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RNAi in X inactivation: contrasting findings on the role of interference

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X inactivation is the process that brings about the dosage equivalence of X-linked genes in females to that of males. This complex process initiated at a very early stage of female embryonic development is orchestrated by long non-coding RNAs transcribed in both sense and antisense orientation. Recent studies present contradicting evidence for the role of small RNAs and RNase III enzyme Dicer in the X inactivation process. In this review, I discuss these results in the overall perspective of X inactivation and gene silencing.

Keywords: Dicer; RNA interference; small RNA; X inactivation; Xist

Introduction

X chromosomal inactivation is an evolutionarily conserved process of silencing of genes on one of the female X chromosomes so as to equalize the expression of X-linked genes to that of their male counterparts.[1] This process commences at a very early stage of female embryonic development and is mediated by *cis* coating of a long non-coding RNA termed Xist (X inactive specific transcript) onto the future inactive X chromosome (Xi). This primarily triggers a cascade of silencing events including recruitment of polycomb proteins, histone modifications such as H3K27 methylation, H3K9 methylation, macroH2A incorporation, *etc*.[2] On the active X chromosome (Xa), the chromosome that escaped the inactivation process, these silencing events are thought to be countered by another non-coding RNA named Tsix (antisense to Xist) transcribed in the antisense orientation of Xist. Tsix prevents the transcription of Xist,[3] its coating of the X chromosome and the ensuing spread of silencing. RNA interference (RNAi) machinery is intricately involved in the silencing of yeast centromeric chromatin *via* small RNA generated from the pericentric sense and antisense non-coding RNA and specialized protein complex named RNA-induced transcriptional silencing (RITS).[4,5] Maintenance of heterochromatin domains by double-stranded RNA (dsRNA) binding proteins and small RNA has also been reported in plants and *Drosophila*. Up to now, unlike the case for lower eukaryotes, in mammals there is no direct evidence linking RNAi machinery proteins and small RNA to the establishment or maintenance of either constitutive or facultative heterochromatin. It has long been speculated that several non-coding RNAs in both the sense and antisense orientations transcribed from the mammalian X inactivation centre (XIC) might be substrates for RNAi machinery to initiate the heterochromatin on one of the X chromosomes. Recently, three different groups[6–8] have reported in depth analyses of small RNA generation from the Xist locus and a role of Dicer in the initiation and establishment of inactive X chromosome.

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Interlink between Xi establishment and Dicer

In an elaborate study of the involvement of RNAi in X inactivation, Ogawa *et al*.[6] looked for small RNA arising from the Xist locus by performing strand-specific northern blots from distinct regions within the Xist RNA and its promoter. They were able to detect distinctly sized small RNA of 24–42 nucleotides corresponding to Repeat A, Exon-7 and promoter regions of Xist upon differentiation of ES cells. These small RNAs named Xi RNA were more robust in the Tsix orientation than in the Xist orientation. The formation of Xi RNAs required Tsix transcription with their levels dropping dramatically in Tsix-deficient ES cells.[9,10] This observation led the authors to identify the existence of double-stranded Xist::Tsix RNA duplexes in the ES cells using a modified RNase protection assay. The amount of Xist::Tsix duplexes showed inverse correlation to Xi RNA with higher amounts of RNaseA-protected dsRNA prior to differentiation (Pre-XCI) compared to a significant reduction in their level during ES cell differentiation (corresponding to the onset of XCI). Although these duplexes have equal probability of being generated from both the X chromosomes initially, an increase in the level of Tsix transcript from future Xa during XCI formation might result in the majority of the duplexes being formed from the Xa. Small RNAs are formed in mammals by the action of an RNase III enzyme, Dicer.[11] So, does Dicer have any role in xiRNA generation? Previous studies[12,13] have reported that Dicer-deficient ES cells show defects in pluripotency and

