

X Chromosome Inactivation Is Initiated in Human Preimplantation Embryos

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X chromosome inactivation (XCI) is the mammalian mechanism that compensates for the difference in gene dosage between XX females and XY males. Genetic and epigenetic regulatory mechanisms induce transcriptional silencing of one X chromosome in female cells. In mouse embryos, XCI is initiated at the preimplantation stage following early whole-genome activation. It is widely thought that human embryos do not employ XCI prior to implantation. Here, we show that female preimplantation embryos have a progressive accumulation of *XIST* RNA on one of the two X chromosomes, starting around the 8-cell stage. *XIST* RNA accumulates at the morula and blastocyst stages and is associated with transcriptional silencing of the *XIST*-coated chromosomal region. These findings indicate that XCI is initiated in female human preimplantation-stage embryos and suggest that preimplantation dosage compensation is evolutionarily conserved in placental mammals.

Introduction

Mammalian XX females equalize gene dosage relative to XY males by the inactivation of one of their X chromosomes in each cell. The X chromosome inactivation (XCI) center contains several genetic elements essential for the transcription initiation of long noncoding RNAs that are involved in XCI. Initiation of XCI requires the *cis* accumulation of a nontranslated mouse *Xist* RNA or human *XIST* RNA (*Xist/XIST* RNA) (MIM 314670) that coats the X chromosome.^{1–3} This is followed by various epigenetic changes on the future inactive X (Xi) chromosome that contribute to chromosome silencing.⁴ In somatic cells, the Xi chromosome is visible as a region of dense chromatin called the Barr body.⁵

There are two different forms of XCI: random XCI and imprinted XCI. Random XCI of either the maternal or the paternal X chromosome takes place in all somatic cell lineages of eutherian mammals, starting around gastrulation. Random XCI has no specific preference for inactivation of one of the parental X chromosomes.^{6–8} In contrast, imprinted XCI results in preferential inactivation of the paternal X chromosome and occurs in female marsupials and mouse placental tissues.^{9–11} Although expression of *Xist* RNA and a preferential expression of *Xist* from the paternal allele has long been observed in preimplantation mouse embryos, the prevailing view has been that actual inactivation of the X chromosome, and thus dosage compensation, begins only after differentiation of the placental precursor cells.^{12–14} In recent years, however, it has become apparent that XCI of the paternal X chromosome is already present from the 4-cell stage onward in all cells of preimplantation mouse embryos.^{15–17} Imprinted XCI in the mouse persists until the blastocyst stage and continues in the trophoblast and the primitive

endoderm.^{10,11,18} However, the inactive paternal X chromosome is reactivated in the inner cell mass (ICM) that forms the embryo proper^{15–17} and is followed by random XCI in somatic cell lineages.^{5,16,19} It is still unclear exactly how the earlier imprinted XCI in cleavage-stage embryos, trophoblast cells, and primitive endoderm cells is programmed by the parental germline. Evidence exists for a mark on the maternal X chromosome that allows it to remain active.^{20,21} On the other hand, there is also evidence for a preference of *Xist*-mediated inactivation of the paternal X chromosome.^{12,15,22} However, these two mechanisms need not be mutually exclusive.

Data regarding the mechanism of human XCI are not easy to obtain, because of restrictions on the use of human embryos and the generation of human embryonic stem (ES) cell lines. Only a minority of human ES cell lines have two active X chromosomes in their undifferentiated state and will start a process of random XCI upon differentiation, similar to mouse ICM cells and ES cell lines.^{16,23–25} The majority of the undifferentiated human ES cell lines so far examined have already inactivated one X chromosome, evident from the single *XIST* cloud in 20%–70% of the cells and the accumulation of specific chromatin modifications.^{24–27} One study showed that differentiation of a human ES cell line resulted in either random XCI or preferential inactivation of a single allele, depending on the differentiated cell type. Only trophoblast cells showed a preferential inactivation of a single X chromosome, similar to mouse trophoblast tissue.²³ Although the parental origin of the X chromosome could not be identified in this ES cell line, which was generated from anonymously donated embryos, it does suggest that a form of preferential XCI, such as imprinted XCI, may exist during the first stages of human trophoblast development, similar to mouse extra-embryonic tissues.^{10,11,18} Studies of human

