X chromosome inactivation in the cycle of life

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Summary
Female mammalian cells silence one of their two X chromosomes, resulting in equal expression levels of X-encoded genes in female XX and male XY cells. In mice, the X chromosomes in female cells go through sequential steps of inactivation and reactivation. Depending on the developmental time window, imprinted or random X chromosome inactivation (XCI) is initiated, and both processes lead to an inactive X chromosome that is clonally inherited. Here, we review new insights into the life cycle of XCI and provide an overview of the mechanisms regulating X inactivation and reactivation.

Key words: Tsix, X chromosome inactivation, XCI activator, XCI inhibitor, Xist

Introduction
The evolution of the sex chromosomes in placental mammals is associated with potential gene dosage differences at a chromosomal level between female (XX) and male (XY) cells. Thus, to equalize X-encoded gene expression, female somatic cells transcriptionally silence one of the two X chromosomes in a process named X chromosome inactivation (XCI), which leads to mono-allelic expression of most X-linked genes. In mice, two forms of XCI exist. Imprinted XCI of the paternally inherited X chromosome (Xp) is initiated in the female cleavage-stage embryo and is maintained in extra-embryonic tissue and its derivatives (Takagi and Sasaki, 1975). The Xp is reactivated in the inner cell mass (ICM) of the developing female embryo (Mak et al., 2004). Then, shortly after implantation, a second round of XCI is initiated in the embryo proper, which is random with respect to the parental origin of the future inactive X chromosome (Xi) (Lyon, 1961). Random inactivation is also observed in female mouse embryonic stem (ES) cells, which are generated from the ICM, when these ES cells are induced to differentiate. Although random XCI is a very stable form of
epigenetic gene silencing, it is reversed in primordial germ cells (PGCs) of the female germline (Monk and McLaren, 1981; Sugimoto and Abe, 2007; Tam et al., 1994).

The mouse has served as an important model with which to study XCI. However, there are indications that the life cycle of XCI shows differences between mammalian species. For example, imprinted XCI is present in mouse and marsupials but has yet to be confirmed in other mammalian species (Okamoto et al., 2011). Also, most human pluripotent stem cells represent a post-XCI state. Variations in XCI between species might be related to the different growth requirements of their early embryos, which are associated with differences in the expression of regulatory factors, and point to a delicate inter-relationship between XCI, pluripotency and differentiation.

**The X inactivation center: cis-acting elements in random XCI**

In mouse, two non-coding X-linked genes play a crucial role in random XCI. Both genes, Xist and Tsix, are located within the X inactivation center (XIC), a region genetically determined to be required for XCI. Xist encodes a spliced and poly-adenylated 17 kb-long RNA (Borsani et al., 1991; Brockdorff et al., 1991; Brown et al., 1991). Xist transcription is mono-allelically upregulated at the onset of XCI, and the non-coding Xist RNA associates with the future Xi in cis (Brown et al., 1992). This coating of the X is followed by exclusion of the transcription machinery and recruitment of chromatin remodeling complexes (Chaumeil et al., 2006). Tsix fully overlaps with Xist but is transcribed in the antisense direction and it is involved in suppression of Xist on the active X chromosome (Xa) (Lee et al., 1999). A third gene, Xite, is also transcribed into a non-coding RNA and acts to enhance Tsix expression (Ogawa and Lee, 2003). Studies of Xist, Tsix and Xite knockout alleles have indicated that Xist RNA is required for XCI to occur in cis and that all three genes play an important role in determining which X chromosome will be inactivated (Lee and Lu, 1999; Ogawa and Lee, 2003; Penny et al., 1996; Marahrens et al., 1997). Female cells with a heterozygous deletion of a region spanning all three genes always initiate XCI on the wild-type chromosome (Monkhorst et al., 2008). This finding demonstrates that the combination of Xist, Tsix and Xite acts as a cis-acting master-switch locus that is not involved in determining the number of X chromosomes silenced, and also indicates that this counting process is regulated by other factors that are encoded by genes located elsewhere in the genome.

