Species-specific differences in X chromosome inactivation in mammals

Takashi Sado and Takehisa Sakaguchi

Division of Epigenomics and Development, Medical Institute of Bioregulation, Kyushu University, 3-1-1, Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

Correspondence should be addressed to T Sado; Email: tsado@bioreg.kyushu-u.ac.jp

Abstract

In female mammals, the dosage difference in X-linked genes between XX females and XY males is compensated for by inactivating one of the two X chromosomes during early development. Since the discovery of the X inactive-specific transcript (*XIST*) gene in humans and its subsequent isolation of the mouse homolog, *Xist*, in the early 1990s, the molecular basis of X chromosome inactivation (X-inactivation) has been more fully elucidated using genetically manipulated mouse embryos and embryonic stem cells. Studies on X-inactivation in other mammals, although limited when compared with those in the mice, have revealed that, while their inactive X chromosome shares many features with those in the mice, there are marked differences in not only some epigenetic modifications of the inactive X chromosome but also when and how X-inactivation is initiated during early embryonic development. Such differences raise the issue about what extent of the molecular basis of X-inactivation in the mice is commonly shared among others. Recognizing similarities and differences in X-inactivation among mammals may provide further insight into our understanding of not only the evolutionary but also the molecular aspects for the mechanism of X-inactivation. Here, we reviewed species-specific differences in X-inactivation and discussed what these differences may reveal.

Reproduction (2013) **146** R131–R139

Introduction

During the evolution of mammals, two morphologically different sex chromosomes have been generated from a pair of autosomes. While this induced benefits in the mechanisms of sex determination and reproduction, it simultaneously caused a serious problem in the gene dosage of sex chromosome-linked genes between the two sexes, XX females and XY males. Female mammals have evolved a mechanism to resolve this dosage imbalance by transcriptionally inactivating one of the two X chromosomes during early development. This chromosome-wide silencing event, which is referred to as X chromosome inactivation or X-inactivation, is one of the typical forms of epigenetic gene regulations observed in mammals. Among the three mammalian lineages, eutherians, which have a placenta during fetal development, and marsupials, which are born relatively premature and are raised in the pouch of females after birth, have employed X-inactivation as a means for dosage compensation even though they diverged about 130 million years ago (Lyon 1961, Richardson *et al.* 1971). It is of interest to note, however, that Japanese spiny rats have XO (monosomy of X) sex chromosome constitution with no cytogenetically visible Y chromosome in both sexes (Kobayashi *et al.* 2007). This switch to

XO in both sexes also seems to occur in the vole *Ellobius lutescens* (Just *et al.* 1995). This may suggest that they have employed an alternative strategy to reconcile the dosage imbalance between the sexes. The third mammalian lineage is represented by egg-laying monotremes, which are a basal branch of mammals that diverged from the eutherian–marsupial lineage about 166 million years ago. They have a complex sex chromosome system; for example, the platypus has ten sex chromosomes, females have five pairs of Xs and males have five Xs and five Ys (Grutzner *et al.* 2004). A previous study demonstrated that while some genes on the platypus X are not dosage compensated, other genes show some form of compensation via stochastic transcriptional repression (Deakin *et al.* 2008). Therefore, monotremes may not have a mechanism for dosage compensation that is comparable with that of eutherians and marsupials.

Since Lyon proposed this hypothesis in 1961 (Lyon 1961), X-inactivation has been drawing the attention of many researchers for more than half a century. This is at least partly due to this chromosome-wide silencing event being an excellent model to explore epigenetic gene silencing in mammals. The functional significance of many epigenetic modifications such as DNA methylation and posttranslational histone modifications has

been addressed regarding whether or not they are associated with an inactive X chromosome. In addition, the recent discovery of many long noncoding RNAs (lncRNAs) that have been implicated in gene regulation further highlights X-inactivation because X-linked lncRNA, X inactive-specific transcript (XIST), which coats the inactive X chromosome, has been shown to play a central role in chromosome silencing. Our current knowledge on X-inactivation during early embryogenesis mostly comes from studies in mice, which is partly due to the fact that it takes place at a very early phase of mammalian development when the embryo is accessible. In addition, mouse embryonic stem (ES) cells have been used as a major *ex vivo* alternative, which recapitulates cells of epiblast lineage that undergo random X-inactivation upon cellular differentiation. Many studies have described X-inactivation in other mammals such as humans and marsupials. The findings of these studies suggest that what is known about X-inactivation in the mice is not necessarily the case in others. Therefore, identifying similarities and differences in X-inactivation among different species of mammals is of importance. Here, we reviewed species-specific differences in X-inactivation and discussed what these differences reveal.

Imprinted X chromosome inactivation

It has long been known that there are two modes of X-inactivation in the mice. In the extraembryonic lineages, which give rise to the future placenta and some extraembryonic membranes, the paternal X (Xp) chromosome is preferentially inactivated (imprinted X-inactivation) (Takagi & Sasaki 1975), while either X chromosome is randomly inactivated regardless of the parental origin in the embryonic lineage contributing to all tissues of the fetus (random X-inactivation; Lyon 1961). Imprinted inactivation of Xp in the extraembryonic lineages is a feature common in rats (Wake *et al.* 1976), common voles (Shevchenko *et al.* 2009), and possibly in other rodents. Although the nature of imprinted X-inactivation is not fully understood, an imprint laid on the maternal X (Xm) during prophase in the first meiosis has been shown to make it resistant to inactivation (Shao & Takagi 1990, Goto & Takagi 2000, Tada *et al.* 2000). Mouse embryos carrying an extra Xm such as XmXmXp or XmXmY embryos never survive to term due to the detrimental effects caused by the presence of two active copies of Xm, which are imprinted not to undergo inactivation in the extraembryonic tissues. It is not clear whether Xp is oppositely imprinted to undergo inactivation. As both XmO (monosomy of X, which is maternal in origin) and XpO (monosomy of X, which is paternal in origin) embryos can survive to term and produce offspring, Xp may not be programmed to undergo inactivation. However, the development of XpO embryos was shown to be retarded

relative to normal embryos (Thornhill & Burgoyne 1993). This may indicate that a proportion of cells in the extraembryonic lineages of XpO embryos somehow overcome or ignore the imprint laid on Xp and form functional extraembryonic tissues to support the development of embryos. The imprint laid on Xp, if any, may not be as robust as the one laid on Xm.