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placental tissue have shown a variety of patterns of XCI. Some reports describe preferential expression from the maternal X chromosome, similar to mice, suggesting conservation of imprinted XCI.^{28–31} However, other papers report a random XCI or an XCI moderately skewed in favor of an inactive paternal X chromosome.^{32–36} If imprinted XCI occurs in humans, it is possible that human trophoblasts gradually lose their imprint and perform random XCI at later stages, as has been demonstrated in vitro.^{37,38} This may result in an XCI pattern skewed toward an inactivated paternal X chromosome, which would explain the mixed results observed in the analysis of placentas.^{28–36}

Defects in dosage compensation prior to implantation of the embryo lead to abnormal development in a majority of the embryos and early lethality, as demonstrated by analysis of parthenogenetic mouse embryos that have two maternal genomes.^{13,39} Similarly, female mutant embryos that inherit a paternal X chromosome with a deletion of the *Xist* gene are not able to inactivate this X chromosome, resulting in two active X chromosomes and early lethality.^{40,41}

Previous studies using PCR analysis of human preimplantation embryos detected *XIST* expression both in female and in male embryos.^{42,43} Because female-specific *XIST* expression was not detected, it was concluded that *XIST* RNA was not functional at this stage of development and that dosage compensation was not initiated in human preimplantation embryos.^{42,43} However, in mice, a brief “pinpoint” expression of *Xist* from both the paternal and the maternal X chromosome was later reported in male and female preimplantation embryos.^{17,19,44,45} The initial *Xist* expression on the maternal X chromosome subsequently disappears while the *Xist* expression from the paternal X chromosome accumulates in female preimplantation embryos to coat the future Xi chromosome.^{17,44–46} Similar pinpoint signals are also observed during the onset of random XCI in male and female ES cells.^{17,19,46} Thus, the *XIST* expression previously reported in male human embryos could be attributable to a brief window of expression of *XIST* from the paternal X chromosome and does not exclude XCI in female embryos.

We therefore decided to reinvestigate XCI in human preimplantation embryos at the single-cell level to analyze *XIST* RNA localization and the transcriptional and epigenetic features of XCI.

Material and Methods

Collection of Surplus Embryos and Cryopreserved Embryos Donated for Research

Ovarian stimulation, oocyte retrieval, and in vitro fertilization (IVF) and/or intracytoplasmic sperm injection (ICSI) procedures were performed as described previously.⁴⁷ This study was approved by the Dutch Central Committee on Research Involving Human Subjects (CCMO, NL11448) and the local ethics review committee of the Erasmus Medical Center Hospital (MEC 2007-130). Written consent was obtained from the couples for confirmation that the surplus or cryopreserved embryos could be used for research purposes.

RNA FISH, DNA FISH, and Immunofluorescence

Immunofluorescence followed by RNA and/or DNA fluorescence in situ hybridization (FISH) was performed as described previously,¹⁷ with some modifications. The zona pellucida of fresh or thawed cryopreserved embryos was removed with 0.05% pronase (Sigma Aldrich) in calcium- and magnesium-free medium (G-PGD, Vitrolife, Kungsbacka, Sweden). Embryos of the 8-cell and morula stage were incubated in calcium- and magnesium-free medium so that single cells could be obtained. Single cells and blastocysts were washed in phosphate-buffered saline (PBS) and fixed on slides in 1% paraformaldehyde (Sigma Aldrich) containing 0.5% Triton X-100 for 0.5–1 hr. Slides were washed with PBS and stored in 70% ethanol at –20°C. Cumulus cells and amniocytes were fixed similarly. Primary antibodies used for immunofluorescence were H3K27Me3 (Abcam, 1:50), macroH2A.1 (Upstate, 1:100), and H3K9ac (Upstate, 1:100). Secondary antibodies (Invitrogen), used at 1:250 dilutions, were as follows: goat anti-rabbit Alexa 594, goat anti-mouse Alexa 488, and goat anti-mouse Pacific Blue.