**Counting the X chromosomes and initiation of random XCI**

How does a cell ‘count’ the number of X chromosomes present in the nucleus? Studies of women with supernumerary X chromosomes (such as 47,XXX or 48,XXXX), Klintefelter (47,XY) men, and tetraploid fetuses, have indicated that all X chromosomes except one are inactivated per diploid genome (Gumback et al., 1963). Interestingly, a clear correlation was found between the number of X chromosomes present in a nucleus and the probability of XCI being initiated at a given step of differentiation (Monkhorst et al., 2009). This suggested the presence of dose-dependent X-encoded activators, predicted to be counteracted by autosomally encoded inhibitors of XCI. XCI activators activate Xist and/or repress Tsix, whereas XCI inhibitors repress Xist and/or activate Tsix. The sum of the XCI-inhibitor activity provides a threshold that has to be overcome by the total XCI-activator activity to allow spreading of Xist RNA in cis. In the embryo of the female developing embryo, or in differentiating female ES cells, the presence of two active X chromosomes ensures that the concentration of the X-encoded activators is sufficient to initiate XCI. Stochastic initiation together with a robust feedback mechanism could be sufficient to regulate XCI (Monkhorst et al., 2008). From earlier investigations, at least two other mechanisms have been proposed to accommodate a mutually exclusive choice process. First, in the alternate states model, both X chromosomes could harbor epigenetic marks in a random fashion that are read out as opposite marks at the onset of random XCI, leading to the pairing model (Bacher et al., 2006; Masui et al., 2011; Xu et al., 2006). Direct interaction between X chromosomes is thought to be initiated through an X-pairing region (Xpr) located distal to Xist, followed by cross-talk between the Xist and Tsix alleles through an as yet unknown mechanism resulting in a mutual choice (Augui et al., 2007).

**XCI activators**

Until now, the genes Rnf12 (also known as Rlim), Jpx (also known as Enox) and Fix, which are all located in close proximity distal to Xist, have been identified to encode trans-acting activators of XCI. Additional transgenic copies of Rnf12, which encodes the E3 ubiquitin ligase RNF12, induce initiation of XCI on the single X in male cells and force initiation of XCI on both X chromosomes in a high percentage of transgenic female cells (Jonkers et al., 2009). Rnf12 is transiently upregulated during ES cell differentiation, and genetic studies have indicated that Rnf12 mainly regulates Xist (Barakat et al., 2011). This is probably an indirect action involving another protein (or proteins) that is a target of RNF12. Two independent studies confirmed that the frequency of initiation of random XCI is reduced in Rnf12+/– and Rnf12–/– ES cells (Barakat et al., 2011; Shin et al., 2010). In one of these studies, Rnf12+/– ES cells totally failed to activate random XCI, indicating that RNF12 is an important activator of XCI, but probably not the only one, as female Rnf12+/– cells still initiate random XCI (in contrast to male cells).

Two potential additional candidate genes involved in XCI activation are the non-coding genes Jpx and Fix. Both genes are upregulated on both X chromosomes upon differentiation of ES cells and partially escape X inactivation. Knockout studies suggest that Jpx is required for XCI and activates Xist in trans, although a cis-based role cannot be excluded (Tian et al., 2011). The other non-coding gene, Fix, is probably involved in the same or a complementary mechanism, as Fix deletion impairs Xist expression and the expression of other genes located in close proximity to Fix, including Jpx, in male cells (Chureau et al., 2011). Thus far, both Jpx and Fix transgenes, when located elsewhere in the genome, failed to induce ectopic XCI in male and female cells (Jonkers et al., 2009). Therefore, it is likely that X-linked Fix and Jpx predominantly activate Xist in cis, possibly by generating a transcriptionally active environment that spans Xist and other flanking genes.

**XCI inhibitors**

Most of the identified inhibitors of XCI appear to be factors that are also involved in maintaining ES cell homeostasis and that are downregulated upon ES cell differentiation (Boyer et al., 2006). This links repression of XCI to the activities of pluripotency factors and the pluripotent state of a cell, as was first proposed more than 30 years ago (Monk and Harper, 1979). The pluripotency factors...
repress XCI either by stimulating expression of Tsix or by repressing Xist expression (Navarro et al., 2008; Navarro et al., 2010). The latter can be direct or indirect through repression of activators of Xist. The pluripotency factors OCT4 (also known as POU5F1), SOX2 and REX1 (also known as ZFP42), the reprogramming factors KLF4 and cMYC, and the ubiquitously expressed factors CTCF and YY1 all activate Tsix by binding either to the Tsix promoter or to the Tsix enhancers Xite and the Dypsas34 region (Donohoe et al., 2009; Donohoe et al., 2007; Navarro et al., 2010).

