Xist/Tsix expression dynamics during mouse peri-implantation development revealed by whole-mount 3D RNA-FISH

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During peri-implantation development in mice, X chromosome inactivation (XCI) status changes dynamically. Here, we examined the expression of Xist and its antisense partner, Tsix, via whole-mount 3D RNA-FISH using strand-specific probes and evaluated XCI status. The results indicate that Xist expression disappears completely by embryonic day (E) 4.5 without Tsix activation in the ICM and that Xist re-expression occurs at E4.75 in some cells, suggesting that random XCI is already initiated in these cells. Intriguingly, epiblast cells exhibiting biallelic Xist expression were observed frequently (~15%) at E5.25 and E5.5. Immunostaining analysis of epigenetic modifications suggests that global change in epigenomic status occurs concomitantly with the transition from imprinted to random XCI. However, global upregulation of H3K27me3 modifications initiated earlier than other modifications, occurring specifically in ICM during progression of Xist erasure. Although both Xist expression and imprinted XCI are thought to be stable in the primitive endoderm/visceral endoderm and trophectoderm/extraembryonic ectoderm lineages, transient loss of Xist clouds was noted only in a subset of extraembryonic ectodermal cells, suggesting distinct features of Xist regulation among the three different embryonic tissue layers. These results will serve as a basis for future functional studies of XCI regulation in vivo.

In female mammals, one of the two X chromosomes is inactivated for gene dosage compensation between XX females and XY males1. This phenomenon, termed X chromosome inactivation (XCI), is regulated by several factors, such as the noncoding RNA Xist and its antisense sequence Tsix. Xist is exclusively expressed from the inactive X (Xi) and accumulates on it, leading to a chromosome-wide inactivation of gene expression2–5. Tsix, with its repressive effect on Xist expression, is expressed normally from the active X and is silenced on Xi 6,7. Imprinted XCI occurs in preimplantation-stage embryos and Xist is essential for its initiation8. During this process, the paternal X (Xp) is preferentially selected as Xi according to a maternal imprint causing Xist repression on maternal allele8–11. The maternal imprint is now thought to be H3K27me3 modifications laid onto maternal X during oogenesis12. The imprinted XCI is then erased in the embryonic lineage, and XCI is resumed later as random XCI, in which Xi is chosen randomly. The erasure of imprinted XCI initiates in the inner cell mass (ICM) of early blastocysts. This is accompanied by the loss of Xist RNA accumulation, EED/EZH2 association and histone H3 lysine 27 trimethylation (H3K27me3) modifications from the Xp, and derepression of genes that are subjected to inactivation on the Xp13–16. During this erasure process, epigenetic memories for imprinted XCI are thought to be erased and both X chromosomes become epigenetically equivalent. Random XCI takes place after this imprinted XCI erasure. Although these events sequences have been described17, the precise timing of XCI erasure and initiation of random XCI during the development of peri-implantation embryos in vivo is not understood fully.

The reasons for studying the precise kinetics of XCI during embryonic development are at least twofold. First, basic information on the dynamics of XCI will provide clues that will contribute to understanding the regulatory

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mechanisms that operate in vivo. Traditionally, the in vitro differentiation system of embryonic stem (ES) cells has been used extensively in studying XCI. Despite its great experimental advantages, the in vitro ES cell system cannot cover all aspects of the XCI dynamics that occur in vivo. For example, imprinted XCI does not exist in the ES cell system. To reevaluate the usefulness of the ES cell system and to understand the XCI phenomenon in general, studies of embryos developing in vivo are essential, as they add complementary knowledge to the data accumulated from in vitro studies. Second, changes in XCI status are likely to be coupled with epigenomic or nuclear reorganization in developing peri-implantation mouse embryos. ICM and mouse ES cells (mESCs) represent a ground state (naïve state) of pluri potency, whereas epiblasts of post-implantation-stage embryos or epiblast stem cells (EpiSCs) correspond to a “primed” pluripotent state. XCI is one of the key features of EpiSCs. In contrast with female mESCs, where the two X chromosomes are both active, a random XCI operates in female EpiSCs. It is becoming increasingly clear that there is a significant difference in epigenetic status or an “epigenetic barrier” between the naïve and primed states of pluripotent stem cells, and that the imprinted XCI–random XCI conversion that takes place in peri-implantation mammalian embryos might be a reflection of epigenomic reorganizations that are not restricted to X chromosomes. Therefore, we believe that the XCI status could be a useful indicator of large-scale epigenomic reprogramming events that have remained unexplored to date.

