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Recent Advances in X-Chromosome Inactivation

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Abstract

X-chromosome inactivation is a paradigmatic epigenetic phenomenon that results in the mitotically heritable transcriptional inactivation of one X-chromosome in female mammals, thereby equalizing X-linked gene dosage between the sexes. The epigenetic factors and mechanisms that execute X-inactivation overlap with those that regulate embryonic development and disease progression, thus offering a window into the epigenetic processes that regulate development and disease. Here I summarize some recent developments as well as open questions in X-inactivation research.

Cells retain their identity in part by inheriting gene expression profiles of their predecessors. Patterns of gene expression that are transmitted through cell division often propagate through epigenetic modifications of chromatin. Emerging evidence increasingly implicates this epigenetic mode of inheritance in a myriad of developmental processes as well as being a significant contributor to human diseases such as cancer. The epigenetic machinery offers a powerful mechanism to impact heritable changes in the expression of a wide variety of genes, both during normal development and in disease (Berdasco and Esteller, 2010; Sauvageau and Sauvageau, 2010; Surface et al., 2010).

Epigenetic regulation of gene expression is characterized by a number of defined covalent modifications of chromatin (Kouzarides, 2007). For example, methylation of cytosine residue of DNA and methylation of lysine 27 of histone H3 often mark transcriptionally silent genes; and, histone acetylation tags sites of active transcription. DNA and histone modifications are proposed to mediate the stable transmission of gene expression states through mitosis and cell division. Although many chromatin marks correlate with specific gene expression states, the factors and mechanisms that trigger epigenetic changes in gene expression are poorly defined (Bonasio et al., 2010).

X-chromosome inactivation offers a pliable model system to delineate factors and chromatin modifications that initiate epigenetic transcriptional changes (Payer and Lee, 2008). X-inactivation occurs in at least two distinct steps: initiation and maintenance. During the initiation phase, the prospective inactive X-chromosome undergoes epigenetic transcriptional inactivation. In the maintenance phase, replicated copies of the inactive X-chromosome are maintained inactive through multiple rounds of cell division. These two phases of X-inactivation occur during discrete stages of early mammalian embryogenesis and employ multiple and overlapping epigenetic mechanisms that can be molecularly dissected in the mouse (Heard and Disteche, 2006).

In the mouse, the pre-eminent X-inactivation model system, X-inactivation initiates early during embryogenesis when individual cells of the early female embryo undergo

transcriptional silencing of genes along one of the two X-chromosomes (Kalantry et al., 2009; Patrat et al., 2009; Namekawa et al., 2010). Subsequently, with rare exceptions, the cellular epigenetic machinery ensures that replicated copies of the inactive and active X-chromosomes are maintained as such through mitosis and into descendant cells (Fig. 1). Initially, all cells of the pre-implantation-stage mouse embryo undergo exclusive inactivation of the paternal X-chromosome, in a process referred to as imprinted X-inactivation (Fig. 2; Kay et al., 1994). Later, at around the time of implantation, the inactive paternal-X selectively reactivates in the inner cell mass cells destined to form the fetus (Sheardown et al., 1997; Mak et al., 2004). These cells subsequently individually undergo random X-inactivation of either the maternal- or the paternal-X, thereby resulting in females being mosaic for X-linked gene expression (Monk and Harper, 1979; Rastan, 1982; McMahon et al., 1983). The remaining cells, all extra-embryonic in their fate (i.e., precursors of the placenta and the yolk-sac), maintain imprinted X-inactivation of the paternal-X (Takagi and Sasaki, 1975; West et al., 1977; Takagi, 1978).