Direct suppression of Xist by pluripotency factors in ES cells has been proposed to involve recruitment of NANOG, OCT4 and SOX2 to a region in Xist intron 1 (Navarro et al., 2008). In support of this, forced downregulation of Nanog, Oct4 and Sox2 gene expression was found to be accompanied by Xist accumulation in male cells (Navarro et al., 2008). However, deletion of the complete pluripotency factor binding site in Xist intron 1 did not result in Xist accumulation in undifferentiated female ES cells (Barakat et al., 2011). Also, abrogation of Tsix expression alone was found to be insufficient for Xist upregulation in undifferentiated cells (Clerc and Avner, 1998). By contrast, the combined removal of both the intron 1 region and the Tsix major transcriptional start site resulted in a loss of Xist repression of autosomally integrated transgenes, covering Xist and Tsix, in undifferentiated cells (Nesterova et al., 2011). Although these results have to be verified by removal of both regions from the endogenous locus, they do suggest that redundant mechanisms are in place to repress Xist. Downregulation of XCI-activator genes might also be crucial for repression of Xist in the ICM. In accordance with this, overexpression of RNF12 in undifferentiated female ES cells was found to result in activation of XCI, suggesting that the XCI-activator concentration might be a key limiting factor for Xist upregulation (Jonkers et al., 2009). This finding also suggests that XCI-activator genes might be repressed by pluripotency factors. Indeed, genome-wide mapping of pluripotency factor binding has shown that NANOG, OCT4, SOX2, DAX1 (also known as NR0B1) and REX1, all bind to the Rnf12 upstream promoter region (Kim et al., 2008), and recent studies indicate that Rnf12 expression is negatively regulated by Nanog expression (Barakat et al., 2011; Navarro et al., 2011).

The identification of XCI activators and XCI inhibitors highlights that the network regulating random XCI involves intricate relationships between multiple factors that often act in a dose-dependent manner on various target genes. Downregulation of most autosomally encoded XCI inhibitors occurs in both male and female differentiating ES cells, but only the female cells will initiate random XCI owing to a higher level of expression of X-linked XCI-activator genes.

**Imprinted XCI**

In the female mouse pre-implantation embryo, XCI is imprinted and this leads to exclusive inactivation of the paternally inherited X chromosome (Xp). Although the Xp has been reported to be inherited in a partially inactive state (Huynh and Lee, 2003), other evidence suggests that both X chromosomes are active in female embryos after zygotic genome activation followed by initiation of imprinted XCI at the 4- to 8-cell stage (Okamoto et al., 2005; Okamoto et al., 2004). It is likely that parental-specific epigenetic modifications, which are set during gametogenesis, regulate imprinted XCI, and these modifications probably involve an imprint that represses Xist on the maternally inherited X chromosome (Xm). This hypothesis is supported by transgene studies, which indicate that all the epigenetic information required for imprinted XCI is located within a 220 kb region that includes Xist and Tsix (Okamoto et al., 2005).

The molecular mechanisms used by placental mammals to silence an X chromosome in imprinted XCI versus random XCI might partly overlap, with both processes requiring Xist. By contrast, in the imprinted XCI process, repression of Xist on the Xm is independent of Tsix, and might involve OCT4- and SOX2-mediated repression of Xist in the pre-implantation embryo, and possibly other factors in extra-embryonic tissues. Tsix-mediated repression of Xist only starts to play an important role on the Xm later in embryonic development, around the morula stage. This can be concluded from the observation that a maternally inherited Tsix-null allele is embryonic lethal owing to aberrant initiation of XCI on the Xm (Lee, 2000; Sado et al., 2001).

A recent study revealed an important role for RNF12 in imprinted XCI, in addition to its role in random XCI. A maternally transmitted Rnf12 knockout allele caused embryonic lethality only in female offspring, owing to defects in the development of extra-embryonic tissues (Shin et al., 2010). Female ΔRnf12/+ embryos with the deletion on the Xm failed to initiate imprinted XCI on the wild-type Xp at the early cleavage stage. Based on this finding, the maternal storage of RNF12 protein in the oocyte was suggested to play an important role in imprinted XCI (Shin et al., 2010). However, continuous expression of the Rnf12 gene in the early embryo might also be required for activation of Xist expression. For random XCI, it was observed that heterozygous ΔRnf12/+ female ES cells and mice show skewed inactivation of one X chromosome towards the mutated X (Jonkers et al., 2009; Shin et al., 2010). This suggests that ongoing de novo synthesis of RNF12 might be required for persistent expression of Xist both for imprinted and random XCI.

