B, Aicardi's syndrome, and Fabry's diseases.[20]

The existence, in the human, of a genetic component controlling XCI bias has also been postulated, but remains highly controversial in part because of the technical difficulties in assessing skewing, as it varies greatly with the tissues analyzed and age. In most studies, mother-to-daughter transmission of XCI skewing has not been detected.[21] However, a single case of heritable skewed XCI has been reported in a family where the trait seems to segregate independently of the hemophilia A mutation present in the family. In this specific case, only females showing a biased XCI of the wild-type X are affected.[22] Skewing, even in this case, could result from the influence of an additional genetic component conferring a proliferative advantage. Autosomal transcription factors such as CTCF or YY1,[23,24] which bind in an allele-specific manner to some of the XCI key regulators, may be able to modify the 50:50 XCI ratio. It is noteworthy that three autosomal loci, *Xiaf1*, *2*, and *3* (X-inactivation autosomal factors)[25] but only one X-linked locus, the X-controlling-element (*Xce*), have been suggested to influence XCI in mice[26] (for review, see also Ref. 27).

X chromosome inactivation: a paradigm for large-scale regulation of gene expression

X-inactivation during embryogenesis and gametogenesis

XCI represents the best-characterized paradigm of epigenetic reprogramming processes. Its onset and reversal are tightly linked, respectively, to the genome-wide programming events occurring during early embryogenesis and during gametogenesis. Our best knowledge of the timing of XCI during early embryogenesis comes from studies performed on rodent embryos. In mice, the first wave of XCI

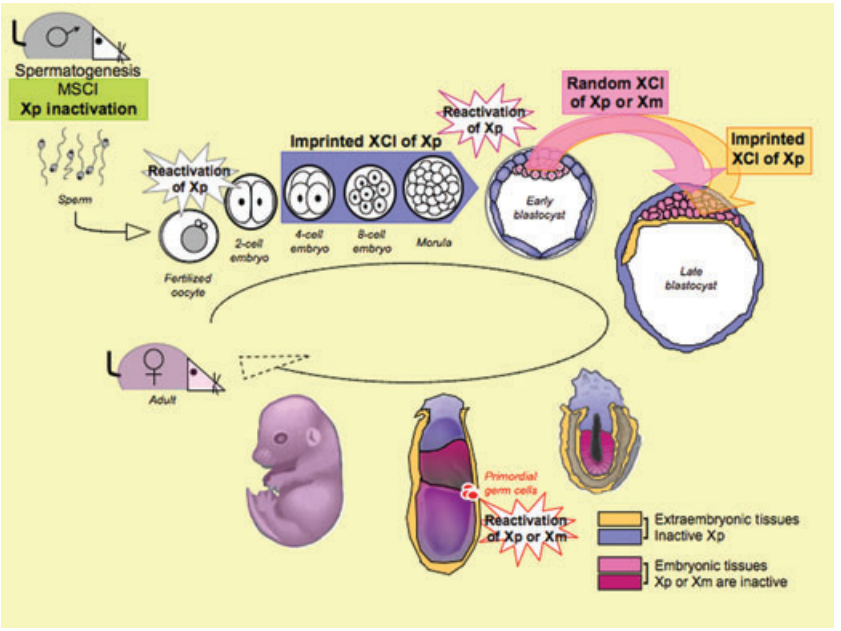

Figure 4. Kinetics of X-inactivation and reactivation during mouse embryogenesis and adult life. During female mouse preimplantation development, the first XCI event occurs around the two-cell stage and consists of an imprinted inactivation of the paternal X (Xp).[28,29] This inactive Xp is maintained in the trophectoderm (blue) whereas in the inner cell mass (ICM, pink) of the early blastocyst the Xp is reactivated to allow for the random XCI of the Xp or of the Xm to take place in the epiblast (pink) of the late blastocyst. Extraembryonic cells of the hypoblast/PrE (yellow) which also derives from the ICM show an inactive Xp which may either result from *de novo* imprinted XCI or from ICM cells where imprinted XCI has been conserved. The Xi (Xp or Xm) is reactivated in female primordial germ cells (precise time remains to be determined) so that the newly formed Xms are active at the time of oocyte fertilization. During male gametogenesis, the Xp is submitted to meiotic sex chromosome inactivation (MSCI) but is somehow reactivated around fertilization.[34] In human, the XCI cycle could be simpler as no imprinted form of inactivation has been reported. *XIST* upregulation could initiate later than in mice, around four- to eight-cell stage, together with zygotic gene activation.