X-inactivation occurs gradually over several rounds of cleavage divisions of the zygote and correlates with an ordered series of epigenetic events, thus providing a window to explore the mechanisms that initiate and propagate gene silencing (Fig. 3). The two Xs in females are initially distinguished by the expression exclusively from the inactive-X of the long non-coding RNA *Xist* (*X-inactive specific transcript*) (Brown et al., 1991). *Xist* upregulation, in fact, presages transcriptional silencing of genes along the prospective inactive X-chromosome. Quite unusually and coincident with silencing of genes along the inactive X-chromosome, *Xist* RNA physically coats the X-chromosome from which its expression is induced (Brown et al., 1992; Clemson et al., 1996). *Xist* RNA accumulation *in cis* on the inactive X-elect is thought to result in recruitment of protein complexes, such as the Polycomb group, whose actions are posited to alter the structure of the chromosome, packaging the DNA into a transcriptionally inert heterochromatin (Fig. 3). While the inactive X-chromosome is distinguished by *Xist* RNA expression, the active-X is characterized by the expression of the *Xist* anti-sense long non-coding transcript *Tsix* (Lee et al., 1999). *Tsix* expression is thought to forestall expression of *Xist* RNA, thereby preventing inactivation of the active-X (Stavropoulos et al., 2001; Navarro et al., 2005; Ohhata et al., 2008).

Both imprinted and random X-inactivation share many of the features that characterize the inactive X-chromosome. Most notably, during both random and imprinted X-inactivation, *Xist* RNA and the Polycomb group proteins decorate the inactive-X early during X-inactivation and *Tsix* transcription is an early hallmark of the active-X. The importance of these factors is further underscored by their absolute requirement during X-inactivation as evidenced by loss-of-function mutations. A defect in *Xist* RNA and components of the Polycomb repressive complex 2 (PRC2) renders the inactive-X prone to being active (Marahrens et al., 1997; Wang et al., 2001; Kalantry et al., 2006; Kalantry et al., 2009); and, vice versa, *Tsix*-deficiency results in inactivation of the normally active X-chromosome (Lee, 2000; Sado et al., 2001).

Combined with the phenotypes in mouse and cell culture models, the early localization of *Xist* RNA and the Polycomb group on the inactive-X have led to the widely held belief that these factors must function during the initiation phase of X-inactivation. In contrast to this widely accepted role for *Xist* and the Polycomb group, however, several lines of evidence suggest that both gene products are dispensable for chromosome-wide silencing of X-linked genes during the initiation phase of X-inactivation. For example, cells of female embryos inheriting a null mutation in *Xist* on the paternal X-chromosome nevertheless transiently initiate silencing of many paternal X-linked genes during imprinted X-inactivation (Kalantry et al., 2009). These data highlight a locus-by-locus variability in *Xist* RNA-dependent

initiation of imprinted X-linked gene silencing and imply the existence of as-yet-undiscovered *cis*-limited triggers of X-linked gene silencing. While imprinted X-linked gene silencing can initiate in the absence of *Xist* RNA, it is not stable. *Xist* is absolutely required to solidify or stabilize the inactive state during imprinted X-inactivation (Marahrens et al., 1997; Kalantry et al., 2009).

The primacy of *Xist* in mediating chromosome-wide transcriptional inactivation is also inferred by the effects of ectopically expressed *Xist* transgenes. While multi-copy *Xist* transgenes can result in a variable degree of silencing of neighboring sequences, single-copy *Xist* expressing transgenes do not induce silencing in their vicinity (Heard et al., 1999). Clearly, *Xist* influences the probability of a sequence to be transcriptionally silenced; but, this effect appears to be dependent on local chromatin context (Chow et al., 2010; Tang et al., 2010).

