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The "lnc" between 3D chromatin structure and X chromosome inactivation

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ABSTRACT

The long non-coding RNA *Xist* directs a remarkable instance of developmentally regulated, epigenetic change known as X Chromosome Inactivation (XCI). By spreading *in cis* across the X chromosome from which it is expressed, *Xist* RNA facilitates the creation of a heritably silent, heterochromatic nuclear territory that displays a three-dimensional structure distinct from that of the active X chromosome. How *Xist* RNA attaches to and propagates across a chromosome and its influence over the three-dimensional (3D) structure of the inactive X are aspects of XCI that have remained largely unclear. Here, we discuss studies that have made significant contributions towards answering these open questions.

Contents

1. Introduction

X Chromosome Inactivation (XCI) is a critical step in the development of eutherian female organisms. In the mouse, XCI is implemented in two distinct waves during embryogenesis. In a process known as imprinted XCI, the cells of early 2–4 cell stage female conceptuses transcriptionally silence the paternal X chromosome (Xp) such that X-linked gene expression is derived almost exclusively from the maternal X (Xm) chromosome [1–3]. The inactive Xp is then maintained in almost all cells of the developing pre-implantation blastocyst, except for the epiblast cells. These cells, which give rise to the embryo proper, reverse silencing and sustain two transcriptionally active sex chromosomes (XaXa) [4]. Upon implantation, the differentiating epiblast cells transition through a second round of XCI, in which one of the two X chromosomes, this time chosen at random, undergoes transcriptional silencing and facultative heterochromatinization that is heritable throughout all subsequent cell divisions [5–12]. Studies in a variety of model systems have revealed important distinctions in the implementation of both imprinted and random XCI across species [13], making it pertinent to emphasize that in this review, we limit our discussion to that of random XCI.

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XCI is attributed with the alignment of X-linked gene dosage in differentiated female cells with that of their male (XY) counterparts [14–16]. Inhibition of XCI in the developing blastocyst affects survival of female embryos and appears to be caused by a failure to instate X-linked gene silencing in the future placenta [17–19]. While this phenotype demonstrates a requirement for imprinted XCI in female embryogenesis, it has hampered efforts to understand whether random XCI is essential for development of the embryo proper, which would require inhibition of XCI specifically in the epiblast but not the trophectoderm cells of an implanting blastocyst. This arrangement of tissue specific control over XCI can be achieved through tetraploid complementation of XCI-deficient epiblast cells, but has not yet been directly tested and as such, the requirement for random XCI in development remains unresolved [20].

The master regulator of XCI is the long non-coding (lnc) RNA *Xist* [21–23]. The *Xist* locus resides upon the X chromosome and is thought to remain inactive throughout the development of male mouse embryos and within the adult male organism [4,17,24–27]. During female development however, *Xist* expression is tightly and dynamically regulated. *Xist* activity becomes detectable in female embryos at the 2–4-cell stage, at which time *Xist* RNA is transcribed solely from the Xp chromosome [13,28,29]. This expression pattern is maintained in cells of the trophectoderm and underlies the imprinted XCI that is observed in these cells and in the extra-embryonic tissues derived from them [30,31]. In later stage pre-implantation embryos, the erasure of imprinted XCI in epiblast cells coincides with a resetting of *Xist* expression, such that the gene appears inactive or is maintained at very low levels of expression [3,13,27,29,31]. As these cells exit from the 'naïve' pluripotent state, one of the two *Xist* alleles is upregulated to produce *Xist* transcripts that spread *in cis* along the X chromosome from which they are expressed, thereby forming a 'coat' that remains stable throughout the interphase portion of the cell cycle [22,32]. How the upregulation of *Xist* is ensured on only one of the two X chromosomes, chosen at random, is a matter of intense debate. Most models posit that *Xist* activation is promoted by the release of multiple inhibitory pathways acting in the naïve pluripotent state as well as by an increase in *Xist* activators due to induction of differentiation, the details of which have been reviewed elsewhere (see Goodrich et al. in this issue, page 3) [33,34]. Through mechanisms that are still largely unclear, *Xist* RNA initiates the eviction of RNA polymerase II from within the *Xist* RNA coated territory [35,36]. The onset of X-linked gene silencing is accompanied by a temporally sequential deposition of repressive H3K27me3 and H3K9me2 marks, enrichment of the repressive macroH2A histone variant, and DNA methylation on the forming Xi [6–12,37,38]. Together these and other epigenetic modifications enable the formation and maintenance of a transcriptionally silent, heterochromatic chromosome, known as the inactive X (Xi) [39].