Whether or not X-inactivation is imprinted in the extraembryonic lineages of humans remains controversial. Some studies demonstrated that X-inactivation was also imprinted in the human placenta (Harrison & Warburton 1986), whereas other studies reported that it was random (Moreira de Mello *et al.* 2010). It should be mentioned, however, that in contrast to mice, an extra X chromosome(s) can be maternal or paternal in origin in Klinefelter syndrome patients (47, XXY). Given that X-inactivation closely couples with cellular differentiation and that the presence of two active X chromosomes is incompatible with normal development in the mice (Monk & Harper 1979) and probably in other mammals, it would be reasonable to assume that one of the two Xms in the placenta of XmXmY individuals may undergo inactivation. This suggests that Xm in humans does not have an imprint comparable to the one laid on Xm in the mice.

While either Xp or Xm is inactivated essentially in a random fashion in the somatic cells of eutherian mammals, Xp undergoes inactivation in all tissues of marsupials (Richardson *et al.* 1971). This represents the first evidence of genomic imprinting in mammals. Although eutherians and marsupials share many features in X-inactivation, marked differences also exist between them, as has been discussed below.

X-linked lncRNAs that coat an X chromosome

An X chromosome undergoing inactivation establishes a unique epigenetic status through epigenetic changes including posttranslational modifications in histones, DNA methylation, higher order chromatin structures, and the recruitment of proteins involved in heterochromatinization. In mice, a series of such epigenetic changes were shown to be triggered by monoallelic upregulation of the X-linked noncoding *Xist* gene (Brockdorff *et al.* 1991, Brown *et al.* 1991) and the subsequent association of its transcripts with the X chromosome from which it originated (Clemson *et al.* 1998). Although the functional significance of *Xist* RNA in X-inactivation has been established based on the studies mainly in mice (Penny *et al.* 1996, Marahrens *et al.* 1997), given the presence of the *Xist* gene among eutherian mammals, it is likely that the function of *Xist* RNA may also be conserved. *Xist* (*XIST* in humans) is located in the chromosomal region referred to as the X-inactivation center (Xic and XIC in mice and humans respectively), which is believed to control the classically defined processes of X-inactivation consisting of

counting, choice, and initiation. *XIST* RNA is one of the most studied lncRNAs of 17–19 kb in length. Comparisons between mouse and human *Xist/XIST* sequences have identified some conserved repeats, termed A to F (Brockdorff *et al.* 1992, Brown *et al.* 1992), among which the A-repeat has been shown to be critical for the silencing function of the RNA by a transgenic approach in mouse ES cells (Wutz *et al.* 2002) and human cultured cells (Chow *et al.* 2007). It has been generally accepted that *Xist/XIST* RNA is required for the initiation of X-inactivation, while it is dispensable for maintenance (Brown & Willard 1994, Csankovszki *et al.* 1999).

An antisense transcript, *Tsix/TSIX*, has been identified at the *Xist/XIST* locus (Lee *et al.* 1999, Migeon *et al.* 2001). Extensive studies on *Tsix* in mouse embryos and ES cells with genetic manipulations have demonstrated that *Tsix* is a negative regulator of *Xist* and its expression prevents the upregulation of *Xist* on the same X chromosome by facilitating the formation of a repressive chromatin configuration at the *Xist* promoter (Lee & Lu 1999, Lee 2000, Sado *et al.* 2001, 2005, Navarro *et al.* 2005). Although it is not clear which of the RNA products or the action of antisense transcription is critical for its function, transcription across the *Xist* promoter appears to be crucial for *Tsix* to exert its negative effects on *Xist* (Ohhata *et al.* 2008). At the onset of X-inactivation, the downregulation of *Tsix* on one X allows the differential upregulation of *Xist* in *cis* and induces subsequent inactivation of the X, whereas continued expression of *Tsix* on the other X prevents the upregulation of *Xist* and keeps the X active. Once X-inactivation is established during development or cellular differentiation, *Tsix* on the active X becomes downregulated, suggesting that *Tsix* is no longer required when the asymmetrical expression pattern of *Xist* is fixed at the relatively early phase of the X-inactivation process. However, while the transcription of *Tsix* spans more than a 40 kb region covering the entire transcription unit of *Xist* in the mice, its human counterpart, *TSIX*, appears to be terminated within intron 4 of *XIST*; therefore, it is truncated before it reaches the *XIST* promoter (Migeon *et al.* 2001). In addition, *TSIX* in humans and cows remains expressed from the inactive X chromosome that expresses *XIST*, but not on the active X in fetal and placental tissues (Migeon *et al.* 2002, Farazmand *et al.* 2004). These observations may suggest that the function of *Tsix* is not conserved in humans and other animals. Although the available evidence strongly suggests that *Tsix* in the mice plays a critical role in the asymmetric regulation of *Xist* on the two X chromosomes in the embryonic, as well as on extraembryonic lineages of female embryos, given our previous observation that the deletion of *Tsix* did not necessarily result in the ectopic upregulation of *Xist* in the embryonic lineage of male embryos (Ohhata *et al.* 2006), the functional significance of *Tsix* is more prominent in the extraembryonic lineages where X-inactivation is imprinted. This

may be relevant to the apparent difference in *Tsix*'s function between mice and humans.