initiates around the four- to eight-cell stages and only affects the paternal X chromosome (Xp)—it is referred to as *imprinted* XCI as opposed to *random* XCI[28,29] (Fig. 4). Interestingly, during this paternal XCI, not all X-linked genes undergo silencing simultaneously—some genes are turned off at the four-cell stage and other at the morula stage.[30] The order of silencing is independent of the position of the genes along the X chromosome but is more likely to relate to the three-dimensional reorganization of the Xi chromosome territory. Nuclear repositioning may bring to close nuclear proximity two genes located far away on the genomic sequence, thereby facilitating the propagation of the silencing signal.[4] At the time of the differentiation of the extra-embryonic trophectoderm (TE) and of the inner cell mass (ICM), the Xp is reactivated in this latter tissue to allow random XCI to occur in the epiblast of the late blastocyst, which will give rise to the embryo proper. In the TE, imprinted XCI is maintained (Fig. 4). The extra-embryonic primitive endoderm (PrE), which delaminates from the ICM, is also characterized by an inactive Xp. However, this Xp shows distinct chromatin features, suggesting that specific chromatin changes are apposed on the Xp in the PrE.[31] Whether the PrE Xi results from a *de novo* imprinted inactivation event or from the migration of ICM cells preprogrammed to participate in the PrE and that have kept an inactive Xp remains to elucidate.

In humans, a precise timing of XCI events during early embryogenesis is lacking due to the rarity of biological material. However, studies of XCI patterns in extra-embryonic tissues suggest that human extra-embryonic cells undergo random and non-imprinted XCI.[32] Earlier imprinted XCI at the morula stage, or before, is not formally excluded but is considered unlikely, as biallelic expression

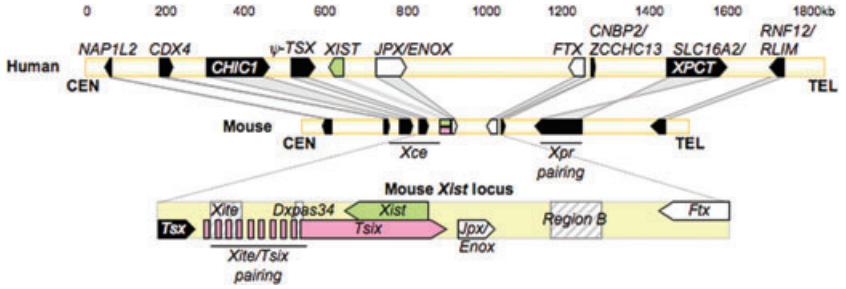

Figure 5. The X-inactivation center. Scaled comparative maps of human and mouse X-inactivation centers.[43] Noncoding and coding genes are shown as open- and black-boxed arrowheads, respectively. A zoom-in of the mouse *Xist* locus showing the best-known players in XCI including the *XIST*/*Xist* noncoding RNA (green) and the *Tsix* antisense transcription[95] (red). Gray-hatched boxes indicate regions of intergenic transcription and regulatory elements.[43,96] The candidate region for the X-controlling element (*Xce*) and the genomic regions involved in X–X pairing events located at the 5′ (*Xpr*) and 3′ (*Xite/Tsix*) of *Xist* are indicated.

of the XCI key-player the *XIST* gene (a prerequisite to the establishment of XCI) is predicted to occur around the time of zygotic gene activation (ZGA), around the four- to eight-cell stage in humans, whereas mouse ZGA initiates as early as the one to two-cell stage.[33]