The *Xist* locus remains active specifically on the Xi throughout the somatic lifetime of a cell. Investigations into the requirement of *Xist* post initiation of XCI defined two distinct phases of the process during differentiation. The initiation phase captures the onset of XCI and is characterized by *Xist* RNA-dependent X-linked gene silencing. During this window of time, which generally correlates with the first 72 h of differentiation, depletion of *Xist* RNA allows for reactivation of silenced X-linked genes and reverses the XCI program. The maintenance phase follows and is characterized by X-linked gene silencing that is largely *Xist*-independent. The loss of *Xist* RNA during this period correlates with the depletion of some enriched heterochromatic marks on the Xi, such as macroH2A and H3K27me3, but extensive reactivation of silenced X-linked genes is not observed [11,39–43].

The *Xist* RNA 'coat' that forms across the Xi has been largely defined by signals observed upon the application RNA Fluorescence In Situ Hybridization (FISH) [44]. Some of the earliest insight into how *Xist* RNA associates with the X chromosome was obtained by treating fixed somatic cells with RNase H, an enzyme that degrades RNA chains involved in RNA:DNA heteroduplexes [32]. RNase H does not act on double- or single-stranded RNAs. When the application of RNase H preceded the hybridization of fluorescent DNA FISH probes to *Xist* RNA, the *Xist* RNA coat over the Xi territory was observed to be intact. This finding suggested that the *Xist* transcripts were not engaged in extensive, if any, RNA:DNA heteroduplexes with genomic DNA. However, to ensure that the *Xist* RNA can indeed bind DNA, the authors also applied RNase H treatment after the DNA probe hybridization step. This reversed order of experimental steps resulted in loss of the FISH signal due to degradation of *Xist* RNA by RNase H and confirmed that, although the *Xist* transcripts are capable of hybridizing to DNA, they do not directly base pair with DNA in the cell. An extension of this conclusion is that *Xist* RNA instead spreads via associations with chromatin and/or the nuclear matrix. In support of this, it has been shown that *Xist* transcripts undergo extensive *cis*-medicated chromosome spread when expressed from cDNA transgenes integrated at ectopic sites on the X or autosome, or in the context of X:autosomal translocations [41,45–48]. This property of *Xist* was recently exploited in a human pluripotent cell model of Down's Syndrome (trisomy of chromosome 21) [49]. *XIST* expressed from a transgene integrated on one of three chromosome 21's in these cells effectively implemented heritable autosomal inactivation *in cis*. This work not only confirmed that *Xist/XIST* RNA spreads independently of X-linked genomic sequences, but also demonstrated the competence of chromosome 21 to be silenced, while also uncovering a potential therapeutic approach towards the treatment of trisomic disorders that catapulted *Xist* RNA biology into the realm of translational medicine.

In the first half of this review, we will focus on the data that suggests that 3D chromatin conformation determines the spread of *Xist* RNA across the X chromosome. We will also cover the identification of trans-activating factors and the putative roles(s) they play in mediating this process. It has long been appreciated that, once formed, the Xi is structurally distinct from that of the Xa [50]. In the second part of this review, we will address differences in the 3D structure of the Xi in relation to the Xa from cytological, biochemical and genomic perspectives. Our aim is to highlight how the *Xist* RNA, by exploiting the 3D architecture of the genome to exert its function, also critically impacts genome organization.

2. Modeling X chromosome inactivation *in vitro*

The study of XCI in implanting blastocysts has been limited, at least in part, by their availability, difficulty of genetic manipulation, and low cell number for biochemical analyses. Embryonic stem cells (ESC), derived from epiblast cells of the mouse pre-implantation blastocyst, expand indefinitely in culture and provide an essentially limitless supply of starting material [51,52]. These cells grow clonally, which is convenient for the isolation of targeted genetic mutants. Like the epiblast cells of the blastocyst, female mouse ESCs maintain two active X chromosomes and upon differentiation, reliably recapitulate random XCI [4]. Thus, this *in vitro* system has become a tractable and genetically pliable paradigm for the molecular dissection of XCI. As the findings discussed within this review are based largely upon mouse models of XCI, we provide a short discussion on the benefits and caveats of current mouse cell culture model systems used to study XCI, as this is information that will benefit the understanding of later sections.

Cultured female ESCs are the prevailing model system for the study of random XCI. However, most female ESC lines are unstable with regards to the X chromosome and within a few cell cycles in culture become genetically XO [53]. For reasons that are unclear, genetic stability is retained if the X homologues harbor