Xist RNA clouds or coatings (i.e., the accumulation of Xist RNA over the entire Xi) are one of the indicators of whether cells are in XCI state22–24, and the accumulation of Xist RNA is lost during imprinted XCI erasure13,14,16. As Xist RNA is essential for the establishment of XCI, re-expression of Xist is thought to be the sign of random XCI commencement. However, the repression of Xist itself might not represent an active state of the X chromosome, because it is known that the expression/repression status of Xist does not necessarily coincide with the expression status of other X-linked genes. For example, it has been reported that Xist expression is dispensable for X inactivation in mouse embryonic fibroblasts (MEFs)25 or in developing primordial germ cells (PGCs)26. Moreover, it has been demonstrated that several X-linked genes located on the imprinted Xi are reactivated even in the presence of Xist coatings15,16. Therefore, it is necessary to examine both Xist repression and activation of X-linked gene(s) to judge whether XCI reversal occurs or not. Among the numerous X-linked genes, we have paid particular attention to Tsix, an antisense RNA partner of Xist that plays a repressive role in Xist expression27,28. It is known that continued expression of an inducible Tsix allele causes stable repression of Xist in imprinted XCI during preimplantation development28. However, whether Tsix has an active role in induction of Xist repression during imprinted XCI erasure in vivo is not fully understood. Furthermore, in female ES cells, Tsix is expressed at high levels from both alleles, and it is proposed that this biallelic Tsix expression induces changes in histone modifications along the Xist locus that ensure the epigenetic equivalency of both X chromosomes in undifferentiated pluripotent cells29, implying that the cells that have undergone X reactivation will show biallelic expression of Tsix both in vitro and in vivo. Therefore, we decided to track the kinetics of both Xist and Tsix expressions to know whether Tsix reactivation trigger the initiation of Xist repression and make a careful estimate of XCI status during XCI reversal and random XCI establishment. In addition, as Tsix reactivation would not necessarily reflect the overall state of the X chromosome, expression statuses of other X-linked genes (Lamp2 and Pgk1) were also examined to confirm progression of X reactivation at the developmental stage when biallelic expression of Xist became predominant. To perform a quantitative expression analysis in peri-implantation-stage embryos, we used whole-mount RNA fluorescence in situ hybridization (FISH), which has been successfully utilized for studying X reactivation in PGCs27. RNA-FISH is better suited to assessing the on- and off-state of transcription because it can detect nascent RNA and not the steady-state level of RNA. Furthermore, the whole-mount technique, which does not destroy embryonic structures, enables the unambiguous detection of RNA expression in different embryonic lineages, such as the trophectoderm/extraembryonic ectoderm (TE/ExE), primitive endoderm/visceral endoderm (PE/VE), and ICM/epiblast lineages.

The results presented here demonstrate Xist/Tsix dynamics during peri-implantation development at an unprecedented resolution, implying the period of imprinted XCI erasure and the timing of random XCI commencement in the ICM/epiblast lineage in vivo. Moreover, we found differences in Xist/Tsix dynamics and changes in epigenetic status among the three different lineages of embryos. The information regarding Xist/Tsix dynamics in vivo obtained in this study will serve as a basis for future functional studies on XCI regulation.

