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Review

X-chromosome inactivation in development and cancer

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ABSTRACT

X-chromosome inactivation represents an epigenetics paradigm and a powerful model system of facultative heterochromatin formation triggered by a non-coding RNA, Xist, during development. Once established, the inactive state of the Xi is highly stable in somatic cells, thanks to a combination of chromatin associated proteins, DNA methylation and nuclear organization. However, sporadic reactivation of X-linked genes has been reported during ageing and in transformed cells and disappearance of the Barr body is frequently observed in cancer cells. In this review we summarise current knowledge on the epigenetic changes that accompany X inactivation and discuss the extent to which the inactive X chromosome may be epigenetically or genetically perturbed in breast cancer.

1. Introduction

In mammals, dosage compensation for X-linked gene products between the sexes is achieved by X-chromosome inactivation (XCI) in females [1]. This process leads to the highly regulated transcriptional silencing of one of the two X-chromosome during early development, leading to the formation of the heterochromatic Barr body. X inactivation is an outstanding example of chromosome-wide epigenetic regulation involving the developmental silencing of approximately one thousand genes. XCI shares many of the features of others epigenetic mechanisms such as a mosaic cellular phenotype; mitotic heritability but developmental reversibility of X-chromosome inactivity; asynchronous DNA replication timing compared to the rest of the genome; and finally a combination of several epigenetic mechanisms – including DNA promoter methylation, histones post-translational modifications, and an unusual nuclear organization. These various features are believed to act synergistically to maintain the X inactive state. Thus, the inactive X chromosome represents a remarkable illustration of the numerous epigenetic mechanisms that can underlie the formation and maintenance of facultative heterochromatin throughout the lifetime of mammals. Pathologists have long noted that the heterochromatic structure of the Barr body was frequently absent in breast cancer cells, particularly in the most aggressive tumors ([2,3]). This observation was later found to be due to the frequent genetic loss of the Xi, with reduplication of the Xa also occurring in some cases in cancer cells resulting in a double dose of X-linked genes. However, another mechanism for Barr body loss in cancer that has been proposed involves the decompaction of its heterochromatic structure, which may be accompanied by X-linked gene reactivation.

Here we provide a brief overview of the current knowledge pertaining to the establishment and maintenance of X inactivation, as well as highlights of some of the known perturbations of the inactive X chromosome's state in a cancer context. This review does not provide an in depth analysis, for this, the reader is referred to other recent reviews [4–6].

2. Setting up inactivation of the X chromosome in mammals

The X-inactivation center (Xic) is a key locus that is required for the initiation of X inactivation. Studies involving X-chromosome deletions and X-autosome translocations in mice and humans mapped the precise region which contains the essential gene for triggering the silencing process, the non-coding RNA *Xist* (X-inactive-specific-transcript) (for review [7]). *Xist* is a 17 000 nucleotide (19 kb in Human), spliced, untranslated regulatory transcript that coats the X chromosome from which it is expressed in *cis* [8,9]. Perhaps surprisingly, *Xist* is present only in eutherian mammals, with no orthologous described in marsupials or monotremes to date. However in marsupials, another non-coding RNA *Rsx* (RNA on the silent X) was recently discovered, that appears to have similar properties to *Xist* even though its sequence is unrelated to *Xist* [10]. In the mouse, deletions and transgenes have demonstrated that *Xist* is required for both the imprinted and

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random forms of X inactivation [11–13]. Nevertheless, *Xist* alone cannot recapitulate all the roles of the Xic which include "sensing/competence", whereby a cell initiates XCI only when more than one Xic is present; "counting", where only one X chromosome stays active per diploid autosome set; and "choice", whereby one of the two X chromosomes is chosen for inactivation while the other remains active. Some of these functions seem to be ensured by other elements of the Xic, in the neighborhood of *Xist*. For example, in mice the *cis*-acting *Xist* antisense transcription unit, *Tsix* [14], plays a key role in the choice of which chromosome will be inactivated. However, several additional *cis*-acting elements and *trans*-acting factors are involved in the dosage-sensitive, monoallelic regulation of *Xist* expression during early differentiation. For example, the *Rnf12* gene lies upstream of *Xist* and its protein product, an ubiquitin ligase that leads to the degradation of the *Xist* repressor protein Rex1, has been shown to have a key role in the XX-dosage dependent activation of *Xist* [15,16]. A recent study also shed light on the precise chromosomal folding of the Xic region in ESCs, revealing that the *Xist* and *Tsix* promoters lie in separate Topologically Associated Domains (TADs) [17]. The *Xist* promoter and all of *Xist*'s known positive regulators, including *Rnf12*, lie in a single 500 kb TAD, while the *Xist* repressor *Tsix* and its regulators lie in the neighboring 220 kb TAD, with the Xist/Tsix overlapping gene bodies across the boundary between the two TADs. The spatial separation between the *Xist* and *Tsix* promoters is likely to be essential for the finely tuned reciprocal regulation and expression of these two keys players in the XCI process [18,19].