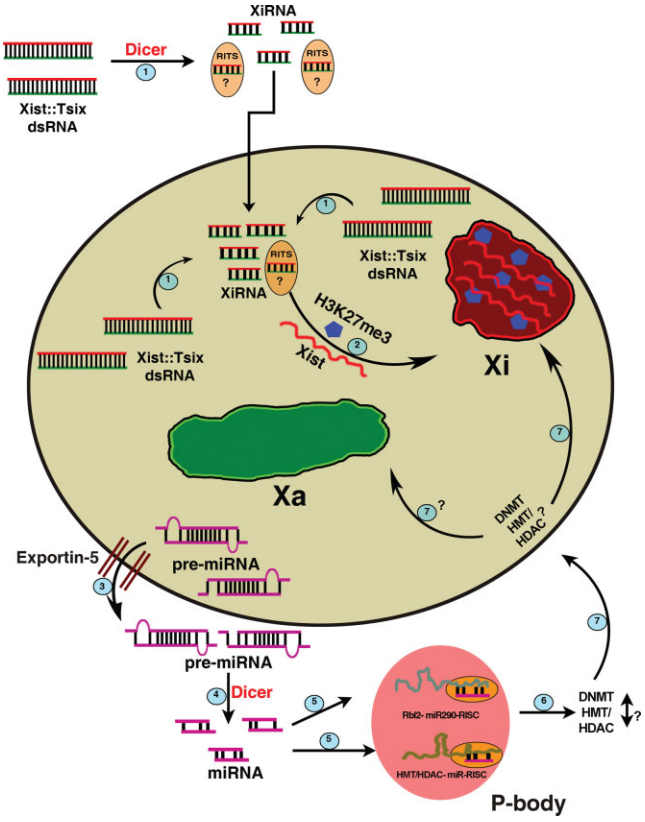

Figure 1. RNase III enzyme Dicer can have either direct or indirect effects on X inactivation. Direct: Xist and Tsix form dsRNA; these Xist::Tsix duplexes are (**1**) processed by Dicer either in the nucleus or in the cytoplasm into small 24–42-nucleotide xiRNA. xiRNA alone or in complex with proteins similar to RITS (RNA-induced transcriptional silencing) of yeast that contain Argonaute-1 and/or Argonaute-2 proteins can mediate (**2**) the localization of Xist and histone methyl transferases like EZH2 to inactive X chromosome (Xi). Indirect: Precursor microRNA (pre-miRNA) transcribed and processed by the Drosha-containing microprocessor complex within the nucleus are (**3**) exported into cytoplasm *via* Exportin-5. These pre-miRNAs are (**4**) processed into mature 21–23-nucleotide miRNA by Dicer in the cytoplasm. **5:** These miRNAs like the miR-290 cluster or other miRNAs can regulate the protein levels of Dnmt transcriptional repressor proteins like Rbl-2 (retinoblastoma-like-2) and heterochromatin-modulating proteins like histone methyl transferases (HMTase) and histone deacetylases (HDAC) or their regulators *via* post-transcriptional repression that localize to processing bodies (P-body). **6** and **7:** Altered levels of Dnmts and/or HMTase and HDACs due to impaired miRNA biogenesis in the absence of Dicer can differentially regulate the inactive X chromosome and its heterochromatic modifications (? indicates that experimental proof is needed).

cellular differentiation. Therefore, the authors had partially overcome the differentiation defects caused by Dicer KO, by expressing Dicer transgene at very low levels ($\ll$5%) in Dicer$^{-/-}$ ES cells. In these Dicer$^{-/-}$ cells, the xiRNA levels were found to be significantly reduced upon differentiation and more importantly Dicer$^{-/-}$ ES cells showed a lack of Xist and H3K27 trimethylation foci characteristics of Xi chromosome. This lack of Xist foci was accompanied by an increase in the steady-state levels of Xist RNA in both male and female Dicer$^{-/-}$ ES cells. Surprisingly, even though there is an increase in the Xist RNA levels, for some unknown reasons these Xist RNAs were not able to coat the Xi. Is it possible that the defective Xist localization and H3K27 trimethylation on Xi are related to general differentiation defects associated with Dicer removal? Considering that the Dicer$^{-/-}$ clones used in this study continued to show defects in repression of pluripotency factor expression even after 10 days into differentiation, the above notion could be a possibility. To delineate the direct effect of Dicer on Xi, a mutation was created in the Tsix locus, producing truncated Tsix in the Dicer$^{-/-}$ cells. ES cell clones carrying these double mutations (Dicer$^{-/-}$ Tsix$^{-/+}$) were able to partially restore Xist localization (12.3%) compared to Dicer$^{-/-}$ (0%). Surprisingly, these cells were not able to restore H3K27 that generally accompanies Xist coating during XCI in wild-type ES cells, indicating that Xist localization and H3K27 incorporation are functionally separable. In conclusion, Ogawa *et al.* provided evidence for the existence of small RNA generated from Xist:Tsix duplexes mediated by Dicer processing and Dicer$^{-/-}$ female ES cells show defects in Xist, H3K27 incorporation onto Xi that are genetically separable from generic differentiation-specific defects associated with the absence of Dicer.