The reversal of XCI patterns during female gametogenesis is mandatory to the initiation of a new XCI cycle at the next generation. Just like Xp reactivation in the ICM, the reactivation of the Xi during oogenesis accompanies genome-wide reprogramming events and is likely to involve the same molecular mechanisms although reactivation of the Xi during oogenesis has not been extensively studied as yet. In contrast, male gametogenesis is characterized by a specific form of XCI called meiotic sex chromosome inactivation (MSCI), which involves a sequestration of X and Y chromosomes into the sex vesicle (except for the paired PARs), where both chromosomes are silenced. This MSCI is thought to be a special example of a more general mechanism called meiotic silencing of unsynapsed chromatin (MSUC). MSUC prevents illegitimate recombination events between unpaired regions of the chromosomes during meiosis by inducing the heterochromatinization of chromosomes that fail to pair with their homologous partners. As heterochromatic chromatin cannot recombine, this mechanism protects against aneuploidy in subsequent generations (for further details on the molecular mechanisms of MSCI, see Ref. 34).

Molecular controls of the initiation of random X-inactivation

The initiation steps in XCI are under the control of an X-linked genomic region called the X-inactivation center (*XIC*). The *XIC* contains the *XIST* gene (Fig. 5). At the time of cell differentiation, *XIST* is upregulated from the future Xi and produces a large noncoding RNA that accumulates on the presumptive Xi chromosome creating a repressive nuclear compartment. The dissection of the molecular mechanisms underlying the initiation of random XCI was mostly realized in the mouse model, especially through the use of female embryonic stem (ES) cells. Female ES are derived from the ICM of the early blastocyst and faithfully recapitulate all the steps of random XCI upon *in vitro* differentiation. In this model, a major *Xist* regulator is the *Tsix* noncoding gene. *Tsix* is transcribed in the antisense orientation to *Xist* and is one of the *cis*-repressor of *Xist* expression in undifferentiated ES cells.[35] At the onset of differentiation *Tsix* is repressed on the future Xi, allowing for *Xist* upregulation and *Xist* RNA accumulation[35–37] (Fig. 6). The molecular mechanisms underlying *Tsix*-dependent regulation of *Xist* transcription involves complex chromatin modifications at the *Xist* locus (for review, see Ref. 38). Essentially, the loss of *Tsix* transcription on one X chromosome creates a permissive chromatin environment at the *Xist* promoter, which becomes enriched for euchromatic marks such as H3K4 di- and tri-methylation and H3K9 acetylation, thereby facilitating the recruitment of the transcription machinery[39,40] (Fig. 6). In terms of putative transcription factors involved in *Xist*/*Tsix* regulation, the best candidates are YY1 and CTCF, which have both been described to play a role in the regulation of monoallelic gene expression at other imprinted loci[41] and bind to both the *Xist* and *Tsix* promoter regions in ES cells.[23,24] Their precise mode of action during initiation of XCI has