While the function of *Xist* may be well conserved among eutherians, its homolog does not seem to be present in marsupials. Based on the partial sequence homology in the syntenic region, the *Xist* gene was suggested to have evolved from a protein-coding gene, *PDZRN3* (*LN3*), which is found in more ancestral classes of animals than in marsupials such as birds, snakes, and amphibians (Duret *et al.* 2006). Although this may imply that X-inactivation in marsupials is regulated by a completely distinct mechanism from the *Xist*-dependent one seen in eutherians, the recent finding of *Rsx* RNA in the opossum has raised an interesting and exciting possibility that marsupials still employ a mechanism synonymous with *Xist* RNA-mediated silencing (Grant *et al.* 2012). *Rsx* RNA encoded on an X chromosome is another spliced lncRNA as long as 27 kb in length with no sequence similar to *Xist* RNA and is expressed only in females and coats the inactive X chromosome to form an *Xist*-like cloud. An *Rsx* transgene integrated on an autosome in mouse ES cells was shown to silence genes in *cis*, implying that *Rsx*, although evolved independently, is a functional counterpart of eutherian *Xist*. It would be of interest to see whether the homolog of *Rsx* is present in other marsupials.

Recently, another X-linked lncRNA, termed *XACT*, has been identified in humans, which appears to be transcribed from a more than 250 kb genomic region as a polyadenylated transcript (Vallot *et al.* 2013). Unlike *Xist/XIST* and *Rsx*, *XACT* RNA is unique in that it is expressed only from an active X chromosome and coats it in *cis* in both XX and XY human ES cells. Interestingly, *XACT* is not expressed in human fibroblasts, which suggests that it is specific to human pluripotent stem cells. The function of *XACT* has yet to be explored.

Epigenetic modifications in an inactive X chromosome

DNA methylation is one of the classical epigenetic modifications known to be involved in gene silencing and almost exclusively occurs at the cytosine residue of CpG dinucleotide sequences in mammals. While CpG islands, which are often found in the vicinity of the regulatory regions in the genome such as promoters and enhancers, are essentially unmethylated in general, those on the inactive X chromosome are known to be more highly methylated than their counterparts on the active X in both humans and mice. However, as far as genic regions are concerned, the methylation levels of CpG sites were shown to be higher on the active X than on the inactive X chromosome in humans (Hellman & Chess 2007), which is consistent with other studies demonstrating that the methylation status of CpG sites in the gene body correlated with transcriptional activity rather than

repression (Zhang *et al.* 2006, Ball *et al.* 2009, Aran *et al.* 2011). While the role of such a gene body methylation is largely unknown, the methylation of CpG islands on the X chromosome has been considered to be important for the maintenance, rather than initiation and propagation, of the inactivated state. Analyses of X-inactivation in mice deficient for DNA methyltransferases have demonstrated that there is a marked difference in the impact of a large reduction in global DNA methylation with X-inactivation between embryonic and extraembryonic tissues (Sado *et al.* 2000); unlike embryonic tissues, X-inactivation in the extraembryonic tissues seems to tolerate the global loss of DNA methylation and maintains the inactive state. This may correlate with lower levels of global DNA methylation in the extraembryonic lineages relative to the embryonic lineage (Chapman *et al.* 1984). Although the number of studies available to date is rather limited, CpG islands on the inactive X chromosome were not methylated in marsupials and this may explain why X-inactivation in marsupials is less complete and reactivation of the hitherto inactivated X spontaneously occurs in cultured cells (Kaslow & Migeon 1987, Hornecker *et al.* 2007).

The acetylation of particular lysine residues in the histone tail, especially histones H3 and H4, has been implicated in transcriptional activity. This has been markedly excluded from an inactive X chromosome in not only humans and mice (Jeppesen & Turner 1993) but also two marsupial species, the opossum and tammar wallaby (Chaumeil *et al.* 2011). In contrast, two repressive histone modifications, histone H3 trimethylated at lysine 27 (H3K27me3) and H3K9me2, were enriched on the inactive X chromosome in all these animals (Heard *et al.* 2001, Plath *et al.* 2003, Silva *et al.* 2003, Mahadevaiah *et al.* 2009, Chaumeil *et al.* 2011). These observations may suggest that histone modifications could represent a more ancestral regulation for X-inactivation than DNA methylation. However, some species-specific differences have been reported in the histone modifications associated with the inactive X chromosome (Table 1). H3K9me3 and H4K20me3, both of which characterize the constitutive

heterochromatin formed at the pericentromeric region and telomere in various species of animals, are also enriched on the inactive X chromosome in humans and marsupials but are apparently absent in mice (Chadwick & Willard 2004, Rens *et al.* 2010, Chaumeil *et al.* 2011, Zakharova *et al.* 2011). Consistent with this finding, heterochromatin protein 1 (HP1), known to colocalize to the constitutive heterochromatin with these modifications, also highlights the inactive X chromosome in humans and marsupials (Chadwick & Willard 2004, Rens *et al.* 2010). It appears that the inactive X chromosome in humans and marsupials shares many features in terms of histone modifications with the constitutive heterochromatin. Although this may imply that an inactive X chromosome in mice is uncommon among mammals, it is not necessarily the case as an inactive X chromosome in the elephant displays no enrichment of H3K9me3 and H4K20me3, similar to that in mice (Chaumeil *et al.* 2011). In addition, the enrichment of H4K20me1, another modification found on an inactive X chromosome in mice and not in marsupials, was also evident in the elephant (Chaumeil *et al.* 2011). It is not known whether it is enriched on a human inactive X chromosome.

The novel HP1-binding protein, HBiX1, which was recently identified in human cultured cells, is localized to the inactive X chromosome and interacts with SMCHD1 (Nozawa *et al.* 2013), whose mouse homolog has been shown to be essential for the maintenance of X-inactivation in the embryo (Blewitt *et al.* 2008). HBiX1 and SMCHD1 appear to be involved in the compaction of the inactive X chromosome by associating with H3K9me3 and H3K27me3 domains respectively in a manner independent of the polycomb repressive complex 2-mediated pathway, which has been considered to participate in the maintenance of facultative heterochromatin such as the inactive X chromosome. Given the essential role of Smchd1 in X-inactivation in the mouse, it is of interest to see whether the mouse homolog of HBiX1 localizes to the inactive X chromosome, which unlike that in humans is not apparently enriched with H3K9me3.

Table 1 Comparison of the epigenetic status of the inactive X chromosome among different species of mammals.