For the detection of *XIST* RNA, a 16.4 kb plasmid covering the complete RNA sequence of the *XIST* gene⁴⁸ was used on nondenatured cells. *Cot1* RNA detection was performed with the use of labeled *Cot1* DNA (Invitrogen) as a probe. For the detection of chromosomes X, Y, and 15, the following DNA probes were used: X centromere (pBAMX5), Y chromosome heterochromatin (RPN1305X), and chromosome 15 satellite III region f (D15Z1). The Y probe occasionally produces a diffuse signal because of the highly polymorphic heterochromatin region.⁴⁹ Overlapping *CHIC1* fosmid clones (G248P389032C3 and G248P86549C3) were located with the UCSC genome browser (UC Santa Cruz) and obtained from BACPAC Resources (Oakland). RNA and DNA FISH probes were labeled by nick translation with fluorochromes Alexa 594 and 555 (Molecular Probes, Invitrogen, Leiden, The Netherlands), diethylaminocoumarin-5-UTP (NEN Life Science Products, Boston, MA, USA), or Bio-16-dUTP (Roche). Probes were validated in cultured lymphocytes of a normal XY male. A significant distance between the signals of the X centromere probe and *XIST* RNA was often observed, because *XIST* is located 80 Mb distal to the centromere. *XIST* expression was detected as a pinpoint signal, a small cloud, or a full cloud. The distinction between the three was usually easy to detect. A pinpoint signal was equivalent in size and intensity to a locus-specific DNA FISH signal, a small cloud was 10–20 times the size of a pinpoint signal, and a full cloud was 100 or more times the size of a pinpoint signal.

Slides were examined with a Zeiss Axioplan 2 epifluorescence microscope equipped with appropriate filters (Chroma, Rockingham, VT, USA). Images were captured with the ISIS FISH Imaging System (MetaSystems, Altlussheim, Germany), and background correction was applied via Adobe Photoshop CS2 when necessary. For each embryo, the positions of all nuclei were mapped in detail, which allowed an accurate analysis of each nucleus. Chromosomally chaotic embryos (ascertained on the basis of chromosome X, Y, and 15 analysis) were excluded from the analysis, and mosaic embryos were included only if less than 50% of the cells were aneuploid for X and Y.

Results

Donated cryopreserved and surplus embryos from in vitro fertilization (IVF) treatments were dissociated and fixed at the 8-cell, morula, and blastocyst stages. RNA FISH with a human *XIST* probe was performed for the detection of