Results

Kinetic changes in Xist/Tsix status during development of the embryonic lineage. Xist and Tsix expression in ICM/epiblast, PE/VE and TE/ExE lineages were examined by using strand-specific RNA-FISH (see Methods for details of the strand-specific probe preparation) combined with immunofluorescence against those cell lineage markers during a period between E3.5 and E5.5 at intervals of 6 h (Figs 1, 2, Table 1 and Supplementary Fig. S1a). Embryonic lineage cells were marked by an antibody against the pluripotent cell marker POU5F1 (OCT3/4) for E3.5 blastocysts and for embryos sampled between E4.75 and E5.5. As POU5F1 expression was observed in both the primitive endoderm and the epiblast at stages from E3.75 to E4.5, anti-NANOG was used instead to stain embryonic lineage cells (Supplementary Fig. S1a). Images were acquired on a confocal microscope, and z-stack images were used to analyze Xist/Tsix expression patterns in each cell of the whole embryo (Fig. 1b–i; see Supplementary Video S1). We used this three-dimensional (3D) whole-mount RNA-FISH technique27 to determine the kinetic changes in Xist/Tsix expression in almost all the cells that constituted entire embryos at E3.5–5.5, and the analysis was also performed at E6.5 (Table 1, 4,127 nuclei in 107 embryos in total). Xist-positive cells were classified into four categories: cells with one Xist cloud per nucleus, cells with single pinpoint or dispersed signals, cells with two Xist signals, or cells with no Xist signal. Tsix-positive cells were categorized into cells with two, one, or no signals. To understand the relationships between the expression patterns of Xist and Tsix in the same individual cells, a matrix illustrating proportional data (%) for each category of Xist- or Tsix-positive cells was constructed (Fig. 1a).
In E3.5 blastocysts, more than 80% of ICM cells possessed one Xist cloud and one pinpoint Tsix signal per nucleus (Fig. 1b), indicating that most ICM cells were still subjected to imprinted XCI at this stage. From E3.75 onward, Xist cloud signals began to disappear and cells showing biallelic expression of Tsix began to appear (Fig. 1c,d).

In E4.5, all epiblast cells were Xist negative. A large proportion of the cells (58%) showed a single Tsix signal, whereas 11% and 31% of cells exhibited no or two Tsix signals, respectively. This indicates that the erasure of Xist precedes the derepression of Tsix from the silent allele and that Tsix activation might not be a prerequisite for the disappearance of Xist. The numbers of cells showing Tsix biallelic expression increased after E4.5,
whereas the numbers of Tsix single-positive cells decreased. Most cells (>53%) exhibited biallelic expression of Tsix and no expression of Xist (Fig. 1h) at E4.75 and E5.0, and the numbers of cells showing Tsix biallelism then decreased from 72% at E5.0 to 29% at E5.25. These results suggest that reactivation of the silent Tsix allele

Figure 2. Xist/Tsix expression kinetics in extraembryonic lineages. (a,b) The matrices represent Xist and Tsix expression patterns in PE/VE lineage cells (a) and TE/ExE lineage cells (b) in each developmental stage, respectively. (c,d) Representative images of Xist (green)/Tsix (magenta) RNA-FISH combined with GATA6 (for PE/VE lineage (c)) and CDX2 (for TE/ExE lineage (d)) immunofluorescence with nuclear DNA staining (white). (e) Image of an E4.5 embryo. Cell 1 exhibited a Xist cloud, whereas cell 2 was negative for the Xist signal. (f,g) Serial sections of cells 1 (f) and 2 (g) respectively.
In both cases, the cell exhibited two signals on the three different positions and looked for such cases. In this analysis, we hardly observed the cells, suggesting that it is highly likely that those cells exhibiting the two signals were diploid cells.

To check the possibility of triploidy in this study, we directly combined RNA and DNA-FISH analyses with whole-mount in situ hybridization analysis (Table 1). Instead, we reasoned that potential triploid cells exhibit two signal, because the random XCI process had already begun in those cells. These Xist weakly positive cells were not clustered or positioned in any specific region of the epiblast. These results suggest that switching from the XCI reversal to random XCI had already been initiated in some nuclei at E4.75–5.0 before all the epiblast cells had completed XCI reversal. The numbers of Xist-positive cells increased further so that at E5.0, 26% of the epiblast cells showed weak Xist expression as a pinpoint signal (Fig. 1e), whereas more than half of the cells (55%) exhibited Xist signals at E5.25. Although most of the cells showed dispersed Xist signals (Fig. 1f), cells displaying Xist clouds began to appear from this stage onward, suggesting that the expression level of Xist increased gradually between E4.75 and E5.25. During this period, the proportion of cells with two Xist signals and one Xist signal decreased, whereas the numbers of cells with one Xist and one Xist signal increased, implying that elevated expression of Xist seems to cause Xist silencing. At E5.5, a large proportion (>50%) of the cells showed one Xist cloud. Xist /Tsix expression patterns in embryonic lineage at E6.5 were also examined and the result showed that almost all of the cells displayed a single Xist cloud with or without one Xist signal and cells with biallelic Xist expression were absent (Fig. 1a and Table 1). This suggests that Xi had already been chosen in almost all the epiblast cells by E6.5.