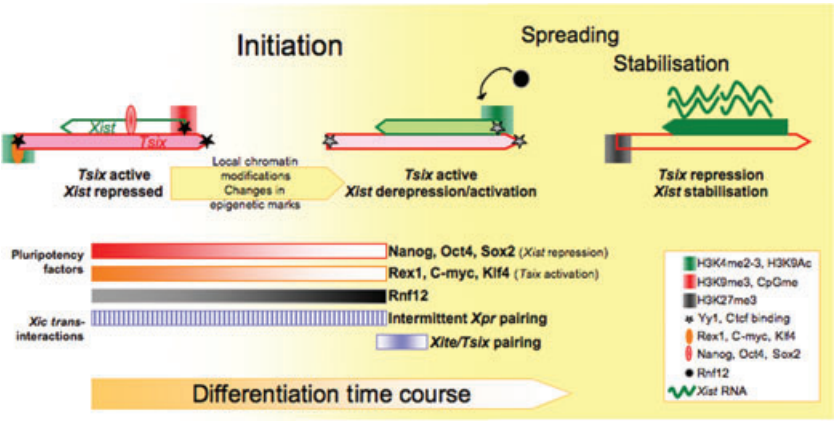

Figure 6. Initiation of random X-inactivation in mouse embryonic stem cells. Before differentiation, *Xist* is repressed by Nanog, Oct4, and Sox2 bound on intron1[45] and by *Tsix* expression under the control of Rex1, Klf4, and c-Myc.[46] At the onset of differentiation the loss of pluripotency factors lifts Xist repression and X–X pairing events introduce an asymmetry between the two Xs,[52] possibly through monoallelic changes in chromatin structures and/or in Yy1/Ctfc binding at *Xist* and/or *Tsix* promoters.[38] At the same time, Rnf12 activates either directly or indirectly the *Xist* gene.[54] Later on during differentiation, *Tsix* is repressed by the addition silent chromatin marks (H3K27me3) at the promoter.[40]

however yet to be fully characterized (for review, see Ref. 38). In addition to *Tsix*, several other genomic elements lying within the mouse *Xic* are involved in the regulation of *Xist* expression (Fig. 5, for review, see Ref. 42). It is important to underline that most of these regulators are not conserved in human. For example, the human *TSIX* gene is either absent[43] or truncated so that it does not overlap *XIST*,[44] suggesting that other molecular mechanisms are at work during the initiation of human XCI.

A long-standing enigma in the field of XCI has been to identify the molecular link between cellular differentiation and the XCI triggering. The answer came recently through the discovery that pluripotency factors Nanog, Oct3/4, and Sox2 bind to *Xist* intron 1 to prevent *Xist* upregulation in undifferentiated mouse ES cells,[45] whereas the pluripotency factors Rex1, Klf4, and c-Myc occupy the *Tsix* promoter to activate *Tsix* expression.[46] At the onset of differentiation, the loss of this pluripotency factor is associated with the induction of *Xist* upregulation (Fig. 6). Interestingly, this important result links repression of XCI and ground state pluripotency, thereby directly connecting the molecular mechanisms responsible for the genome-wide resets occurring in the ICM, and during oogenesis to the reactivation of the Xi at these same stages (for review, see Ref. 47). In agreement with this idea, efficient experimental reactivation of the inactive state has only been obtained using the transfer of a somatic nucleus into an enucleated oocyte,[48] the forced expression of specific factors in somatic cells to generate induced pluripotent stem (iPS) cells,[49] and the fusion of hematopoietic and ES cells,[50] three strategies involving a genome-wide reprogramming toward the pluripotent state.

Other intriguing features underlying the initiation of random XCI are the ES cell's ability to count the number of X chromosomes and "choose" which one(s) to inactivate. Recent findings have shed some light on the molecular mechanisms lying behind these functions. The first one was the discovery that the two X chromosomes come into close nuclear proximity both before and at the very beginning of the differentiation process. These two X–X pairing events involve two specific regions within the *Xic*, respectively, the *Xpr*,[51] located within the *Xpct* gene,[52] and the *Tsix–Xite* region[53] (Fig. 5). Dynamic nuclear contacts between these regions are thought to mediate the *trans*-sensing of the two X chromosomes, thereby participating in the counting process, although stringent proof of this is lacking (Fig. 6). As a result of this *trans*-sensing, distinct modifications could be apposed on each allele and resolved in the determination of the Xa and of the Xi. Another factor potentially involved in X chromosome counting is the U3 ubiquitin ligase produced by the X-linked *Rnf12* gene,[54] which is conserved in human (Fig. 5). Interestingly, the Rnf12 protein acts on the initiation of XCI in a dose-dependant manner: overexpression

of *Rnf12* induces ectopic inactivation of the single X chromosome in male cells and of both X chromosomes in female cells. This observation suggests that the dose of Rnf12 produced by the single X chromosome in male cells is not sufficient to trigger XCI, whereas a double dose of Rnf12 in female cells is just enough to initiate XCI on a single X (Fig. 6). The exact chronology of *Rnf12*'s intervention in the cascade of events leading to the initiation of XCI remains to be established.