Xist-independent imprinted X-inactivation appears to be the ancestral form of X-inactivation. Marsupial mammals, which shared a common ancestor with placental mammals about 150 Mya, display imprinted X-inactivation in all their cell types and also lack *Xist* (Davidow et al., 2007; Hore et al., 2007; Shevchenko et al., 2007). *Cis*-acting elements other than *Xist* must therefore mediate imprinted X-inactivation in marsupials. Evolutionarily, the appearance of *Xist* coincides with the divergence of eutherians and marsupial mammals and the origins of random X-inactivation that is unique to eutherians (Wallis et al., 2007). X-linked gene silencing during random X-inactivation is preceded by a choice step whereby the cellular machinery targets one of the two Xs to undergo inactivation. *Xist* presence on an X-chromosome is absolutely necessary for that X to be *chosen* to be inactivated. If an X-chromosome lacks *Xist*, the other X-chromosome is invariably chosen for inactivation (Marahrens et al., 1998). Formally, this result does not necessarily support (or oppose, for that matter) a role for *Xist* in *initiating* X-linked gene silencing: the mutant X is preempted from attempting to initiate silencing because it fails to first be chosen to be inactivated. Whether *Xist* triggers X-linked gene silencing during random X-inactivation, therefore, requires the generation of homozygous *Xist*-mutant embryos, where both Xs now have an equal chance of being chosen for inactivation.

That X-linked gene silencing is subject to regional regulation, as opposed strictly to a central element such as *Xist*, is also consistent with the notion that X-linked genes became subject to dosage compensation gradually. X-inactivation is thought to be a by-product of sexual differentiation of a pair of homologous chromosomes into an X- and a Y-chromosome (Jegalian and Page, 1998). Due to a declining ability of the Y to recombine with the X, it has progressively degenerated. The gradual loss of genes from the proto-Y during X-Y differentiation is believed to have driven silencing of homologous X-linked genes in females in a piecemeal fashion (Jegalian and Page, 1998; Lahn and Page, 1999). If *Xist* RNA is the sole trigger of imprinted X-inactivation, the expectation would be that X-chromosomal genes would have to await its arrival before undergoing dosage compensation. This idea is clearly inconsistent with evidence suggesting that *Xist* has originated subsequent to the loss of Y-linked genes and hence the proposed advent of dosage compensation resulting in silencing of X-linked genes (Lahn and Page, 1999).

Xist RNA expression and accumulation on the presumptive inactive-X precedes the enrichment of Polycomb group proteins on the inactive-X (Okamoto et al., 2004). As detailed in the review by Trask and Mager in this issue, Polycomb group genes encode an evolutionarily conserved set of proteins that mediate cellular memory in diverse settings, including during X-chromosome inactivation. Female embryos and cells lacking the essential Polycomb group protein EED, resulting in a complete absence of histone methylation mediated by the PRC2 Polycomb complex, harbor defects in imprinted X-

inactivation (Wang et al., 2001; Kalantry et al., 2006). These *Eed*^{-/-} embryos nevertheless initiate imprinted X-inactivation; the defect is restricted to the maintenance phase of imprinted X-inactivation (Kalantry et al., 2006). During random X-inactivation, PRC2 appears to be dispensable for both the initiation as well as the maintenance phases of X-inactivation (Wang et al., 2001; Kalantry and Magnuson, 2006). It remains to be determined whether in the absence of PRC2, the sister PRC1 complex can compensate and enact X-inactivation (Schoeftner et al., 2006). These data nevertheless underscore both the differences in the epigenetic machinery required to mediate imprinted versus random X-inactivation as well as the need to definitively delineate epigenetic factors that function in *trans* to initiate and maintain X-inactivation.