Intriguingly, a significant number of epiblast cells exhibited biallelic expression of Xist at E5.25 (13.9%) and E5.5 (9.3%) (Fig. 1a). Cells with two Xist clouds were also detected, and the frequency of such cells increased at E5.5 (Figs 1g and S3). Combined analysis of RNA and DNA-FISH can directly address whether those cells were normal diploid or tri-/higher-ploid cells, but such combined analysis is not compatible with our whole-mount protocol (see Discussion).

As differentiation of the PE and epiblasts could not be delineated clearly by the lineage markers NANOG and GATA6 at this stage (Supplementary Fig. S1b), PE/VE cells with weaker Xist expression might represent precursor cells of the epiblast lineage, in which expression appears to be stable in the PE/VE lineage during peri-implantation development.

Most of the cells (88–98%) in the PE/VE lineage marked by GATA6 expression (Supplementary Fig. S1a) exhibited one Xist cloud per nucleus throughout the developmental period examined here (Fig. 2a.e). This result suggests that the imprinted repression of maternal Xist and paternal Xist expression are basically maintained in this lineage from E3.5 to E5.5. However, pinpoint signals for Xist were lost specifically at E4.0 in nearly 50% of the cells. Loss of Xist expression of this kind has never been observed in ExE cells. At E4.0 and E4.25, some cells (12.1% at E4.0 and 4.4% at E4.25) exhibited weak or no signals for Xist. As differentiation of the PE and epiblasts could not be delineated clearly by the lineage markers NANOG and GATA6 at this stage (Supplementary Fig. S1b), PE/VE cells with weaker Xist expression might represent precursor cells of the epiblast lineage, in which Xist will disappear.

Transient loss of Xist clouds in a subset of ExE cells around the implantation stage. The TE or ExE cells were marked by CDX2 expression (Supplementary Fig. S1a and Supplementary Video S2). At all stages examined, most of the TE/ExE cells (70–95%) possessed one Xist cloud and a single Xist spot (Fig. 2b.d–f), suggesting that the imprinted XCI persisted throughout the development of this lineage. However, careful examination of the expression of Xist revealed that cells with no Xist signals were present and that the number of these cells increased transiently, reaching a peak at around E4.75 (21% of all the ExE cells showed no Xist expression;
These Xist-negative cells were not clustered or positioned in a specific region of ExE tissues. Most of these Xist-negative cells had either a single or no Tsix signal, and very few cells showed two Tsix signals. This suggests that the transient loss of Xist expression occurs independently of the status of Tsix expression from the Xi.

Epigenetic dynamics during peri-implantation development. To examine global epigenomic changes during peri-implantation development, we performed immunofluorescence analysis on modifications of DNA, i.e., 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) (Fig. 3a) as well as histone modifications such as di- and trimethylation of histone H3 lysine 9 (H3K9me2 and me3) (Fig. 3b) and trimethylation of histone H3 lysine 27 (H3K27me3) (Fig. 3c).

As shown in Fig. 3a,b, the levels of 5mC, 5hmC, H3K9me2 and H3K9me3 in ICM/epiblasts were comparable with or somewhat lower than those in ExE at E4.5 and E4.75, whereas the signals appeared to be more prominent in epiblasts relative to ExE at E5.25–5.5. The results suggest that changes in the four epigenetic modifications showed a similar trend. Namely, global levels of the modifications increase after E4.75.

In contrast to the four modifications mentioned above, the H3K27me3 modification behaves differently (Fig. 3c). At E3.5, H3K27me3 staining signals were detected as a single, heavy deposit probably corresponding to Xi over a relatively weak background in each ICM and TE cell. However, at E4.5, H3K27me3 modifications were globally upregulated specifically in ICM and this global upregulation was continuously observed at E4.75 and E5.25–5.5. Therefore, upregulation of global H3K27me3 level in epiblast initiated earlier compared to the increase in other four modifications. Interestingly, the H3K27me3 staining was seen as particulate structures distinct from chromocenters as shown in Fig. 3c.