As a concluding remark, it should be pointed out that the existence of such molecular mechanisms and the role of most of the abovementioned genomic elements during human XCI are unclear. The recent development of human ES cells showing the same properties as mouse ES cells should allow the characterization of initiation during human XCI.[55]

New advances in deciphering the spreading of the inactivation signal along the X chromosome

A unique property of the *Xist* RNA consists in its ability to coat the Xi thereby creating a repressive nuclear compartment, which allows, in humans (*XIST*), the silencing of 75–85% of X-linked genes depending on the tissue considered[56] (in the mouse, 97% of X-linked genes are inactivated).[57] This implies that the association of *Xist* RNAs with the Xa or with autosomes directly adjacent to the Xi in the nuclear space must somehow be prevented. Recent efforts to identify the molecular partners responsible for the *Xist* RNA/Xi specific interaction have followed three different axes: (1) the search for X-specific regions involved in *Xist* RNA recruitment, (2) the definition of the subregion(s) of the 15-kb *Xist* RNA engaged in Xi coating, and (3) the identification of the factors (if any) which bridge *Xist* RNAs to the Xi.

Originally, the spreading of the XCI signal along the Xi had been hypothesized to involve specific regions relaying the XCI signal.[58] The first clues supporting this idea came from the analysis of X:A translocations and *Xic* trangenes on autosomes that showed different extents of propagation of XCI along the targeted autosomes.[59,60] A correlation was established between the spreading ability of X-inactivation within specific genomic contexts and its enrichment in long interspersed repeats (L1)—the X being relatively enriched in L1 sequences compared to autosomes[61]—suggesting that these repetitive elements may assume the function of "way stations" (Fig. 7A). A recent analysis of the mode of action of L1 repeats during the XCI process suggests that silent L1s participate in the formation of a specific heterochromatic nuclear compartment during the first phase of XCI[62] (Fig. 7B), whereas "young" and transcribed L1s may facilitate the spreading of the silencing signal across regions resistant to XCI.[62,63] The initial accumulation of *Xist* RNAs on the Xi may also be ensured by a region located just upstream of the *Xist* gene and enriched in H3K27me3 and H3K9me2 that would act as "nucleation center" of the XCI signal.[64,65] The link between this "hot spot" of histone methylation and L1 sequences remains to be characterized.

Does the coating of the Xi by *Xist* RNAs involve a specific sequence carried by the transcript? The ectopic expression of different forms of *Xist* cDNA in male differentiating ES cells showed that several subregions of the transcript act synergistically to efficiently "paint" the Xi.[66] This may indicate that a specific folding engaging these RNA sequences may be required for a proper association with the Xi. The matrix protein hnRNP U/SP120/SAF-A mediates, at least in part, the association of *Xist* with the Xi,[67] suggesting that *Xist* RNA coating could also involve attachment to the nuclear matrix.[68] Altogether these data favor a model where *Xist* RNAs would first accumulate on the *Xic* of the future Xi at the level of the histone methylation hot spot and then recruit silent L1s interspersed along the Xi, thereby creating a three-dimensional heterochromatic compartment. Active L1s would, then, mediate the spreading of the silencing signal across X-linked regions escaping XCI[62] (Fig. 7B). These different levels of interaction—with the methylation hot spot, the silent and active L1s, and the nuclear matrix—might be ensured by specific foldings of *Xist* RNAs that would expose the RNA sequences necessary for each interaction (Fig. 7A).