	Eutherians			Marsupials	
	Mouse	Human	Rabbit	Opossum	Tammar wallaby
Active marks					
H3ac	x	x	x	x	x
H4ac	x	x	ND	x	x
Repressive marks					
H3K9me2	o	o	o	o	o
H3K9me3	–	o	ND	o	o
H3K27me3	o	o	o	o	o
H4K20me1	o	ND	ND	–	–
H4K20me3	–	o	ND	o	o

o, enriched on Xi; x, excluded from Xi; –, no enrichment/exclusion; ND, not determined.

When and how does X-inactivation initiate?

X-inactivation starts with the accumulation of *Xist* RNA on Xp in each blastomere at around the 4- to 8-cell stages in mice (Okamoto *et al.* 2004). This paternal expression of *Xist* is maintained in not only the trophectoderm that emerges as the first differentiated tissue but also the primitive endoderm originating from a subset of cells in the inner cell mass (ICM) of the blastocyst, both of which belong to the extraembryonic lineages giving rise to those tissues that undergo imprinted X-inactivation. An *Xist* cloud coating Xp, on the other hand, disappears in the remaining undifferentiated cells in the ICM (Mak *et al.* 2004, Okamoto *et al.* 2004), which are thought to contribute to the epiblast lineage. Xp then becomes transiently reactivated, and either X subsequently becomes inactivated again at random as cells differentiate. However, the loss of the *Xist* cloud may not necessarily be a direct cause of reactivation in the epiblast lineage (Williams *et al.* 2011). In contrast to these situations in mice, a recent study of preimplantation embryos in humans and rabbits revealed that they use diverse strategies to initiate X-inactivation.

In rabbits, the accumulation of *Xist* RNA first occurs at the morula stage (60–70 cells) in a subset of cells and the proportion with *Xist* expression then increases toward the blastocyst stage (Okamoto *et al.* 2011). Interestingly, a significant proportion of cells in the blastocyst transiently exhibited two *Xist* clouds, which apparently silence an X-linked gene on the respective Xs. This does not seem to necessarily result in cell death and most of cells in the late blastocyst end up with only one *Xist* cloud. Given that the activity of both Xs can be initially affected, it is likely that there is no imprinting for X-inactivation in the rabbits.

In the human embryos, one study reported that while *XIST* RNA in females starts to accumulate at around the morula stage as a single cloud and causes transcriptional silencing of X-linked genes by the blastocyst stage, it does not accumulate to form a cloud in males (van den Berg *et al.* 2009). Another more recent study demonstrated that *XIST* RNA, when examined at the blastocyst stage, was detected as either a single or double cloud in a significant proportion of cells in males and females respectively within which X-linked genes were still transcribed (Okamoto *et al.* 2011). Therefore, although the situation appears to be controversial, the latter is consistent with earlier studies demonstrating that *XIST* expression in human embryos is detectable by RT-PCR in both sexes at the blastocyst stage (Daniels *et al.* 1997, Ray *et al.* 1997). Another study in the rhesus monkey demonstrated that, based on RT-PCR not RNA-FISH, *XIST* is expressed not only from both X chromosomes in females but also from a single X in males at the blastocyst stage (Tachibana *et al.* 2012). In addition, X-linked *G6PD* was apparently coexpressed with *XIST*, suggesting that *XIST* RNA expressed in the blastocyst had not yet induced

silencing in the rhesus monkey. If *XIST* RNA is expressed from any X chromosomes at the blastocyst stage in humans and rhesus monkeys, regardless of their gender, but its coating does not accompany silencing of the X-linked genes, the kinetics of X-inactivation following the accumulation of *XIST* RNA in primates may differ from that in mice and rabbits. It is currently unknown when *XIST* is eventually shut off on the X chromosome that stays active in humans and rhesus monkeys.

The difference in the timing of *Xist/XIST* upregulation between mice and others such as rabbits and humans may indicate that our current knowledge about X-inactivation, which basically comes from studies using mouse embryos, is not necessarily applicable to many other eutherian mammals. In contrast to the mouse, *Xist/XIST* expression and subsequent X-inactivation do not seem to be imprinted at the preimplantation stage in rabbits and humans. A difference was also observed in the timing of zygotic gene activation (ZGA) in these animals. While ZGA in the mouse becomes detectable at as early as the 1- to 2-cell stages (Bouniol *et al.* 1995), it becomes detectable at the 4- to 8-cell stages, a few rounds of cleavage behind, in rabbits (Manes 1973) and humans (Braude *et al.* 1988). This markedly early onset of ZGA in the mouse may make this animal peculiar in that *Xist* is expressed only from the Xp at the preimplantation stage (Fig. 1). It is tempting to speculate that *Xist* exhibits apparently imprinted expression at this stage because only the paternal but not the maternal allele is permissive to transcription following the initiation of ZGA in the mice. This is in fact consistent with the previous finding that in XX androgenetic embryos, *Xist* became upregulated to form the clouds on both X chromosomes derived from the father at the early cleavage stage (Okamoto *et al.* 2000). While the paternal genome undergoes global remodeling of the chromatin soon after fertilization, which could make the paternal *Xist* ready for transcription, the chromatin structure of the maternal allele may require further cell division to become permissive to transcription. This lag in the initiation of ZGA in rabbits and humans relative to mice should allow the embryo to undergo some additional rounds of cell division by ZGA. This may be enough to alter the chromatin structure of maternal *XIST* and make it equivalent to that of the paternal allele at the time when *XIST* is upregulated, resulting in the biallelic expression of *XIST* at the blastocyst stage. In other words, ZGA takes place so early in the mouse embryo that the inaccessible chromatin structure of the maternal *Xist* allele has not yet been remodeled enough by the time *Xist* is scheduled to be upregulated during development, and as a result, *Xist* expression is confined to the paternal allele in mice. In a sense, we might not need to assume a germline imprint that prevents expression of maternal *Xist* in the early cleavage stage embryo and the paternal *Xist* is simply expressed by default. The maternal deletion of

Figure 1 Respective timing of ZGA and *Xist* upregulation relative to the developmental stages of the preimplantation embryo was compared between mice (extraembryonic lineages) and humans.