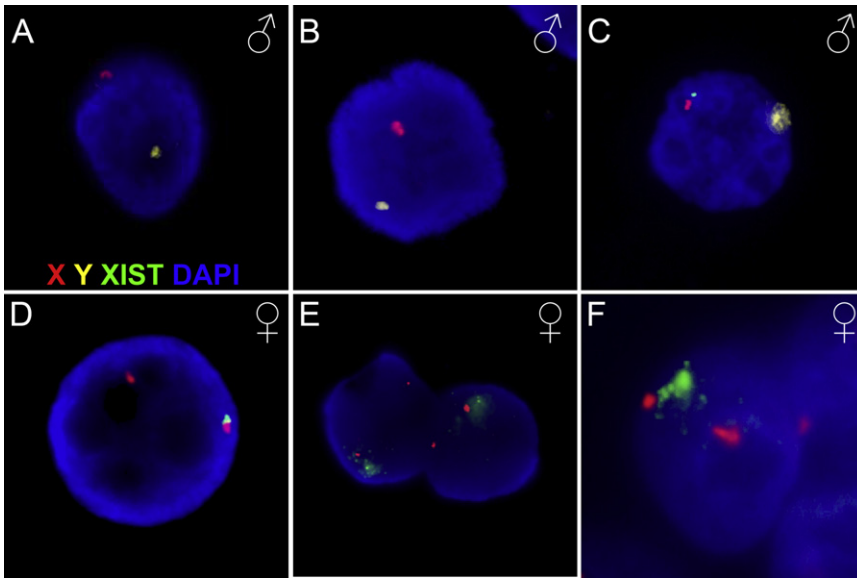


Figure 1. *XIST* Expression in Male and Female Human Preimplantation Embryos RNA and DNA FISH staining with probes detecting *XIST* RNA (green), the X (red) and Y (yellow) chromosome, and DAPI counterstain. Human male embryos (A–C) do not generally show *XIST* signals at the 8-cell stage (A) or at the morula stage (B). A minority of male cells at the morula stage show a pinpoint of *XIST* staining (C). Female embryos (D–F) show an *XIST* pinpoint in the majority of embryos at the 8-cell stage (D). Two cells at the morula stage each show a beginning cloud of *XIST* on one of their two X chromosomes (E). At the blastocyst stage, this has further accumulated to a full cloud on one of the two X chromosomes (F). A third diffuse red signal is an X chromosome from an adjacent cell that is in a different focal plane.

the Xi chromosome in single cells, followed by DNA FISH with chromosome X-, Y-, and 15-specific probes for the identification of the sex and diploid status of the embryos (see Figure S1, available online).

XIST Expression in Early Human Embryos

Only a fraction of the blastomeres from male embryos showed *XIST* RNA signals. These signals were small “pinpoint”-like signals reminiscent of the expression of unstable *XIST* RNA.^{44,46} This form of *XIST* expression was observed mostly at the morula stage, and the pinpoints never accumulated to a “cloud”-like signal in blastocysts, indicating the absence of XCI in male preimplantation embryos (Figures 1A–1C and Table 1). In contrast, female embryos showed a different *XIST*-staining pattern: the majority of cells had pinpoint signals for *XIST* RNA at the 8-cell stage. The *XIST* signal gradually accumulated to a full cloud on one of the X chromosomes at the late morula and blastocyst stages in female embryos (Figures 1D–1F). Distinct patterns of *XIST* expression were observed for different developmental stages (Table 1): In 8-cell-stage embryos, 65% of the blastomeres displayed one pinpoint signal of *XIST*, 4% of the blastomeres had a small cloud

of *XIST*, and 19% showed two pinpoints of *XIST* RNA. The remaining 12% of blastomeres had no expression of *XIST*. In morulas, *XIST* expression had progressively accumulated, resulting in 49% of the cells displaying a single *XIST* cloud, suggesting that XCI was initiated in these cells, and 19% of the cells had a pinpoint of *XIST* RNA. The percentage of cells with two pinpoints was reduced from 19% at the 8-cell stage to 2% in morulas. The rest of the blastomeres had no *XIST*, two small clouds of *XIST*, or a pinpoint together with a cloud signal. Different cells of the same embryo regularly showed variable levels of *XIST* and distinct *XIST* expression patterns (Figure S2), similar to previous observations in mouse embryos.^{13,44,50,51}

Blastocysts did not disaggregate well during the fixation procedure and were therefore examined as intact embryos. Nuclei that could be visualized showed a full *XIST* cloud in 90% of the cells (Figure 1 and Table 1). The remainder of cells either had no *XIST* signal or had two small cloud signals. In summary, whereas female embryos at the 8-cell stage showed a clear single pinpoint or small cloud of *XIST* that accumulated to a proper *XIST* cloud at the late morula and blastocyst stage, male embryos showed only brief expression of *XIST* in a minority of the cells.

Table 1. *XIST* Patterns in Human Embryos at Different Developmental Stages

Embryo Stage	No. of ♀ Embryos (No. of Cells)	<i>XIST</i> Pattern in ♀ Embryos						No. of ♂ Embryos (No. of Cells)	<i>XIST</i> Pattern in ♂ Embryos	
		(-)	(•)	(••)	(•●)	(-●)	(••)		(-)	(•)
8-cell	5 (26)	12	65	19	-	4	-	6 (30)	94	6
Morula	13 (89)	19	19	2	6	49	4	9 (91)	86	14
Blastocyst	6 (>100)	5	-	-	-	90	5	5 (98)	93	7

Percentages of analyzable cells that have the indicated pattern of *XIST* signals. Signals in blastomeres were scored as negative (-), pinpoint signal (•), small cloud (•●), or full cloud (●).