Xist RNA expression is countered by transcription of the Xist anti-sense RNA Tsix. In fact, the exclusive function of *Tsix* is to repress *Xist* (Payer and Lee, 2008). The act of *Tsix* transcription modulates *Xist* chromatin and thereby is thought to repress *Xist* expression (Stavropoulos et al., 2001; Navarro et al., 2005; Sado et al., 2005). During random X-inactivation, *Tsix* functions in a complementary manner to *Xist* to mediate the choice of an X-chromosome to be inactivated. A *Tsix*-mutant X-chromosome is invariably chosen for inactivation, coinciding with Xist RNA being more readily induced from that X-chromosome (Lee and Lu, 1999; Luikenuis et al., 2001; Sado et al., 2001; Kalantry and Magnuson, 2006). During imprinted X-inactivation, *Tsix* is expressed from the active maternal-X (Lee and Lu, 1999; Sado et al., 2001); and, its absence on the maternal-X predisposes the maternal-X to becoming inactivated (Lee, 2000; Sado et al., 2001). *Tsix* has therefore been postulated to be a component of the germline mark established in the oocyte that prevents inactivation of the maternal-X in the resultant embryo (Lee, 2000). However, if *Xist* is not absolutely required to initiate chromosome-wide silencing of X-linked genes during imprinted X-inactivation, then the role of *Tsix* in preventing imprinted X-inactivation is brought into question. If *Xist* and *Tsix* function in a mutually exclusive manner to establish the epigenotype of the inactive paternal- and the active maternal-X chromosomes during imprinted X-inactivation, then it is reasonable to expect that a paternally inherited *Xist* and maternally inherited *Tsix* mutation would fully complement each other. Yet, this appears to not be the case (Sado et al., 2001). This finding therefore implies that a *Tsix*-independent mechanism may serve to establish the maternal epigenotype in the oocyte.

The mechanism by which a cell counts the number of X-chromosomes has long fascinated X-inactivation enthusiasts. Before a cell can commence the process of inactivating an X, it must first count the number of X-chromosomes it harbors. Only if the number of X-chromosomes exceeds one per diploid set of autosomes will stable X-inactivation occur (Avner and Heard, 2001). This equation is especially true during random X-inactivation and may also apply, albeit perhaps less stringently, during imprinted X-inactivation (Goto and Takagi, 2000; Okamoto et al., 2000). One tempting hypothesis proposed to explain counting is that the two Xs sense the existence of each other by physically pairing prior to the triggering of X-inactivation (Marahrens, 1999). Since such pairing would only occur in XX females, XY males would be immune from inactivation. The X-inactivation center—a region of the X-chromosome containing *Xist*, *Tsix* as well as several hundred kilobases of sequence on either side—is thought to be the segment that mediates counting (Clerc and Avner, 2003). Although this hypothesis is quite attractive and is supported by several recent reports describing the physical pairing of the two Xs via elements in the X-inactivation center, there are a number of observations to the contrary (Bacher et al., 2006; Augui et al., 2007; Xu et al., 2007; Monkhorst et al., 2008; Jonkers et al., 2009). The primary counterpoint is that deletions encompassing a large region of the X-inactivation center, including *Xist* and *Tsix*, nevertheless result in proper inactivation of the wild-type X-chromosome (Monkhorst et al., 2008). Moreover, evidence implicating X-inactivation center segments in X-chromosome pairing is not consistently replicated (Jonkers et al., 2009).

In light of the discord in the pairing data, very recently and quite excitingly the protein encoded by the X-linked *Rnf12* gene has been put forth as a factor that senses the number of X-chromosomes and triggers *Xist* expression in a dose-dependent manner during random X-inactivation (Jonkers et al., 2009). This discovery would also potentially explain why X-inactivation occurs in XX female but not in XY male cells. Since *Rnf12* is X-linked, the two active-Xs in female cells would express twice as much RNF12 protein prior to X-inactivation compared to XY male cells. The increased RNF12 levels would then induce X-inactivation only in female and not in male cells; RNF12 would therefore serve to “count” the number of X-chromosomes. Indeed, overexpression of RNF12 in male embryonic stem (ES) cells—an *in vitro* system to model random X-inactivation—leads to ectopic *Xist* expression from the single X-chromosome in a percentage of the transfected male cells (Jonkers et al., 2009). In the converse, loss-of-function experiment, the reduction in the level of RNF12 in female cells to that found in male cells by deleting one copy of *Rnf12* the female ES cells, however, did not result in the expected absence of X-inactivation (Jonkers et al., 2009). Thus, while RNF12 appears to be sufficient to trigger X-inactivation *in vitro*, it may not be necessary.