Tsix, which results in ectopic activation of maternal *Xist* around the time of implantation (Lee 2000, Sado *et al.* 2001), implies that *Tsix* is required to prevent the upregulation of *Xist* on the Xm once the maternal *Xist* becomes permissive to transcription. It would be worth noting that in cloned mouse embryos generated by somatic cell nuclear transfer (SCNT), *Xist* often became upregulated on every X chromosome in both sexes beginning at the 4-cell stage (Inoue *et al.* 2010). Assuming that reprogramming both active and inactive X chromosomes undergo in ooplasm upon SCNT reverts their chromatin to the default state equivalent to the one of the Xp at the early preimplantation stage, it would not be surprising to see *Xist* upregulation on every X regardless of the previous activity in the donor cell in the absence of *Tsix*. X-inactivation was shown to be imprinted in preimplantation bovine embryos; however, as this was based on the allelic expression analysis of a few X-linked genes by RT-PCR (Xue *et al.* 2002) and ZGA in the cow and was shown to start at around the 8-cell stage in one study (Frei *et al.* 1989), but at the 2-cell stage in other study (Memili *et al.* 1998), it is of interest to examine the expression of *XIST* and other X-linked genes by RNA-FISH.

Concluding remarks

Since the discovery of *XIST* in humans and the subsequent isolation of the mouse homolog, *Xist*, the molecular mechanisms of X-inactivation have been more fully elucidated from studies using genetically manipulated mouse embryos and ES cells. However, marked differences have been observed in X-inactivation between mice and other mammals such as imprinted X-inactivation in the extraembryonic lineages. We have discussed the potential relationship between the selective upregulation of paternal *Xist* at the early preimplantation stage and the timing of ZGA. Given that implantation takes place on around embryonic day 4 in mice, while it does on around day 9 in humans (Wilcox *et al.* 1999) and on day 7 in rabbits (Lutwak-Mann 1962), it may be reasonable to assume that mice need earlier ZGA than humans and rabbits to prepare for implantation. The timing of implantation may be related to the length of gestation: it is about 20 days in mice, rats, and voles, all of which manifest imprinted inactivation of Xp in the extraembryonic lineages. As far as we are aware, it is not known whether X-inactivation in the extraembryonic lineages is also imprinted in other

rodents with a longer gestation period. It is also of interest to know when ZGA initiates in marsupials, which manifests Xp-specific inactivation in all tissues.

Although we have discussed the potential impact of ZGA on the differences of X-inactivation in the extraembryonic lineages between rodents and other mammals, it may be also interesting to note the potential role of RNF12 (RLIM) in the oocyte. A maternal store of RNF12 was shown to be important for the upregulation of paternal *Xist* during preimplantation development in mice (Shin *et al.* 2010). Although it is not known whether RNF12 exists in the oocyte of other mammals that do not have imprinted X-inactivation, if it does not, this could be an alternative speculation to the timing of ZGA. As our knowledge about X-inactivation in other mammals is rather limited, further studies using other eutherian mammals will provide further insights in terms of evolutionary and molecular aspects into our understanding of not only X-inactivation but also epigenetic regulation in general.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review reported.

Funding

This work was supported by the grant-in-aid for scientific research on innovative areas from the Ministry of Education, Science, Sports, and Culture of Japan to T Sado.