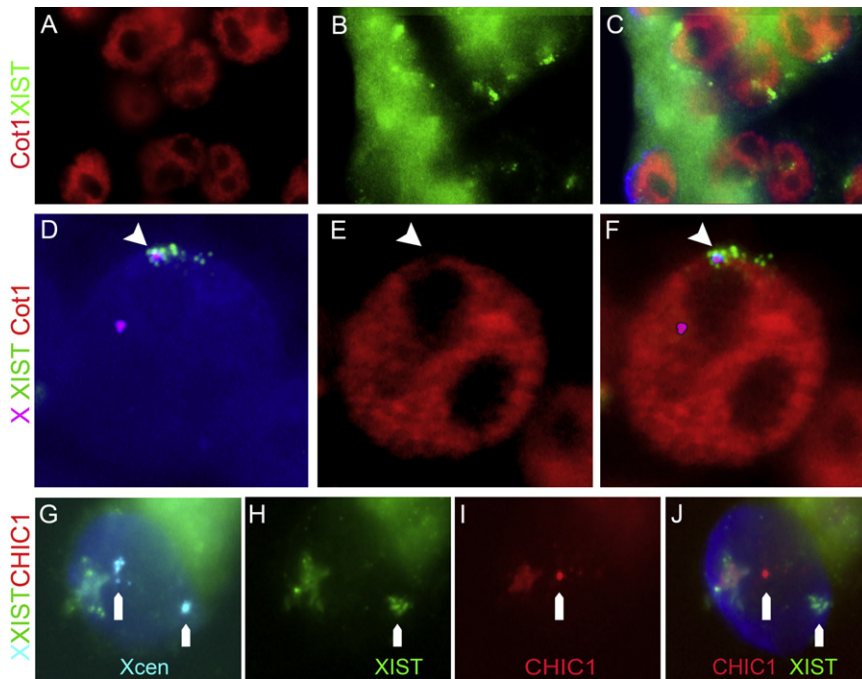


Figure 2. Transcriptional Changes on the Inactive X Chromosome

(A–F) *Cot1* exclusion around *XIST*. (A–C) Cells of a female blastocyst embryo, stained for *Cot1* RNA (red in A) and *XIST* RNA (green in B) showing depleted regions of *Cot1* RNA around the *XIST* signals indicating the position of the Xi chromosome (merged in C). (D–F) Representative cell of a female blastocyst with staining for the X centromeres and *XIST* RNA (D; Xcen in magenta, *XIST* in green) together with *Cot1* (red in E). Transcription of *Cot1* RNA was absent in a region that overlaps with *XIST* RNA staining (F), whereas the active X without *XIST* staining overlaps with a *Cot1*-positive region.

(G–J) Female blastocyst cell with two X centromeres (cyan in G) has a single *XIST* cloud on one X chromosome (green in H) and monoallelic expression of *CHIC1* on the other X chromosome (red in I, merged in J). A dust spot is visible in all colors and is therefore nonspecific staining.

Thus, a clear difference in the timing, duration, and level of *XIST* expression between male and female human embryos suggests the occurrence of XCI in female preimplantation embryos.

Absence of Transcriptional Activity on the *XIST*-Coated Chromosomal Region

We analyzed the transcriptional activity together with *XIST* staining, because *XIST* RNA accumulation on the X chromosome itself does not automatically imply an inactivated status of the X chromosome. Transcriptional activity was investigated by *Cot1* RNA FISH staining, which highlights areas of ongoing hnRNA transcription; transcriptionally silent nuclear compartments, such as an Xi chromosome, are devoid of *Cot1* RNA.^{15,17} Human embryos were stained by RNA and DNA FISH with probes for *Cot1* RNA, *XIST* RNA, chromosome 15, and the X and Y chromosomes. Cumulus cells of human follicle complexes were used as positive controls in each experiment (Figures S3A–S3C). Cells of human blastocysts showed *XIST* RNA clouds corresponding to areas where *Cot1* RNA (Figures 2A–2F) was excluded. Figures 2D–2F show a representative cell with an *XIST*-coated X in a *Cot1*-depleted region and the second X chromosome in a *Cot1*-positive area. The *Cot1* exclusion coincided with the *XIST* signal in 89% of the cells ($n = 47$). Comparable percentages of *Cot1* exclusion from *Xist/XIST*-positive areas have been observed in mouse embryos and differentiating human ES cells.^{15,22,24} The initiation of transcriptional silencing was confirmed with RNA FISH detection of nascent transcripts of the X-linked *CHIC1* gene. Blastocyst cells showed a single spot of *CHIC1* expression close to one X centromere and an *XIST* cloud on the other Xi chromosome (Figures 2G–2J). Biallelic expression was not observed in blastocysts