So far, most studies of XCI during early development have been carried out in mice, which are more amenable to a genetic approach and where access to embryonic material is easier. In this species, XCI occurs in two phases during embryogenesis, an initial imprinted form and a later random form. In the imprinted form, *Xist* itself is imprinted such that only the paternal allele is expressed from two cell stage, whereas the maternal allele is repressed by an as yet undefined maternal imprint that is established during oogenesis. The paternal X chromosome (Xp) becomes silenced during pre-implantation development and remains inactive in extra-embryonic tissues. However the Xp is reactivated in the inner cell mass (ICM) of the mid-stage blastocyst, that will give rise to the embryo proper [20]. XCI is then reinitiated and occurs randomly by up-regulation of *Xist* from either the paternal or the maternal X chromosome. This second wave of XCI, can be recapitulated in vitro in differentiating mouse ESCs which provide a powerful model system for the study of the molecular mechanisms underlying random XCI. Remarkably, the differentiation process is delayed or paused in female ES cells until one of the two X chromosomes has become silenced, suggesting that a double dose of X-linked genes interferes directly with differentiation, thus creating a coupling between XCI and development [21]. Unexpectedly, in other mammals recent studies have revealed that the timing and pattern of XCI initiation observed in rodents is rather different to that seen in other eutherian mammals such as humans and rabbits [22,23], where *XIST* is not imprinted but is expressed from all X chromosomes in males and females from the 4 or 8 cells stages [24]. In rabbits, a significant proportion of blastomeres display *XIST* RNA coating of both X chromosomes in early blastocysts. This situation is rapidly resolved at slightly later stages, where most cells display only one *XIST* RNA-coated chromosome. In humans, the onset of XCI appears to be much slower than in mice and rabbits, as the two X chromosome remain active both in the ICM and the trophectoderm even though *XIST* coating is present. By contrast, early XCI appears to be absolutely essential in mice, in order to ensure proper dosage compensation given their fast embryonic development [25]. The exact reasons for this evolutionary diversity are still not clear but the rapid evolution of rodent genomes may mean that they have acquired mechanisms for finely regulating *Xist*, such as an early maternal imprint to prevent premature XCI, as well as reactivation of the Xp to allow for random XCI (see [21,24] for discussion).

The mechanisms underlying the early events during XCI are still not fully understood. Nevertheless, gene silencing is clearly triggered by *Xist* RNA coating of the future inactive X chromosome. It has been proposed that the *Xist* transcript might bind to high affinity sites on the chromosome, thereby inducing local heterochromatinisation, which can then facilitate the spread of XCI. LINE1 (Long Interpersed Nuclear Elements 1) repeat elements, which are highly enriched on the X chromosome compared to autosomes, have been proposed to facilitate the spread of XCI, by participating in the local propagation of inactivation and facilitating the recruitment of genes into the silent nuclear compartment formed by *Xist* RNA [26]. However, direct genetic evidence proving that L1s are required for efficient spreading or gene silencing is still lacking. More recent findings, based on mapping of *Xist* RNA along the X chromosome and of the nuclear organization of the *Xist* RNA coated inactive X, suggest that *Xist* propagation and coating are highly related to the three-dimensional conformation of the inactive X chromosome, and possibly also to the binding of Polycomb Repressive Complex 2 (PRC2) protein complex [27–29]. However, although studies comparing PRC2/H3K27me3 density and *Xist* RNA mapping suggest co-localisation [27,28], this is not in agreement with super-resolution *Xist* RNA FISH/immunofluorescence studies, where PRC2 and *Xist* RNA are found to be spatially segregated [30]. Furthermore, these studies do not demonstrate that the regions of the X associated with *Xist* RNA are directly involved in its spreading or silencing functions. Indeed, the mechanisms underlying *Xist*'s *cis*-limited chromosome coating capacity, as well as its ability to inactivate genes, remain mysterious.

Several studies have focused on the early changes in chromatin structure that occur during the initiation of XCI with the hope of providing insight into the role of *Xist* and the establishment of the silent state of the inactive X. Loss of euchromatin-associated histone modifications (such as H3K9Ac, H3K4me2 and H3K4me3) are amongst the earliest chromatin changes that occur following *Xist* RNA coating [20,31,32] (Fig. 1). Global H4 hypoacetylation occurs shortly afterwards [33]. Passive histone-loss during replication or else active removal (either by enzymatic activity, proteolytic activity or histone exchange) may underlie the early disappearance of those histones modifications. In addition to these early chromatin changes, the disappearance of factors associated with transcription, such as RNA polymerase II and loss of nascent transcripts are observed on the Xi immediately after *Xist* RNA coating. One or two cell cycles later, several new histone modifications appear on the *Xist*-coated chromosome. These include H3K27me3, H3K9me2, H4k20me1 and H2Ak119ub1 (for review [22]) which all become enriched with rather similar kinetics of Xi enrichment during random XCI in differentiating ESCs. In pre-implantation embryos, H3K27 tri-methylation precedes H3K9 di-methylation on the X chromosome undergoing inactivation [20]. Intriguingly, H3K27me3 and H3K9me3 seem to be enriched in different regions of the human inactive X chromosome in somatic cells, suggesting the existence of two different types of heterochromatin [34]. In murine differentiating female ESCs and somatic cells, H3K27me3 and H3K9me2 are enriched rather uniformly along the Xi, though slightly more in gene-rich regions [35]. The Xi profile of H2Aub1 has not yet been described.

The factors that lay down or bind to the different chromatin marks present on the Xi are being unraveled in mice. Polycomb repressive complex 1 (PRC1) mediates mono-ubiquitination of histone H2A lysine 119 (H2Aub1) [36,37] and PRC2 catalyzes the trimethylation of H3K27 [38,39]. *Xist* RNA is believed to have a role in the targeting of both PRC1 and PRC2 to the Xi, though whether this is direct or indirect is still unclear. In the case of PRC2, the cofactor

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