Establishment of X-linked gene silencing and stability of the inactive state

The chromosomal coating by *Xist* RNAs is closely associated with the accumulation of polycomb repressor complex 2 (PRC2) on the Xi and subsequent deposition of the inactive histone mark H3K27me3.[69] Interestingly, a single specific subsequence of the *Xist*

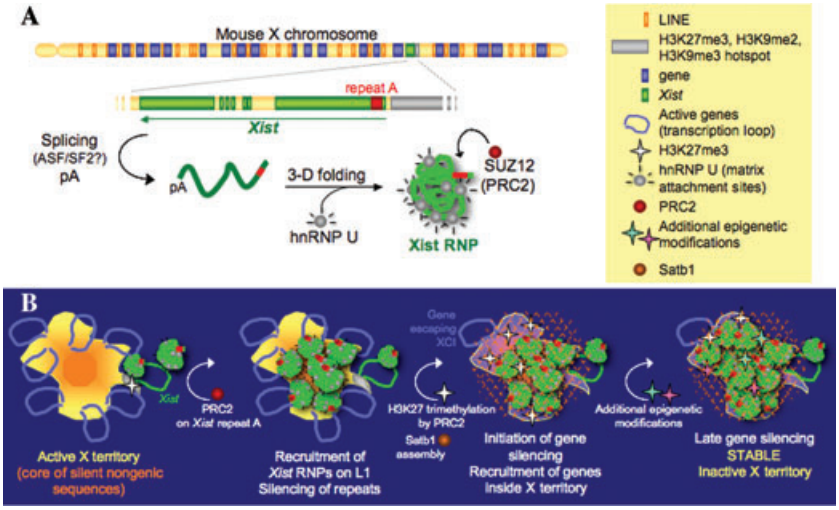

Figure 7. Spreading of the inactivation signal along the X-chromosome and establishment of X-linked gene silencing. (A) Schematic representation of the whole X chromosome showing LINE 1 repeats acting as "way stations" (orange), gene units (blue), the *Xist* gene (green), and the associated H3K27/H3K9 methylation hot spot (gray) which may serve as a "nucleation centre" for the propagation of *Xist* transcripts along the X chromosome. A zoom-in of the region of the *Xist* gene depicting *Xist* exonic structure and the location of the A repeat is also shown. *Xist* transcription is associated with splicing (mediated by the association of ASF/SF2 on repeat A[97]) and polyadenylation of the transcripts. The three-dimensional folding of *Xist* RNAs allows for the A repeat and other functional sequences (binding sites for hnRNP U) to be accessible and to form the *Xist* RNPs (ribonucleotide particle). (B) Hypothetical scenario of the propagation of XCI on the X chromosome territory. At the onset of *Xist* accumulation, active X chromosomes consist in a core of silent nongenic sequences (yellow/orange) with residual transcription surrounded by active gene units (blue). *Xist* upregulation is accompanied by the association of *Xist* RNPs on the methylation hot spot and progressive accumulation of *Xist* RNPs on LINE 1 sequences that trigger the silencing of X-chromosome repeated sequences. PRC2 assembly on *Xist* A repeat induces global H3K27me3 of the chromosome. H3K27me3 together with Satb1 factors surrounding the *Xist* domain mediate the silencing and relocation of genes into the Xi chromosome territory. Other epigenetic modifications and attachment to the nuclear matrix stabilize the inactive state.

RNA (repeat A), consisting of a conserved repeat located at the 5′ extremity of *Xist* is responsible for the silencing function of *Xist*[66] (Fig. 7A). This A repeat folds into a specific two-dimensional structure able to bind the Suz12 protein and other PRC2 components,[70] suggesting that X-linked gene silencing is mediated by the recruitment of the PRC2 complex to region A of *Xist* RNAs and the subsequent propagation of the repressive histone mark H3K27me3 to the entire Xi (Fig. 7B). However, it is noteworthy that X-linked silencing, especially silencing of repeats, initiates before the recruitment of PRC2 to the Xi,[71] indicating that other mechanisms must be responsible for these early silencing events. In addition, the differential kinetics of silencing of several X-linked genes during early development[30,4] point to additional gene-specific regulation.