No role for Dicer in Xist and H3K27 recruitment on to Xi

In contrast to the above study, Kanellopoulou *et al.*[8] found that the Dicer KO ES cells did not exhibit defects in the proper localization of Xist, initiation of Xi silencing and recruitment of polycomb proteins. ES cells of male origin do not have an inactive X chromosome and therefore do not express Xist. However, regulated ectopic expression of Xist in male ES cells efficiently coated the X chromosome and initiated X chromosomal inactivation by recruiting all the Xi characteristic heterochromatin factors.[14] So, to study the effect of Dicer on the Xi initiation, Kanellopoulou *et al.* expressed Xist from the male X chromosome under the control of Tet regulatable promoter in control and Dicer$^{-/-}$ male ES cells. Upon induction with doxycycline, Xist expression and coating was found to be similar in the control and Dicer$^{-/-}$ cells. Moreover, enrichment of polycomb proteins, Eed, Ezh2, Suz12 and H3K27 trimethylation that are recruited to Xist-coated Xi was also found to be comparable between the control and Dicer-deficient male ES cells, indicating that Dicer is dispensable for Xi localization of Xist, enrichment of polycomb and H3K27 trimethylation. In this male Dicer$^{-/-}$ ES cell model, similar to the control ES cells, there was proper exclusion of phosphorylated RNA polymerase, a marker for active

transcription, as well as absence of transcription of the X-linked gene pgk1 upon induction of Xist silencing through Dox. These results indicated that there is proper X inactivation in the absence of Dicer in the male ES cells induced to express Xist from the X chromosome. More importantly, Kanellopoulou *et al*. showed that, although Xist levels are increased in the Dicer$^{-/-}$ male ES cells, this increase in Xist level was apparently a post-transcription event, as unspliced Xist RNA levels remained constant between control Dicer$^{-/-}$ ES cells. Further, to determine whether Dicer is required for Xi establishment in native conditions in female ES cells, the authors established female dcr$^{fl/fl}$ ES cells from embryos of B6 dcr$^{fl/fl}$ mice. The Dicer$^{-/-}$ female ES cell lines derived from this genetic background had only one X chromosome rather than a normal XX karyotype. Of more significance is the finding that in hybrid (CAST/Ei $\times$ C57BL/6) female ES cells that retained stable XX karyotype, Dicer deletion did not perturb the process of X chromosomal inactivation and was indistinguishable from the control ES cells.