In addition, we noted that the heterochromatin organization, revealed by DAPI staining, changed dramatically in different cell lineages at different developmental stages (Fig. 3d). The number and morphologies of DAPI-dense chromocenters were roughly similar to each other between ICM, PE, and TE cell lineages at E4.5. However, at E5.5, each cell in different lineages appeared to develop unique heterochromatin organization. In epiblasts, chromocenters were distributed over the entire nucleus, whereas VE lineage cells were characterized by smaller chromocenters distributed along the nuclear membrane and ExE lineage cells showed relatively few and large chromocenters. These observations suggest that distinct epigenetic status and chromatin organizations characteristic to these three lineages are established in a relatively short period during peri-implantation development.

Discussion

We traced Xist/Tsix expression during peri-implantation embryonic development and attempt to address assessment of XCI dynamics in vivo using whole-mount 3D RNA-FISH (Fig. 4). The results showed that Xist erasure began at E3.75 and was completed by E4.5, whereas the cells that exhibited biallelic expression of Tsix and no expression of Xist became dominant at the following stage between E4.75 and E5.0. The expression patterns of two X-linked genes, Pfk1 and Lamp2, were also examined at E4.75. Proportions of cells showing biallelic expression of these genes were very similar to that of cells exhibiting Tsix biallelic expression, indicating that X reactivation certainly occurred for X-linked genes other than Tsix. Borensztein et al. clearly demonstrated that the timing of reactivation varied among individual X-linked genes and classified X-linked genes into ‘early reactivated (reactivated from E3.5)’, ‘late reactivated (from E4.0)’ and ‘very late reactivated’. The ‘very late reactivated’ genes were not reactivated even at E4.0. Pfk1 gene is classified as late reactivated gene, and not fully reactivated at E4.0. Here, we demonstrated that Pfk1, one of the late reactivated genes, was activated in about half of the embryonic lineage cells at E4.75. Tsix was activated in only ~15% of the ICM cells at E4.0–E4.25 and ~50% at E4.75, suggesting that Tsix is categorized into the ‘late reactivated’ genes or possibly later than ‘late reactivated’ genes. Combined, reactivation of Pfk1, Tsix and Lamp2 likely occurs at the late-to-very late stage of XCI reversal. The cells exhibiting two Tsix without Xist signals may not always mean X-reactivated state. Nevertheless, as all of X-linked genes including Tsix should be bialleically expressed in the cells subjected to XCI reversal, biallelic expression of Tsix without Xist is, at least, necessary condition for X-reactivated state. Therefore, based on the Xist/Tsix expression pattern as well as results of two other X-linked genes, we think that the proportion of X-reactivated cells in the embryonic lineage appears to peak at E4.75–5.0. We found that, already at E4.75, a few epiblast cells exhibited weak Tsix expression while the same cells continued to show Tsix biallelism. Such cells are considered to have initiated random XCI. These cells showing ‘early’ Tsix expression were not clustered or positioned spatially in specific regions of the embryos. The numbers of these cells further increased until E5.0, and then Tsix biallelic expression declined at later stages.

At E6.5, almost all of the cells displayed a single Tsix cloud and cells with biallelic Tsix expression were absent. Using the same RNA-FISH technique, it was demonstrated that there was no epiblast cell exhibiting biallelic expression of a X-linked gene, Me2p2, at E6.5. These results suggest that random XCI is established by E6.5. Previous cytogenetic studies based on Kanda staining or DNA replication timing demonstrated that random XCI is established by E5.5 or around E6.5, respectively. Those results were generally consistent with ours based on Xist/Tsix expression. The slight differences in the timing found between studies might have been caused by differences in the genetic background of the strains used or could be a reflection of the use of different indices representing different stages of the random XCI process.