Interestingly, the *Xist* A repeat is also necessary for the relocation of silent X-linked genes inside the Xi chromosome territory.[71] The precise mechanism by which this chromosomal reorganization operates is still unclear. An association of newly silenced genes with the X-chromosome core enriched in silent nongenic sequences[72] is a possibility. In this case, large-scale chromatin compaction of H3K27trimethylated regions may mediate the nuclear reorganization of the genes (Fig. 7B). Alternatively, the AT-rich binding protein Satb1, which has been shown to be required for proper X chromosome silencing both during mouse ES cell differentiation and in lymphoma[73] could also be involved. Indeed, the nuclear distribution of Satb1 suggests a role in restricting *Xist* RNAs and X-linked genes to the bulk of the Xi (Fig. 7B).

Further stabilization and maintenance of the inactive state are ensured by additional layers of sequential chromatin modifications,[74] which include association with the histone macroH2A variant and/or CpG island methylation (Fig. 3, for review see Ref. 33).

Current and future employments of X-chromosome inactivation as diagnostic or therapeutic tools

Skewed X-inactivation as a diagnostic tool to detect asymptomatic carriers of X-linked mutations

A current use of XCI concerns the identification of heterozygous carriers of X-linked recessive mutations, which is of crucial importance for family counseling and planning medical treatment (when available) of potentially affected offspring. Although heterozygous carriers of autosomal mutations can be detected by laboratory testing or in-depth physical examination for some genetic diseases, in the case of most X-linked mutations female carriers appear normal in all respects. This results often from the selection process referred to above, in which survival of cells that have inactivated the mutant allele is favored in tissues where the gene responsible for the disease is expressed. A corollary of this is that skewed XCI can be a robust indicator of carrier status. Assays to examine XCI skewing require the presence of a polymorphism able to distinguish the two Xs and a means of determining which X is active. Although examination of RNA and/or protein expression provides the most direct measure of X chromosome activity, specific DNA methylation patterns associated with the Xi are more often used as a surrogate because DNA is easier to extract, store, and analyze. An assay monitoring DNA methylation of the highly polymorphic CAG trinucleotide polymorphism at the 5′ end of the gene encoding the androgen receptor (AR) has become a popular and widely adopted method for measuring XCI skewing.[21] Because skewing does not always affect all tissues similarly, it is important to ascertain as many cell types as possible by testing different readily available proband biological samples. These samples will typically include peripheral blood leukocytes, oral mucosal cells, and muscle biopsies. It is important to stress that this test, which is based on the quantitative measure of methylation levels of a single gene, may not always be accurate.

Recurrent miscarriages are a major health concern for women, affecting some 17% of couples who wish to have a child. It has been assumed that a large proportion of these pregnancy losses have a genetic origin. Causes involving the X chromosome, such as mutations in X-linked genes implicated in placenta formation, may in part be responsible (Fig. 2; for review on imprinted XCI see Ref. 75). However, the most likely event involves the presence of an X-linked lethal trait in the mother, which would be transmitted with a 25% probability to a male fetus. For these mothers, the risk of spontaneous abortion is increased from the general population risk of 15–20% to a combined risk of up to 40% at each pregnancy ($[0.25 \times 1] + [0.75 \times 0.20] = 0.4$). It is predicted that female carriers of such X-linked lethal traits will show extremely skewed XCI against the mutated X, which could be detected using the abovementioned test.

More generally, this simple test could provide us with a method for predicting the carrier status of every woman suspected, on the basis of her pedigree, to be a carrier of an X-linked mutation. For this reason it constitutes an important breakthrough in the field of genetic diagnosis, allowing for the scanning and detection of mutations corresponding to up to 5% of the genome in a single test. However, it is important to keep in mind that skewed XCI has to occur in the biological tissues that are under test in order for carrier status to be detected. Moreover, potential lineage specific differences in X-inactivation status and/or underlying mechanisms cannot be excluded. Currently, this test is used to detect X-linked forms of immunodeficiencies and X-linked enzymatic defects, where early disease diagnosis is required to initiate treatment as soon as possible and to prevent symptoms appearing in the child (e.g., in the case of lysosomal storage disorders like Fabry's disease and Hunter's syndrome).[76,77] Because XCI profiles are clonal, this test can be applied on tumor biopsies to assess cancer propagation and the origin of the metastases. For example, in the case of tumors affecting multiple organs, if all the tumors show the same XCI profile, this will increase the probability that the tumors likely originated from the same initial transformation, thereby suggesting advanced metastasis. In contrast, several XCI profiles would be indicative of independent mutational events which might lead to a different appreciation of the clinical treatment.