References

- Aran D, Toperoff G, Rosenberg M & Hellman A 2011 Replication timing-related and gene body-specific methylation of active human genes. *Human Molecular Genetics* **20** 670–680. (doi:10.1093/hmg/ddq513)
- Ball MP, Li JB, Gao Y, Lee JH, LeProust EM, Park IH, Xie B, Daley GQ & Church GM 2009 Targeted and genome-scale strategies reveal gene-body methylation signatures in human cells. *Nature Biotechnology* **27** 361–368. (doi:10.1038/nbt.1533)
- van den Berg IM, Laven JS, Stevens M, Jonkers I, Galjaard RJ, Gribnau J & van Doorninck JH 2009 X chromosome inactivation is initiated in human preimplantation embryos. *American Journal of Human Genetics* **84** 771–779. (doi:10.1016/j.ajhg.2009.05.003)
- Blewitt ME, Gendrel AV, Pang Z, Sparrow DB, Whitelaw N, Craig JM, Apedaile A, Hilton DJ, Dunwoodie SL, Brockdorff N *et al.* 2008 SmcHD1, containing a structural-maintenance-of-chromosomes hinge domain, has a critical role in X inactivation. *Nature Genetics* **40** 663–669. (doi:10.1038/ng.142)
- Bouniol C, Nguyen E & Debey P 1995 Endogenous transcription occurs at the 1-cell stage in the mouse embryo. *Experimental Cell Research* **218** 57–62. (doi:10.1006/excr.1995.1130)
- Braude P, Bolton V & Moore S 1988 Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature* **332** 459–461. (doi:10.1038/332459a0)
- Brockdorff N, Ashworth A, Kay GF, Cooper P, Smith S, McCabe VM, Norris DP, Penny GD, Patel D & Rastan S 1991 Conservation of position and exclusive expression of mouse *Xist* from the inactive X chromosome. *Nature* **351** 329–331. (doi:10.1038/351329a0)
- Brockdorff N, Ashworth A, Kay GF, McCabe VM, Norris DP, Cooper PJ, Swift S & Rastan S 1992 The product of the mouse *Xist* gene is a 15 kb inactive X-specific transcript containing no conserved ORF and located in the nucleus. *Cell* **71** 515–526. (doi:10.1016/0092-8674(92)90519-I)
- Brown CJ & Willard HF 1994 The human X-inactivation centre is not required for maintenance of X-chromosome inactivation. *Nature* **368** 154–156. (doi:10.1038/368154a0)
- Brown CJ, Ballabio A, Rupert JL, Lafreniere RG, Grompe M, Tonlorenzi R & Willard HF 1991 A gene from the region of the human X inactivation centre is expressed exclusively from the inactive X chromosome. *Nature* **349** 38–44. (doi:10.1038/349038a0)
- Brown CJ, Hendrich BD, Rupert JL, Lafreniere RG, Xing Y, Lawrence J & Willard HF 1992 The human *XIST* gene: analysis of a 17 kb inactive X-specific RNA that contains conserved repeats and is highly localized within the nucleus. *Cell* **71** 527–542. (doi:10.1016/0092-8674(92)90520-M)
- Chadwick BP & Willard HF 2004 Multiple spatially distinct types of facultative heterochromatin on the human inactive X chromosome. *PNAS* **101** 17450–17455. (doi:10.1073/pnas.0408021101)
- Chapman V, Forrester L, Sanford J, Hastie N & Rossant J 1984 Cell lineage-specific undermethylation of mouse repetitive DNA. *Nature* **307** 284–286. (doi:10.1038/307284a0)
- Chaumeil J, Waters PD, Koina E, Gilbert C, Robinson TJ & Graves JA 2011 Evolution from *XIST*-independent to *XIST*-controlled X-chromosome inactivation: epigenetic modifications in distantly related mammals. *PLoS ONE* **6** e19040. (doi:10.1371/journal.pone.0019040)
- Chow JC, Hall LL, Baldry SE, Thorogood NP, Lawrence JB & Brown CJ 2007 Inducible *XIST*-dependent X-chromosome inactivation in human somatic cells is reversible. *PNAS* **104** 10104–10109. (doi:10.1073/pnas.0610946104)
- Clemson CM, Chow JC, Brown CJ & Lawrence JB 1998 Stabilization and localization of *Xist* RNA are controlled by separate mechanisms and are not sufficient for X inactivation. *Journal of Cell Biology* **142** 13–23. (doi:10.1083/jcb.142.1.13)
- Csankovszki G, Panning B, Bates B, Pehrson JR & Jaenisch R 1999 Conditional deletion of *Xist* disrupts histone macroH2A localization but not maintenance of X inactivation. *Nature Genetics* **22** 323–324. (doi:10.1038/11887)
- Daniels R, Zuccotti M, Kinis T, Serhal P & Monk M 1997 *XIST* expression in human oocytes and preimplantation embryos. *American Journal of Human Genetics* **61** 33–39. (doi:10.1086/513892)
- Deakin JE, Hore TA, Koina E & Marshall Graves JA 2008 The status of dosage compensation in the multiple X chromosomes of the platypus. *PLoS Genetics* **4** e1000140. (doi:10.1371/journal.pgen.1000140)
- Duret L, Chureau C, Samain S, Weissenbach J & Avner P 2006 The *Xist* RNA gene evolved in eutherians by pseudogenization of a protein-coding gene. *Science* **312** 1653–1655. (doi:10.1126/science.1126316)
- Farazmand A, Basurur PK, Stranzinger G, Graphodatskaya D, Reyes ER & King WA 2004 Expression of *XIST* sense and antisense in bovine fetal organs and cell cultures. *Chromosome Research* **12** 275–283. (doi:10.1023/B:CHRO.0000021864.66235.81)
- Frei RE, Schultz GA & Church RB 1989 Qualitative and quantitative changes in protein synthesis occur at the 8–16-cell stage of embryogenesis in the cow. *Journal of Reproduction and Fertility* **86** 637–641. (doi:10.1530/jrf.0.0860637)
- Goto Y & Takagi N 2000 Maternally inherited X chromosome is not inactivated in mouse blastocysts due to parental imprinting. *Chromosome Research* **8** 101–109. (doi:10.1023/A:1009234217981)
- Grant J, Mahadevaiah SK, Khil P, Sangrithi MN, Royo H, Duckworth J, McCarrey JR, VandeBerg JL, Renfree MB, Taylor W *et al.* 2012 *Rx* is a metatherian RNA with *Xist*-like properties in X-chromosome inactivation. *Nature* **487** 254–258. (doi:10.1038/nature11171)
- Grutzner F, Rens W, Tsend-Ayush E, El-Mogharbel N, O'Brien PC, Jones RC, Ferguson-Smith MA & Marshall Graves JA 2004 In the platypus a meiotic chain of ten sex chromosomes shares genes with the bird Z and mammal X chromosomes. *Nature* **432** 913–917. (doi:10.1038/nature03021)
- Harrison KB & Warburton D 1986 Preferential X-chromosome activity in human female placental tissues. *Cytogenetics and Cell Genetics* **41** 163–168. (doi:10.1159/000132221)