We also found that X-reactivation seemed to progress in a cell division-independent manner. Although NANOG-positive ICM cells did not proliferate actively from E3.75 to E4.5 (Table 1), imprinted XCI reversal can progress at these stages. Conversely, after E4.75, when the epiblast cells resume Tsix expression to establish random XCI, cell proliferation was accelerated dramatically (Table 1). Such a XCI reversal event is also observed during PGC development and is accompanied by genome-wide epigenetic reprogramming. Here, we demonstrated that a large-scale change in epigenetic modifications and in chromatin organizations also occurred between E3.5 and E5.5, in particular during E4.75 and E5.25–5.5 when Xist accumulation on one of the two X chromosomes is progressively established. Interestingly, H3K27me3 upregulation in the ICM/epiblast lineage,
Figure 3. Epigenetic dynamics during peri-implantation development. (a–c) Immunofluorescence analysis of epigenetic modifications in peri-implantation embryos using antibodies against 5mC, 5hmC (a) H3K9me2, H3K9me3 (b) and H3K27me3. (c) The green and magenta colors indicate immunofluorescence signals of 5mC and each other modification, respectively and white dotted circles indicate the location of the ICM/epiblast lineage cells. 5mC and 5hmC staining were performed in the same embryos. The images of H3K9me2, H3K9me3, and H3K27me3 are overlapped with nuclear staining (white). Magnified views of epiblast and ExE cells at E5.25–5.5 (a,b) and E4.5, 4.75, and E5.25–5.5 (c) are shown on the right side (a,b) and bottom of each image (c) respectively. (d) The images of DAPI staining at E4.5 and 5.5. Magnified views of 1: ICM/epiblast, 2: PE/VE and 3: TE/ExE cells at each developmental stage are shown. The yellow and white scale bars indicate 25 and 5 μm, respectively.
in contrast to all the other epigenetic modifications examined, was observed between E3.5 and E4.5. This developmental period exactly matches the period of Xist erasure. It has been reported that H3K27me3 is required for Tsix-independent Xist repression in naive ES cells. In addition, Inoue et al. demonstrated that H3K27me3 is required for maternal Xist silencing in imprinted XCI during pre-implantation development\cite{21}. Therefore, it is possible that paternal Xist repression observed in ICM/epiblasts may be caused by H3K27me3 modifications laid onto paternal Xist regulatory regions after E3.5. These results indicate that the major de- and reprogramming events of the epigenetic status occur at around the peri-implantation stage, and thus the conversion of XCI regulation from imprinted to the random process seems to be one of those major epigenomic events.

Here we showed that Xist erasure occurred without any activation of Tsix from the Xi, suggesting that Tsix expression is not involved in initiating the erasure of the Xist RNA. Forced induction of Tsix expression caused Xist repression in cis, resulting in X reactivation in a lineage- and stage-specific manner\cite{22}. In contrast with this ‘gain-of-function’ type of experiment, Maclary et al. showed that XCI reversal in the ICM could occur in the absence of functional Tsix, suggesting that Tsix is dispensable for X chromosome reactivation at this stage\cite{23}. Moreover, Payer et al. observed a loss of H3K27me3 enrichment on the Xi of epiblast cells in Tsix-null mutant embryos, although the loss was significantly delayed in the mutants compared with normal embryos\cite{24}. The results of these two studies based on Tsix null mutants were consistent with our findings. Thus, the onset of the loss of inactive markers of the Xi can occur in the absence of Tsix expression. Pasque et al. demonstrated that Tsix expression is dispensable for XCI reversal during the reprogramming of MEFs to induced pluripotent stem cells (iPSCs)\cite{25}. Therefore, is Tsix not essential for X chromosome reactivation at all? Considering that the X reactivation process can be divided into at least two phases—the initiation of release from the inactive state and the establishment of epigenetic equivalency between the two X chromosomes—Tsix is probably not involved in the former phase, but is likely to be involved in the latter, thus assuring the epigenetic parity of both X chromosomes, as suggested by Navarro et al.\cite{26}.