In a separate study, Shin et al. (2010) tested the *in vivo* requirement of *Rnf12* in X-inactivation by generating mice harboring a conditionally mutant *Rnf12* allele. Consistent with the previous study, cells that would normally undergo random X-inactivation commenced X-inactivation when devoid of or deficient in *Rnf12* (albeit with slight differences compared to wild-type samples). However, upon deletion of both alleles of *Rnf12* in mouse oocytes the authors strikingly discovered defects in *Xist* RNA induction and X-linked gene silencing from the paternal X-chromosome in the resulting early female embryos; this defect coincided with lethality of the mutant embryos (Shin et al., 2010). Thus, while random X-inactivation can occur in the absence of RNF12, imprinted X-inactivation requires RNF12. Moreover, the authors suggest that this RNF12 function is oocyte-derived (Shin et al., 2010). However, animals generated from oocytes where one but not both *Rnf12* alleles is subject to deletion argue against this conclusion. While female embryos derived from these oocytes that inherit a mutant *Rnf12* allele perish in utero, presumably due to a defect in imprinted X-inactivation, females that inherit the wild-type *Rnf12* allele are born at expected frequencies. If in fact maternally deposited RNF12 present in the oocyte drives imprinted X-inactivation in the early embryo, then its deficiency should impact embryos that inherit either the mutant or wild-type *Rnf12* allele from heterozygous oocytes equally. One explanation for this seeming discrepancy may be that zygotically active maternal *Rnf12* allele, perhaps in addition to oocyte-derived RNF12 protein is necessary for proper imprinted X-inactivation.

Formally, though, neither of the two aforementioned studies necessarily reveals a role for RNF12 in the cellular mechanism that counts the number of X-chromosomes. For example, parthenogenetic embryos harboring two maternal-Xs and no paternal X do not typically undergo X-inactivation (Matsui et al., 2001); presumably, these embryos harbor similar levels of RNF12 compared to wild-type female embryos yet neither X is inactivated. The *Rnf12* findings by Shin et al. (2010) highlight that the maternal X-chromosome in the early embryo is immune from X-inactivation. The answer to how RNF12 selectively targets the paternal-X and avoids the maternal X-chromosome for *Xist* expression may impinge on the nature of the germline mark placed on the maternal-X during oogenesis that forestalls its inactivation in the early embryo (Tada et al., 2000).

Since the discovery of *Xist*, the field of X-inactivation has progressed rapidly. By harnessing the power of newer technologies, including (but not limited to) mouse mutagenesis, genome sequencing efforts, improved techniques to detect X-linked gene expression, and high-resolution microscopy, many groups have contributed to the molecular dissection of the

epigenetic machinery that controls how a female cell stably inactivates one of the two X-chromosomes. These findings have broad implications, as the molecular themes that operate during X-inactivation apply to autosomal gene regulation during development and are increasingly being found to go awry during disease progression. Insights into the inactivation of an X-chromosome therefore promise to provide a broader understanding of normal and abnormal epigenetic regulation of gene expression. Despite the many advances in X-inactivation in the past two decades, however, some key questions remain to be answered. The coming years promise to provide a stream of exciting new findings in X-inactivation.

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Fig. 1.

The mosaic coat color of the calico cat exemplifies X-chromosome inactivation (Lyon, 1961). Calico cats are almost exclusively female and comprise of two colors of fur, black and orange, on an otherwise white background. The black and orange patches are due to mutually exclusive expression of two different alleles of the same X chromosome-linked gene. In fact, in the alternating patches, genes along only one of the two Xs are active while most genes on the other X-chromosome are inactive. The discreteness of the patches is due to clonal expansion of melanocytes whose precursors had inactivated one or the other X-chromosome during embryogenesis; the patches therefore reflect the epigenetic stability of X-inactivation.