Cell replacement therapies involving X-inactivation

A major area of therapeutic research and development concerns cell replacement, which relies on

collecting cells from the patient and compensating or repairing the effects of the mutation *ex vivo*. "Repaired" cells are subsequently grafted into the disease-affected tissue, theoretically without risk of rejection of the autograft. Until now, cell replacement therapies were employed mainly to treat categories of disease such as immune system defects, allowing the collection and growth of the target cells: in this case, adult hematopoietic stem cells. Interestingly, immature hematopoietic precursor cells have been shown to conserve the ability to initiate ectopic XCI, suggesting that their X-chromosome chromatin structure is still permissive enough to allow XCI patterns to be changed.[78] Restoration of a normal phenotype, by either forcing the XCI of the mutated X chromosome or the recreation of a balanced cellular mosaicism among hematopoietic stem cells prior to reimplantation, represent possibly interesting options for the treatment of autoimmune diseases.

Recent revolutionary advances in the field of stem cell research have shown that it is possible to reprogram somatic cells toward a pluripotent state. Ectopic expression of only four key factors in a human somatic cell is sufficient to obtain these so-called iPS (induced pluripotent stem) cells, which recapitulate most of ES cell characteristics, such as their ability to contribute to any adult tissue.[79,80] The challenge is now to specifically and routinely direct the differentiation process into a desired lineage.[81] Encouraging progress has been made recently in the controlled differentiation of cardiac,[82,83] neural,[84] or hematopoietic lineages.[85] In terms of XCI, the genome reprogramming associated with somatic cell de-differentiation is accompanied by a reactivation of the Xi in mouse iPS (miPS) cells[86] but not in human iPS (hiPS) cells.[87] However, because hiPS derived from mosaic human fibroblasts (with either a paternal or maternal Xi) are clonal, this makes possible the selection of hiPS populations that have inactivated the X chromosome carrying a mutation. The next step will be to differentiate these "repaired" cells toward the desired cell type and to reimplant them into the patient. More generally, a combination of genome sequencing using high-throughput technologies to identify mutations, nuclear reprogramming, and *in vitro* mutation repair should allow the treatment of most genetic diseases through cell replacement therapy.

X-inactivation as a mean to an end

Theoretically, it should be possible to use the chromosome-wide properties of the XCI mechanism to induce long-distance gene silencing. Indeed, it has been shown that inserting the human *XIC* into a mouse autosome triggers long-range gene repression in *cis*.[88] Along this line, one could imagine that targeted insertions of the *XIC* into a specific chromosome or a specific genomic region associated with appropriate genetic manipulation, such as the utilization of insulators to restrict the spreading of the XCI signal to the targeted region, could be used to treat cases of mosaic trisomy and/or some types of abnormal genomic amplifications/duplications. Another long-term application consists in using XCI as a model to assess the efficiency and accuracy of genome wide reprogramming during early embryogenesis.[86] It should, moreover, be possible to screen libraries of synthetic molecules to identify new drugs involved in, or potentiating, gene reprogramming on the basis of their ability to prevent XCI to occur or, in contrast, to induce ectopic XCI.

Conclusion

As we understand more about X-chromosome genetics and X-inactivation at the molecular level, unforeseen layers of complexity appear. Major progress will almost certainly be conditioned by, and will in turn influence, our general understanding of chromatin structure and chromosome function. But it is more and more obvious that lessons from X-inactivation, which is now unanimously considered as one of the major paradigms of epigenetic regulation of gene expression, will be applicable to other related regulation systems such as genomic imprinting and, more generally, to global genome reprogramming events.

Conflicts of interest

The authors declare no conflicts of interest.

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