Intriguingly, here we showed that the biallelic expression of Xist, including two Xist clouds, could take place at the onset of random XCI during normal mouse embryonic development. In general, to examine the ploidy of those cells, a combined analysis of RNA- and DNA-FISH can be used. For the combined analysis, samples are fixed and anchored on a glass slide so that DNA-FISH images are superimposed to the recorded RNA-FISH signals. However, as the embryos examined in our whole-mount protocol were not anchored, we could not perform a combined analysis of RNA- and DNA-FISH. Alternatively, we searched potential triploidy or higher ploidy cells exhibiting one or more Tsix signals localized distantly from two Xist clouds. However, we rarely found such cases. Therefore, we think that the cells with two Xist clouds were likely to be normal diploid cells. This conclusion is also supported by in vitro studies performed by other groups: biallelic expression of Xist was detected in a subset of differentiating diploid ES cells\cite{26,27}; live-cell imaging of the fluorescently tagged nascent Xi in vitro showed survival of some cells with two Xi signals\cite{28}; and two Xist signals could be also detected in diploid ICM cells allowed to differentiate in vitro\cite{29}. In addition, ectopic expression of Xist was also observed in epiblast cells...
of male embryos at the same stage (Supplementary Fig. S4) although frequency of detection was much lower in male (1.2 cells per embryo at E5.5 (n = 5)) than in female (6 cells per embryo at E5.5 (n = 4)). This trend is also true for differentiating ES cells. Therefore, it is possible that such biallelic expression of Xist in female, and also ectopic Xist expression in male, can occur in the normal diploid cells during peri-implantation development in mice. Similar results have been reported recently by Sousa et al. At E4.75 when re-expression of Xist began to be observed in the ICM/epiblast cells of female embryos, the cells exhibiting biallelic expression of Xist already appeared. In addition, the percentage of the cells showing asymmetric expression of Xist, e.g. one pinpoint (or dispersed) and one cloud, was lower than that of the cells showing two Xist pinpoints (or dispersed) signals or two clouds (Supplementary Fig. S3). Therefore, it appears that the Xist biallelism is caused by simultaneous Xist activation from both alleles rather than an occasional up-regulation of the second Xist allele in the cells that have already initiated Xist expression from one allele. It was unexpected and intriguing observation, because Xist RNA accumulation on the future Xi is supposed to occur after determining the number of X chromosomes and election of the future Xi and Xa. Since the present study only provides snap shots of initiation process of random XCI, longitudinal observations using different techniques will be needed to delineate how exactly random XCI initiates or to determine the fate of cells with Xist biallelic expression. Biallelic Xist activation can be found in a high proportion of cells during early development in human and rabbit which have no imprinted XCI mechanism. Although these phenomena are superficially similar to the one in mice, Xist biallelism in mice may have different biological significance. Proportion of the epiblast cells exhibiting Xist biallelic expression in post-implantation epiblast of mice is low compared to those in human pre-implantation embryo (at most 15% in mice vs >80% in human) and cells showing Xist biallelic expression were only transiently detected (from E5.25 and E5.5), whereas Xist biallelism is observed in relatively long period of human preimplantation development, implying that the transient Xist biallecal expression may be harmful for mouse chromosomes. On the other hand, Xist accumulation does not induce chromosome-wide XCI in human preimplantation embryos. Interestingly, as described above, ectopic expression of Xist in mice was observed at almost the same stage in both female and male euploid cells. It is thus possible to speculate that such ectopic expression in mice might be a reflection of large-scale changes in epigenetic states and/or in nuclear architecture that occur in both male and female embryos at a similar developmental stage corresponding to the beginning of random XCI establishment in female embryos.

Our in vivo analysis revealed that Xist/Tsix expression dynamics were significantly different among the three different embryonic lineages studied: ICM/epiblast, TE/ExE, and PE/VE. Interestingly, Xist expression was lost transiently in some ExE cells, from E4.0 to E5.0, reaching a peak of 21% at E4.75. One possible explanation for the loss of Xist clouds is that those cells were in early G1 phase because Xist clouds may not be observed in early G1 phase depending on pretreatment (permeabilization) of the cells prior to RNA-FISH. As another explanation, we speculate that loss of Xist clouds is possibly linked to the change in regulatory mechanism of imprinted XCI in the ExE lineage. Imprinted Xp inactivation is thought to continue in ExE cells. However, the imprinted XCI can be reversed in a subset of female ExE cells that carry a paternal derived Xist mutation, suggesting that the primary imprint for imprinted XCI, which is likely to be H3K27me3 modification in maternal Xist locus, might be erased in ExE cells. Oikawa et al. demonstrated that the primary imprint mark must be erased in the TE/ ExE cells of E4.5 embryos by nuclear transfer experiments. These results suggest that imprinted XCI is somehow maintained during ExE development, although its regulatory mechanism might be converted to a different one. The timing of the transient loss of Xist in ExE cells, shown in this study, roughly matches the transition period of the mechanism for imprinted XCI regulation in ExE cells suggested by Oikawa et al. Therefore, we speculate that the transient loss of Xist observed in the ExE might be involved in the conversion of the imprinted XCI regulatory mechanism in the ExE lineage.