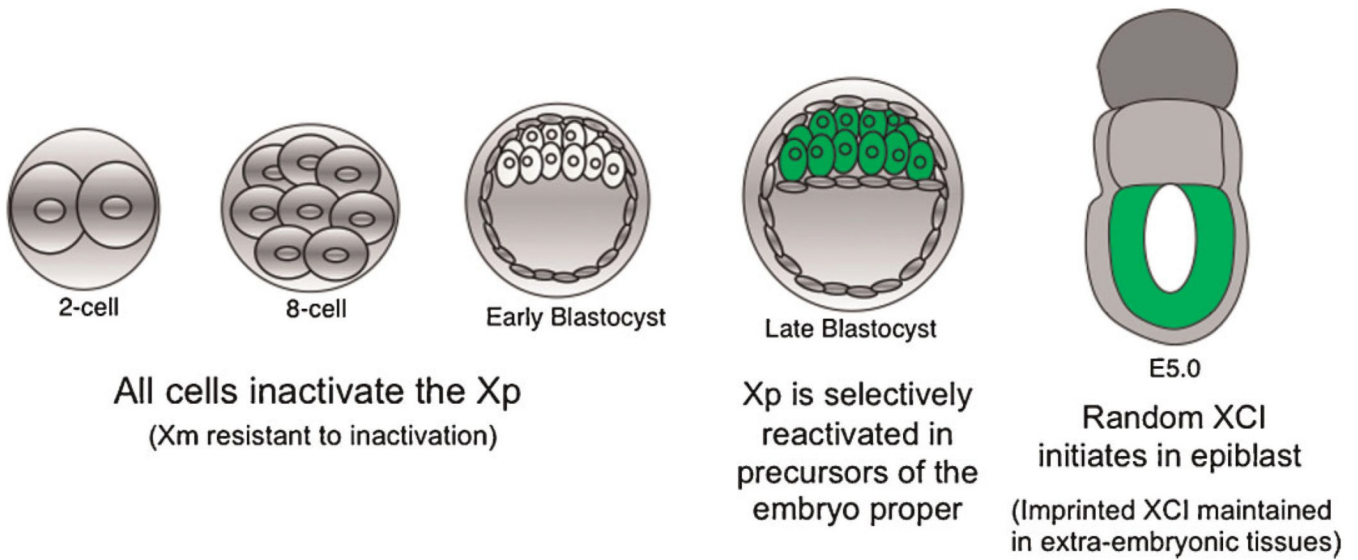


Fig. 2.

Two forms of X-inactivation, imprinted and random, occur in the mouse embryo. During early pre-implantation development of the embryo, all cells undergo imprinted inactivation of the paternal X-chromosome. During the late blastocyst stage, however, the cells of the inner cell mass (ICM; in green) reactivate the Xp. These cells contain the precursors of the embryo proper or the fetus. The cells in gray comprise either the trophoblast or the yolk-sac, which give rise to the placenta or the yolk-sac, respectively. After the embryo implants, the ICM descendants randomly inactivate either the maternal- or the paternal-X. The remaining cells, all extra-embryonic, maintain imprinted X-inactivation of the paternal X-chromosome.