and was not assayed in earlier stages. Thus, the X chromosome that is at least partially coated with *XIST* RNA in the human embryo is in a transcriptionally silent area, demonstrating that XCI and dosage compensation commences in preimplantation female human embryos.

Chromatin Conformational Changes on the Inactive X Chromosome

To further explore XCI in human preimplantation embryos, we investigated histone modifications that are established hallmarks of a silenced X chromosome. Specific accumulation of one or more histone modifications forms macrochromatin bodies that indicate the position of the Xi chromosome, as shown for XCI in mouse preimplantation embryos and in differentiating mouse and human ES cells.^{1,4,16,17,25,52–54}

Identification of the chromatin state of X chromosomes was carried out with antibodies that detect hypoacetylation of lysine 9 on histone H3 (H3K9ac), accumulation of trimethylation of lysine 27 on H3 (H3K27Me3), and the enrichment of the histone variant macroH2A,^{1,4,16,17,52,53} followed by DNA FISH for the gender identification of the embryo. As a control, we used cumulus cells in which the Xi chromosome can be identified as a chromatin-dense Barr body or amniocytes in which immunostaining together with *XIST* detection is possible. Human cumulus cells showed DAPI-dense Barr bodies that were positively stained for macroH2A and H3K27Me3, marks associated with inactive chromatin (Figures S3D–S3G). Amniocytes showed the same accumulation of chromatin markers overlaying the *XIST* signal (Figures S3H–S3K). In addition, staining with an antibody against H3K9 acetylation, an active chromatin mark, showed exclusion from Barr bodies in cumulus cells or from *XIST*-stained regions in