Methods
Mice. The mouse strain C57BL/6J was used throughout the experiments. As an exception, embryos with mixed genetic background, BDF1xC57BL/6J, were used for Lamp2 and Pgk1 RNA-FISH experiments, because only very faint or no FISH signals were detected around E4.75 when we used embryos of C57BL/6J strain. E3.5–5.5 embryos were recovered at intervals of 6 h from naturally mated female mice. Noon was set as E0.5 on days when vaginal plugs were detected. We collected embryos between E3.5 and E4.0 by flushing the uterus. In many studies, E4.5 stage embryos were obtained by culturing preimplantation blastocyst or by flushing out the uterus. However, we experienced that the number of embryos collected by flushing out around E4.5 stage is substantially smaller than those obtained at E3.5, indicating that embryos firmly attached (= implanted) to the uterus cannot be recovered by flushing out, and it is likely that only embryos of relatively early stage (not yet firmly attached) were collected. Therefore, embryos after E4.25 were collected by manual dissection. The collected embryos were used immediately for the subsequent analysis without embryo culture. All animal experiments were performed in accordance with the guidelines for the experimental use and care of laboratory animals of the RIKEN Tsukuba Institute with prior approval from the Institutional Animal Experiment Committee of the RIKEN Tsukuba Institute.

Strand-specific probe synthesis for whole-mount 3D RNA-FISH. Strand-specific DNA probes for detecting the Xist, Tsix, Lamp2 and Pgk1 RNAs were prepared according to our published protocol. For details, see the Supplementary Methods.

Expression analysis of Xist/Tsix via whole-mount 3D RNA-FISH combined with immunofluorescence. Whole embryos were dissected then treated with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 10s on ice for permeabilization. After fixation with 4% paraformaldehyde in PBS with 0.1% Triton X-100 for
10 min at room temperature, RNA-FISH and immunofluorescence analyses were performed. Details of the procedure, including the antibodies used, are provided in the Supplementary Methods.

**Analysis of Xist/Tsix expression patterns.** Xist and Tsix expression patterns in individual cells from each lineage were examined as follows. The whole embryo was subjected to Xist/Tsix RNA-FISH followed by immunostaining with a marker that was specific to a particular lineage: POU5F1 or NANOG for ICM/embryonic ectoderm, GATA6 for PE/VE, and CDX2 for TE/ExE. Images were acquired using confocal microscopy. Optical sections of the confocal images were examined manually, and Xist/Tsix expression patterns in each cell of the embryos were determined by considering the overlaps of cellular images between the z-stack images. Xist expression patterns in each cell were classified into four categories: (1) cells with one Xist cloud per nucleus; (2) cells with single pinpoint or dispersed signals; (3) cells with two Xist signals; or (4) cells with no Xist signal. Tsix-positive cells were categorized into cells with two, one, or no signals. The combinations of Xist and Tsix expression patterns in each cell were determined, and the proportion of each combination was calculated and shown as a matrix. We determined Xist and Tsix expression patterns and their combinations in almost all the cells that constituted a whole embryo at a particular stage. We performed these analyses for nine different embryonic stages and for three different cell lineages of each stage. We repeated these experiments at least three times for each embryonic stage. At E6.5, more than 250 embryonic ectoderm cells in total were randomly chosen from two embryos (115 and 139 cells, respectively) and their Xist and Tsix expression patterns were examined. Confocal image data used in this study have been deposited to Systems Science of Biological Dynamics (SSBD) database (http://ssbd.qbic.riken.jp) and will be open to the public.

**Immunofluorescence analysis of epigenetic modifications.** Whole embryos were dissected, treated with 0.1% Triton X-100 in PBS for 10 s on ice for permeabilization, and fixed with 4% paraformaldehyde in PBS with 0.1% Triton X-100 for 10 min at room temperature followed by immunofluorescence analysis. For details, including antibody information, see the Supplementary Methods.

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Author Contributions
H.S. and K.A. conceived the project and wrote the manuscript. H.S. performed all the experiments.

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