- Heard E, Rougeulle C, Arnaud D, Avner P, Allis CD & Spector DL 2001 Methylation of histone H3 at Lys-9 is an early mark on the X chromosome during X inactivation. *Cell* **107** 727–738. (doi:10.1016/S0092-8674(01)00598-0)
- Hellman A & Chess A 2007 Gene body-specific methylation on the active X chromosome. *Science* **315** 1141–1143. (doi:10.1126/science.1136352)
- Hornecker JL, Samollow PB, Robinson ES, VandeBerg JL & McCarrey JR 2007 Meiotic sex chromosome inactivation in the marsupial *Monodelphis domestica*. *Genesis* **45** 696–708. (doi:10.1002/dvg.20345)
- Inoue K, Kohda T, Sugimoto M, Sado T, Ogonuki N, Matoba S, Shiura H, Ikeda R, Mochida K, Fujii T *et al.* 2010 Impeding Xist expression from the active X chromosome improves mouse somatic cell nuclear transfer. *Science* **330** 496–499. (doi:10.1126/science.1194174)
- Jeppesen P & Turner BM 1993 The inactive X chromosome in female mammals is distinguished by a lack of histone H4 acetylation, a cytogenetic marker for gene expression. *Cell* **74** 281–289. (doi:10.1016/0092-8674(93)90419-Q)
- Just W, Rau W, Vogel W, Akhverdian M, Fredga K, Graves JA & Lyapunova E 1995 Absence of Sry in species of the vole *Ellobius*. *Nature Genetics* **11** 117–118. (doi:10.1038/ng1095-117)
- Kaslow DC & Migeon BR 1987 DNA methylation stabilizes X chromosome inactivation in eutherians but not in marsupials: evidence for multistep maintenance of mammalian X dosage compensation. *PNAS* **84** 6210–6214. (doi:10.1073/pnas.84.17.6210)
- Kobayashi T, Yamada F, Hashimoto T, Abe S, Matsuda Y & Kuroiwa A 2007 Exceptional minute sex-specific region in the X0 mammal, Ryukyu spiny rat. *Chromosome Research* **15** 175–187. (doi:10.1007/s10577-006-1093-y)
- Lee JT 2000 Disruption of imprinted X inactivation by parent-of-origin effects at Tsix. *Cell* **103** 17–27. (doi:10.1016/S0092-8674(00)00101-X)
- Lee JT & Lu N 1999 Targeted mutagenesis of Tsix leads to nonrandom X inactivation. *Cell* **99** 47–57. (doi:10.1016/S0092-8674(00)80061-6)
- Lee JT, Davidow LS & Warshawsky D 1999 Tsix, a gene antisense to Xist at the X-inactivation centre. *Nature Genetics* **21** 400–404. (doi:10.1038/7734)
- Lutwak-Mann C 1962 Glucose, lactic acid and bicarbonate in rabbit blastocyst fluid. *Nature* **193** 653–654. (doi:10.1038/193653a0)
- Lyon M 1961 Gene action in the X-chromosome of the mouse (*Mus musculus* L.). *Nature* **190** 372–373. (doi:10.1038/190372a0)
- Mahadevaiah SK, Royo H, VandeBerg JL, McCarrey JR, Mackay S & Turner JM 2009 Key features of the X inactivation process are conserved between marsupials and eutherians. *Current Biology* **19** 1478–1484. (doi:10.1016/j.cub.2009.07.041)
- Mak W, Nesterova TB, de Napoles M, Appanah R, Yamanaka S, Otte AP & Brockdorff N 2004 Reactivation of the paternal X chromosome in early mouse embryos. *Science* **303** 666–669. (doi:10.1126/science.1092674)
- Manes C 1973 The participation of the embryonic genome during early cleavage in the rabbit. *Developmental Biology* **32** 453–459. (doi:10.1016/0012-1606(73)90254-6)
- Marahrens Y, Panning B, Dausman J, Strauss W & Jaenisch R 1997 Xist-deficient mice are defective in dosage compensation but not spermatogenesis. *Genes and Development* **11** 156–166. (doi:10.1101/gad.11.2.156)
- Memili E, Dominko T & First NL 1998 Onset of transcription in bovine oocytes and preimplantation embryos. *Molecular Reproduction and Development* **51** 36–41. (doi:10.1002/(SICI)1098-2795(199809)51:1<36::AID-MRD4>3.0.CO;2-X)
- Migeon BR, Chowdhury AK, Dunston JA & McIntosh I 2001 Identification of TSIX, encoding an RNA antisense to human XIST, reveals differences from its murine counterpart: implications for X inactivation. *American Journal of Human Genetics* **69** 951–960. (doi:10.1086/324022)
- Migeon BR, Lee CH, Chowdhury AK & Carpenter H 2002 Species differences in TSIX/Tsix reveal the roles of these genes in X-chromosome inactivation. *American Journal of Human Genetics* **71** 286–293. (doi:10.1086/341605)
- Monk M & Harper MI 1979 Sequential X chromosome inactivation coupled with cellular differentiation in early mouse embryos. *Nature* **281** 311–313. (doi:10.1038/281311a0)
- Moreira de Mello JC, de Araujo ES, Stabellini R, Fraga AM, de Souza JE, Sumita DR, Camargo AA & Pereira LV 2010 Random X inactivation and extensive mosaicism in human placenta revealed by analysis of allele-specific gene expression along the X chromosome. *PLoS ONE* **5** e10947. (doi:10.1371/journal.pone.0010947)
- Navarro P, Pichard S, Claudio C, Avner P & Rougeulle C 2005 Tsix transcription across the Xist gene alters chromatin conformation without affecting Xist transcription: implications for X-chromosome inactivation. *Genes and Development* **19** 1474–1484. (doi:10.1101/gad.341105)
- Nozawa RS, Nagao K, Igami KT, Shibata S, Shirai N, Nozaki N, Sado T, Kimura H & Obuse C 2013 Human inactive X chromosome is compacted through a PRC2-independent SMCHD1–H3K9me3 pathway. *Nature Structural & Molecular Biology* **20** 566–573. (doi:10.1038/nsmb.2532)
- Ohhata T, Hoki Y, Sasaki H & Sado T 2006 Tsix-deficient X chromosome does not undergo inactivation in the embryonic lineage in males: implications for Tsix-independent silencing of Xist. *Cytogenetic and Genome Research* **113** 345–349. (doi:10.1159/000090851)
- Ohhata T, Hoki Y, Sasaki H & Sado T 2008 Crucial role of antisense transcription across the Xist promoter in Tsix-mediated Xist chromatin modification. *Development* **135** 227–235. (doi:10.1242/dev.008490)
- Okamoto I, Tan S & Takagi N 2000 X-chromosome inactivation in XX androgenetic mouse embryos surviving implantation. *Development* **127** 4137–4145.
- Okamoto I, Otte AP, Allis CD, Reinberg D & Heard E 2004 Epigenetic dynamics of imprinted X inactivation during early mouse development. *Science* **303** 644–649. (doi:10.1126/science.1092727)
- Okamoto I, Patrat C, Thepot D, Peynot N, Fauque P, Daniel N, Diabanguaya P, Wolf JP, Renard JP, Duranthon V *et al.* 2011 Eutherian mammals use diverse strategies to initiate X-chromosome inactivation during development. *Nature* **472** 370–374. (doi:10.1038/nature09872)
- Penny GD, Kay GF, Sheardown SA, Rastan S & Brockdorff N 1996 Requirement for Xist in X chromosome inactivation. *Nature* **379** 131–137. (doi:10.1038/379131a0)
- Plath K, Fang J, Mlynarczyk-Evans SK, Cao R, Worringer KA, Wang H, de la Cruz CC, Otte AP, Panning B & Zhang Y 2003 Role of histone H3 lysine 27 methylation in X inactivation. *Science* **300** 131–135. (doi:10.1126/science.1084274)
- Ray PF, Winston RM & Handyside AH 1997 XIST expression from the maternal X chromosome in human male preimplantation embryos at the blastocyst stage. *Human Molecular Genetics* **6** 1323–1327. (doi:10.1093/hmg/6.8.1323)
- Rens W, Wallduck MS, Lovell FL, Ferguson-Smith MA & Ferguson-Smith AC 2010 Epigenetic modifications on X chromosomes in marsupial and monotreme mammals and implications for evolution of dosage compensation. *PNAS* **107** 17657–17662. (doi:10.1073/pnas.0910322107)
- Richardson BJ, Czuppon AB & Sharman GB 1971 Inheritance of glucose-6-phosphate dehydrogenase variation in kangaroos. *Nature: New Biology* **230** 154–155.
- Sado T, Fenner MH, Tan SS, Tam P, Shioda T & Li E 2000 X inactivation in the mouse embryo deficient for Dnmt1: distinct effect of hypomethylation on imprinted and random X inactivation. *Developmental Biology* **225** 294–303. (doi:10.1006/dbio.2000.9823)
- Sado T, Wang Z, Sasaki H & Li E 2001 Regulation of imprinted X-chromosome inactivation in mice by Tsix. *Development* **128** 1275–1286.
- Sado T, Hoki Y & Sasaki H 2005 Tsix silences Xist through modification of chromatin structure. *Developmental Cell* **9** 159–165. (doi:10.1016/j.devcel.2005.05.015)
- Shao C & Takagi N 1990 An extra maternally derived X chromosome is deleterious to early mouse development. *Development* **110** 969–975.
- Shevchenko AI, Pavlova SV, Dementyeva EV & Zakian SM 2009 Mosaic heterochromatin of the inactive X chromosome in vole *Microtus rossiaemeridionalis*. *Mammalian Genome* **20** 644–653. (doi:10.1007/s00335-009-9201-x)
- Shin J, Bossenz M, Chung Y, Ma H, Byron M, Taniguchi-Ishigaki N, Zhu X, Jiao B, Hall LL, Green MR *et al.* 2010 Maternal Rnf12/RLIM is required for imprinted X-chromosome inactivation in mice. *Nature* **467** 977–981. (doi:10.1038/nature09457)
- Silva J, Mak W, Zvetkova I, Appanah R, Nesterova TB, Webster Z, Peters AH, Jenuwein T, Otte AP & Brockdorff N 2003 Establishment of histone H3 methylation on the inactive X chromosome requires transient recruitment of Eed–Enx1 polycomb group complexes. *Developmental Cell* **4** 481–495. (doi:10.1016/S1534-5807(03)00068-6)
- Tachibana M, Ma H, Sparman ML, Lee HS, Ramsey CM, Woodward JS, Sritanaudomchai H, Masterson KR, Wolff EE, Jia Y *et al.* 2012 X-chromosome inactivation in monkey embryos and pluripotent stem cells. *Developmental Biology* **371** 146–155. (doi:10.1016/j.ydbio.2012.08.009)