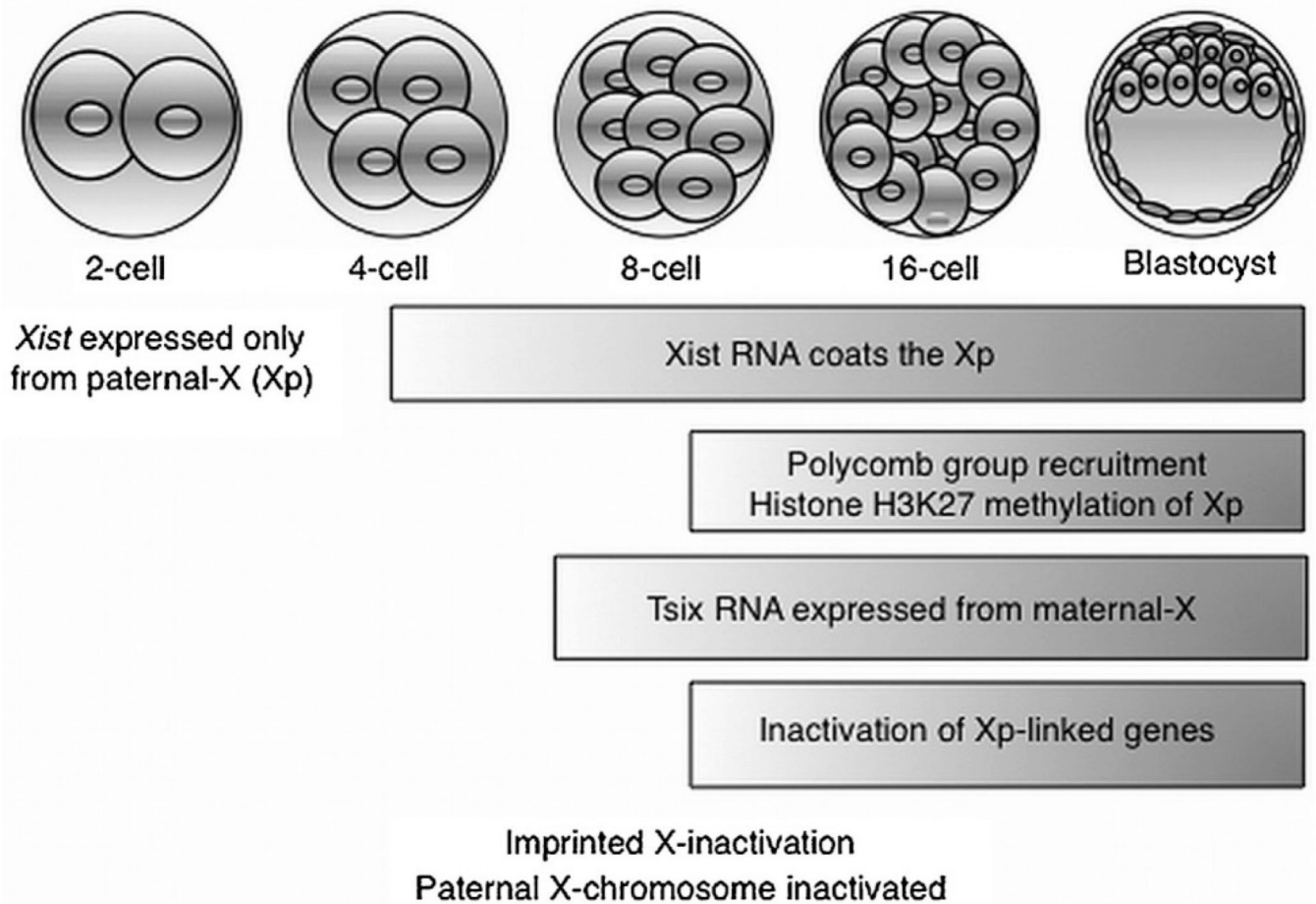


Fig. 3. Dynamics of X-chromosome inactivation in the early mouse embryo. All cells initially undergo imprinted X-inactivation resulting in silencing of genes exclusively along the paternal X-chromosome. A hallmark of X-inactivation is the expression of *Xist* RNA, which is only expressed from the paternal-X at the stages shown. Soon after *Xist* is transcribed, it starts to physically coat the paternal-X, followed closely by accumulation of proteins such as the Polycomb group and associated histone modifications that are posited to bring about silencing of paternal X-linked genes. The active maternal X-chromosome expresses the *Xist* anti-sense RNA *Tsix*, which is proposed to counter inactivation of the maternal-X by suppressing *Xist* expression.