Proper Xist localization in Dicer-deficient embryos

In another interesting study, Nesterova *et al*.[7] found that transcriptional events in the upstream promoter regions of Xist, both in the sense and antisense orientation, correlates with reduced DNA methylation at the Xist promoter regions of future Xi upon differentiation. As transcription was documented both in the sense and antisense orientation at the Xist locus, is there a possible function for Dicer in regulating the methylation status at the Xist promoter? Dicer KO male ES cells are characterized by a hypomethylated Xist promoter and have increased amounts of Xist transcripts. The CpG hypomethylation was not restricted only to the Xist promoter but was widespread across several loci including H19 and Igf2rAir imprinted loci, indicating a global perturbation of DNA methylation. Previous reports demonstrated that Dicer KO ES cells have decreased amounts of *de novo* DNA methyltransferases, Dnmt3a, Dnmt3b and Dnmt3l, most possibly due to defective processing resulting in reduced miRNA 290 levels, which in turn regulate the protein levels of Dnmt transcriptional repressor proteins like Rbl2 *via* miRNA-mediated post-transcriptional silencing.[15,16] Overexpression of either the miR290 cluster or Dnmt transgenes in Dicer KO cells restores Dnmt levels and DNA methylation, suggesting a direct relationship between miRNA and Dnmt in ES cells. So, are the reduced Dnmt levels responsible for Xist promoter hypomethylation and increased Xist transcript levels? Nesterova *et al*. found reduced Dnmt levels in their Dicer KO cells, suggesting that impaired miRNA functions might be responsible for the observed defects in Xist promoter DNA methylation. Similar levels of modified histones, *i.e*. high H3K9me2, H3K27me3 and reduced H3K4me2 that are characteristic of silenced promoters in Dicer KO cells, further indicated a specific defect only in the DNA methylation process but not in the heterochromatin in general. Dicer KO embryos showed early embryonic lethality with most of them not surviving beyond embryonic day (E) 7.5.[17] The authors therefore compared Xist localization in female mouse embryos on E6.5 and found no significant differences in Xist and Tsix staining patterns between wild-type and Dicer KO embryos. However, in Dicer KO embryos Xist cloud intensity was less than that in the controls, which is probably due to activation of apoptotic process leading to embryonic lethality.

Conclusion and future perspectives

Although the study of Ogawa *et al*. shows a correlation between the XiRNA derived most probably from Xist:Tsix duplexes and a role for Dicer in Xi heterochromatinization, recent reports indicate no such direct involvement of Dicer in the Xi initiation steps like Xist coating and H3K27 methylation (Fig. 1). However, all these studies indeed show a role for increased Xist transcript levels in Dicer KO male and female ES cells. Although reduced Dnmt levels may be responsible for this Xist upregulation due to promoter hypomethylation, a direct role for Dicer or Dicer-derived factors in regulating post-transcriptional levels of mature Xist levels has not been established. Effects of Dicer depletion on Tsix transcription and/or stability need to be addressed. It is difficult to rule out the possibility of Dicer having a direct role in Xi heterochromatin structure considering the rescue of Xist localization defects by subsequent Tsix mutation.[6] Even earlier, studies addressing the role of Dicer in centromeric heterochromatin arrived at diametrically opposite conclusions.[12,13] Unlike *Drosophila*, wherein endogenous siRNA and miRNA are processed by two different Dicer enzymes,[18] in mammals, a single Dicer enzyme performs both these functions. As each miRNA regulates multiple target mRNAs, identification of specific proteins that are acting in concert with Dicer to regulate the siRNA or miRNA pathway will help to distinguish between the loss of miRNA-mediated effects and direct effects of Dicer on heterochromatin. In yeast, the RITS complex containing the Argonaute protein Ago1 binds and mediates heterochromatin formation and maintenance in all the known heterochromatic loci.[19] In human cell lines, promoter targeting siRNA are known to recruit Argonaute proteins, Ago1 and/or Ago2 along with Dnmt3a, Dnmt1 and HDAC1.[20–22] It would be interesting to check whether xiRNA and/or Xist, Tsix, or repeat A RNA from the Xist locus can specifically bind to the Argonaute family proteins. Mammalian Dicer is reported to be localized predominantly in the cytoplasm. In this context, several questions including in which cellular compartment the Xist::Tsix pairs form duplexes,

how they become substrates to Dicer protein and whether or not XiRNA shuttle between cytoplasm and nucleus remain to be explored. Moreover, Dicer depletion in cells led to the activation of the DNA damage response pathway with accumulation of proteins such as γ-H2AX on DNA loci.[23] Facultative heterochromatin at the Xi loci is known to be intricately related to DNA damage response proteins ATM, ATR and BRCA1.[24,25] Whether Dicer directly or indirectly regulates the levels or recruitment of these proteins to Xi during replication needs to be investigated.

Until now, these studies in the X inactivation field not only offered glimpses of the role of Dicer in the process of X inactivation but also hinted at the involvement of the RNAi pathway and/or small dsRNA in heterochromatin formation and chromosome stability in mammals. Future studies focused on delineating the regulation of heterochromatin mediated by endogenous, locus-specific siRNA and specific associated proteins will help to distinguish indirect effects mediated *via* miRNA-based mRNA regulation.

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