- Tada T, Obata Y, Tada M, Goto Y, Nakatsuji N, Tan S, Kono T & Takagi N** 2000 Imprint switching for non-random X-chromosome inactivation during mouse oocyte growth. *Development* **127** 3101–3105.
- Takagi N & Sasaki M** 1975 Preferential inactivation of the paternally derived X chromosome in the extraembryonic membranes of the mouse. *Nature* **256** 640–642. (doi:10.1038/256640a0)
- Thornhill AR & Burgoyne PS** 1993 A paternally imprinted X chromosome retards the development of the early mouse embryo. *Development* **118** 171–174.
- Vallot C, Huret C, Lesecque Y, Resch A, Oudrhiri N, Bennaceur-Griscelli A, Duret L & Rougeulle C** 2013 XACT, a long noncoding transcript coating the active X chromosome in human pluripotent cells. *Nature Genetics* **45** 239–241. (doi:10.1038/ng.2530)
- Wake N, Takagi N & Sasaki M** 1976 Non-random inactivation of X chromosome in the rat yolk sac. *Nature* **262** 580–581. (doi:10.1038/262580a0)
- Wilcox AJ, Baird DD & Weinberg CR** 1999 Time of implantation of the conceptus and loss of pregnancy. *New England Journal of Medicine* **340** 1796–1799. (doi:10.1056/NEJM199906103402304)
- Williams LH, Kalantry S, Starmer J & Magnuson T** 2011 Transcription precedes loss of Xist coating and depletion of H3K27me3 during X-chromosome reprogramming in the mouse inner cell mass. *Development* **138** 2049–2057. (doi:10.1242/dev.061176)
- Wutz A, Rasmussen TP & Jaenisch R** 2002 Chromosomal silencing and localization are mediated by different domains of Xist RNA. *Nature Genetics* **30** 167–174. (doi:10.1038/ng820)
- Xue F, Tian XC, Du F, Kubota C, Taneja M, Dinnyes A, Dai Y, Levine H, Pereira LV & Yang X** 2002 Aberrant patterns of X chromosome inactivation in bovine clones. *Nature Genetics* **31** 216–220. (doi:10.1038/ng900)
- Zakharova IS, Shevchenko AI, Shilov AG, Nesterova TB, VandeBerg JL & Zakian SM** 2011 Histone H3 trimethylation at lysine 9 marks the inactive metaphase X chromosome in the marsupial *Monodelphis domestica*. *Chromosoma* **120** 177–183. (doi:10.1007/s00412-010-0300-y)
- Zhang X, Yazaki J, Sundaresan A, Cokus S, Chan SW, Chen H, Henderson IR, Shinn P, Pellegrini M, Jacobsen SE et al.** 2006 Genome-wide high-resolution mapping and functional analysis of DNA methylation in arabidopsis. *Cell* **126** 1189–1201. (doi:10.1016/j.cell.2006.08.003)

Received 23 April 2013

First decision 23 May 2013

Revised manuscript received 3 July 2013

Accepted 11 July 2013