**Reactivation of the Xi**

The tight connection between the pluripotency factor network and suppression of XCI provides a mechanism for proper developmental timing of random XCI in the early female embryo, but might also be instrumental in the observed reactivation of the Xi in the ICM and in PGCs. In female mouse embryos, imprinted XCI of the Xp is reversed in the late blastocyst at embryonic day (E) 4.5 of development. From the late morula stage onwards, Nanog is expressed, and only ICM cells with prolonged Nanog expression show reactivation of the Xi (Silva et al., 2009). Interestingly, Xist repression in the ICM might be required for reactivation of the Xi, but it is not sufficient. This is illustrated by studies involving NANOG overexpression in the ICM, which results in repression of Xist at an earlier stage of development, but does not lead to accelerated X reactivation (Williams et al., 2011). This finding indicates that upregulation of Nanog expression, possibly in conjunction with downregulation of XCI activators, is involved in Xist shutdown, but also that at least one additional mechanism is involved in the reactivation process.

A second wave of Xi reactivation is initiated in developing female germ cells. PGCs arise in the epiblast at ~E7.5 and subsequently migrate through the hindgut to reach the genital ridge at E11.5. In the mouse embryo, XCI is random in the epiblast cells that give rise to the PGCs, and reactivation of the Xi occurs during migration or around the time PGCs enter the genital ridge (Chuva de Sousa Lopes et al., 2008; de Napoles et al., 2007; Sugimoto and Abe, 2007). Reactivation of the randomly inactivated Xi in PGCs seems to require a longer time window than resetting of imprinted XCI in the ICM, which might reflect differences in the composition
of the Xi heterochromatin formed during the random XCI and imprinted XCI processes. OCT4, SOX2, NANOG and REX1 are highly expressed in PGCs and are therefore candidate factors involved in the direct repression of Xist in the germline cells, possibly supported by a low level of expression of XCI activators, including RNF12 (Mise et al., 2008).

Reactivation of the Xi can also be artificially induced during formation of induced pluripotent stem (iPS) cells (Maherali et al., 2007) or by fusion of somatic cells with embryonic carcinoma (EC) cells (Takagi et al., 1983). Neither forced expression of Oct4, Sox2, Klf4 and cMyC to generate IPS cells, nor expression of EC-specific pluripotency genes in hybrid cells, is sufficient to reactivate the Xi instantly (Do et al., 2007; Maherali et al., 2007). In both cases, reactivation of an X-linked GFP reporter gene on the Xi occurs late during the reprogramming process, probably because several layers of epigenetic silencing mechanisms have to be erased in conjunction with a shutdown of the Xist promoter. Epiblast stem cells (EpiSCs) are post-XCI pluripotent stem cells isolated from the epiblast of an E5.5 embryo (Brons et al., 2007; Tesar et al., 2007). Reprogramming of EpiSCs into ES cells by sustained culture in the presence of LIF, or by forced expression of Klf2, Klf4 and Nr5a, also leads to reactivation of the Xi (Bao et al., 2009; Guo and Smith, 2010; Guo et al., 2009). Furthermore, the Xi is reactivated in embryos obtained through somatic cell nuclear transfer (SCNT) (Eggan et al., 2000). Extra-embryonic tissues of cloned female embryos obtained through SCNT retain the Xi that was also inactive in the donor cell, which suggests that the information required for proper imprinted XCI might be reminiscent of the epigenetic marks acquired during random XCI.

Perspectives

Recent advances in XCI research have indicated that direct XCI activators and XCI inhibitors play crucial roles in counting and initiation leading to random XCI. Although several of the factors involved in random XCI have been identified, it is likely that still more genes will prove to encode factors implicated in the regulation of random XCI. In fact, the identification of more regulatory factors is expected, in view of the supposition that regulatory pathways gain stability with a higher number of interlinked interactions. One of the key questions to be answered is how each of the different factors contributes to regulation of the XCI process. Several pluripotency factors regulate each other’s expression and even regulate expression of activators of XCI. Hence, future studies will be complicated and challenging. Although a clear link with random XCI has been established for several of them, it remains to be determined whether a given factor also regulates either imprinted XCI or reactivation of the Xi, or both. In vivo and ex vivo models will be required to answer most of these questions, adding a level of technical complication, but advances are being made. We anticipate that future studies will shed more light on the complex network of factors regulating XCI.

Acknowledgements

We would like to thank J. Anton Grootegoed for his contribution to preparation of the manuscript. We would also like to apologize to colleagues whose work could not be cited owing to space limitations.

Funding

This work was supported by The Netherlands Organisation for Scientific Research (NWO)-TOP, NWO-Vici and European Research Council (ERC) starting grants.

Competing interests statement

The authors declare no competing financial interests.

Development at a Glance

A high-resolution version of the poster is available for downloading in the online version of this article at http://dev.biologists.org/content/139/12/2085.full.

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