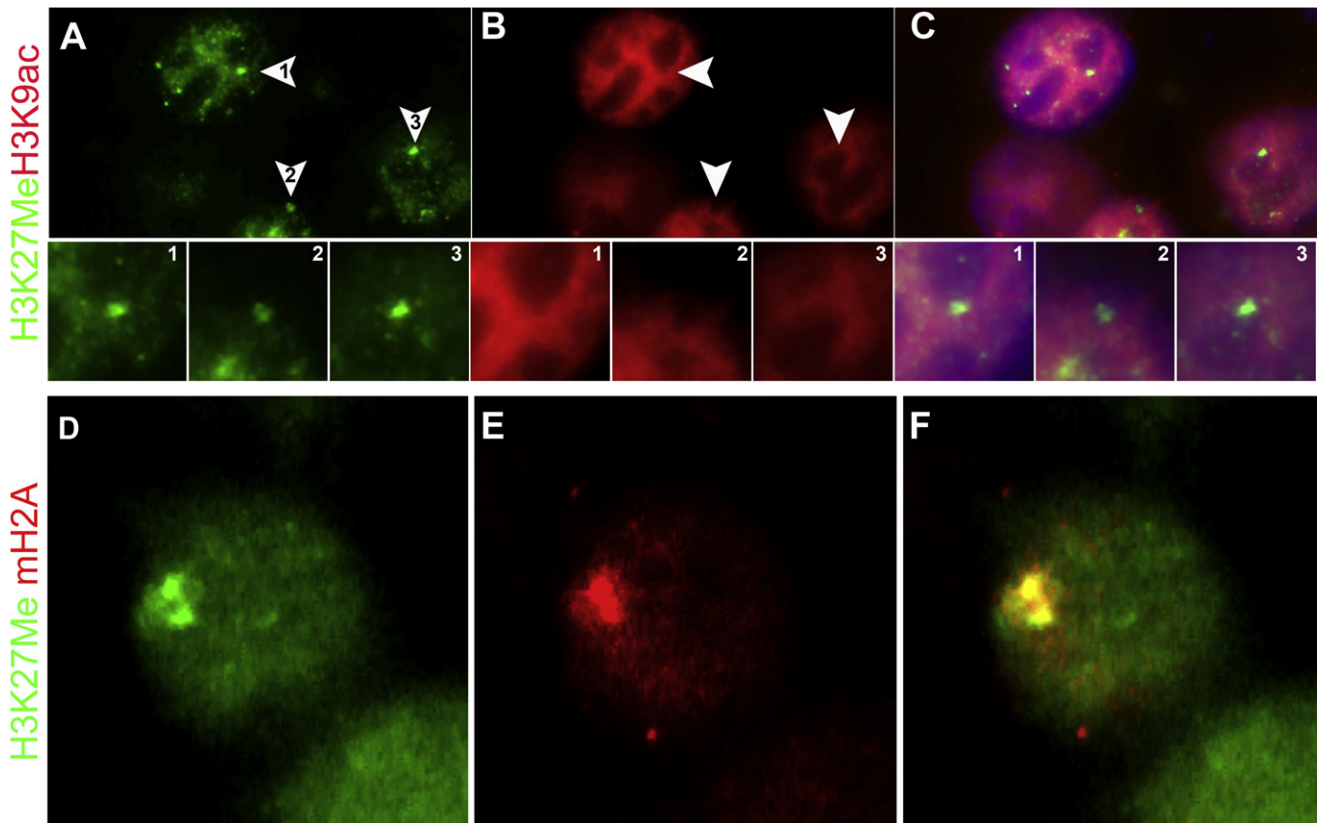


Figure 3. Epigenetic Changes on the Inactive X Chromosome

(A–C) Three adjacent blastocyst cells show a single nuclear domain with H3K27Me3 hypermethylation (arrowheads in J and enlarged panels 1–3), and staining for H3K9 acetylation (B, 1–3) shows an H3K9ac-depleted region overlaying the H3K27Me3 accumulation (C, 1–3), indicating the position of the Xi chromosome.

(D–F) Representative blastocyst cell shows a single nuclear domain with H3K27 hypermethylation (green, D) and enrichment for macroH2A (red, E) with a clear overlap (yellow, F), analogous to the signal around an Xi chromosome.

amniocytes (Figures S3L–S3S). These findings confirm the specificity of the antibodies used to indicate a macrochromatin body as the Xi. Staining of cells from preimplantation blastocysts with the use of the same antibodies showed identical results; i.e., double staining showed a single region where accumulated H3K27Me3 formed an exact overlay with the region of H3K9 hypoacetylation (Figures 3A–3C), indicating the presence of an Xi. Furthermore, macroH2A enrichment and accumulation of H3K27Me3 (Figures 3D–3F) colocalized exactly in blastocyst cells. Up to 30% of the analyzable cells in blastocysts showed a double immunostaining of chromatin marks that are specific for XCI (either H3K27Me3 together with macroH2A or H3K27Me3 in a depleted region of H3K9ac). The other 70% of the cells had no visible accumulation (or depletion, in the case of H3K9ac) of either antibody, and single accumulations were rarely found (<5%). In contrast, male embryos did not show accumulation of H3K27Me3 or macroH2A, and no specific exclusion of H3K9ac was observed (data not shown).

Taken altogether, these observations show that once *XIST* RNA coats the X chromosome in human embryos, epigenetic changes that are known to lead to XCI are

induced, similar to that which has been observed in mouse preimplantation embryos and ES cells.^{4,55,56}

Discussion

In contrast to previous suggestions that XCI may not be present in the human preimplantation embryo,^{4,42,43,55,57} our observations of *XIST* RNA accumulation in female embryos, local accumulation of Xi-specific chromatin modifications, and the absence of active transcription in *XIST* RNA-coated areas indicate that XCI occurs in human female preimplantation embryos, starting from the 8-cell stage. However, the extent of the coating by *XIST* and the extent of transcriptional silencing other than that of the *CHIC1* gene remain unknown.

Conservation of Timing of X Chromosome Inactivation

The developmental stage at which embryonic genome activation occurs in mammalian species varies considerably: at the 1- to 2-cell stage in mice, at the 4- to 8-cell stage in cows and humans, and at the 8- to 16-cell stage in sheep and rabbits.^{58,59} The necessity of dosage compensation by

XCI is likely to start at the onset of genome activation; thus, around the 2-cell stage in mice and around the 8-cell stage in humans. Initiation of XCI can be detected in mice from the 2-cell stage onward by *Xist* RNA FISH, showing a small pinpoint of *Xist* in 67% of female cells, and complete X-associated *Xist* accumulation occurs by the 8-cell stage.^{15,17} In human embryos, we observed pinpoint *XIST* signals in 65% of female 8-cell blastomeres and *cis* accumulation (cloud-like signals) of *XIST* in late morulas and in blastocysts (Figure 1 and Table 1). We could not analyze embryos before the 6- to 8-cell stage because such embryos were not available for research. Given that our observations in 6- to 8-cell-stage human embryos show *XIST* RNA with exactly the same pinpoint signals and at a similar frequency as those in 2-cell-stage mouse embryos (67% versus 65%; Table 1 and ¹⁵), it is likely that the 8-cell stage represents the actual onset of *XIST* expression in humans. The later stage of XCI in humans relative to mice suggests a correlation with the later transition from maternal to embryonic or zygotic gene expression in humans.⁶⁰

XIST patterns were analyzed only in euploid cells, and because similar results were observed in cells derived from normal or mosaic embryos (Table S1), these data were combined. No consistent pattern of *XIST* expression was discernible in the sex aneuploid cells (Table S1). The quality of the embryos did not influence the results; we observed similar patterns of XCI in euploid cells of surplus embryos and cryopreserved embryos. It is likely that aneuploidies other than those detected with the chromosome X, Y, or 15 probes were present in the analyzed cells, because human embryos are known to have high aneuploidy rates.⁶¹ However, aneuploidies may have only a subtle effect on the skewing of the selected X chromosome.⁶²

Comparison of several characteristics of XCI between mouse and human embryos indicates that although the onset of *XIST* expression may occur later in humans than in mice, the timeline and order of *XIST* expression initiation through actual XCI is similar in mice and men (Figure 2 and ⁶³).

Detection of *XIST* in Male Embryos

In contrast to previous reports on the detection of *XIST* in human embryos,^{42,43} we found a clear difference in *XIST* expression between male and female embryos (Figure 1). The previous data on human male embryos were obtained by nested PCR, which, in contrast to our FISH method, detects very low levels of *XIST* RNA. The large number of cycles in this method probably masked the difference in *XIST* levels between male and female embryos, leading to the erroneous conclusion that human embryos do not initiate XCI.^{42,43} In general, large differences in levels of *Xist/XIST* expression are found between embryos and between cells of a single embryo in both mice^{17,50} and humans (Table 1 and Figure S2). PCR detection of RNA expression in pooled embryos or even single embryos

can mask these variations,⁵⁰ and interpretations of these results are therefore unreliable. Thus, although PCR is more sensitive than the FISH method, the ability to localize the transcripts, as in a FISH experiment, is essential for studying the X-inactivation process.

The onset of *Xist/XIST* expression in male embryos, detected as a pinpoint signal by *Xist/XIST* RNA FISH, is slightly later than that in female embryos, both in mice and in humans (^{17,44,45}, and Table 1). Although our *XIST* RNA FISH probe did not overlap with the reported transcribed *TSIX* sequence (MIM 300181),⁶⁴ which is transcribed in the opposite orientation and may be involved in downregulation of *XIST*, we cannot exclude that the transient male and early female pinpoint signals are (in part) *TSIX* RNA. We therefore tested *TSIX* probes that had been used previously to detect *TSIX* in human fetal cells,⁶⁴ but we failed to obtain specific signals on either embryos or control fetal amniocytes from routine amniocentesis. Single-strand *XIST* and *TSIX* RNA probes produced too much background. Thus, whether human *TSIX* plays a role in the initiation of XCI in humans remains to be investigated.

Similar to those in mouse embryos, the pinpoint signals in human male embryos never accumulated to a complete cloud, indicating that initial *XIST* and/or *TSIX* expression does not lead to actual inactivation of the X chromosome in male embryos. (^{17,44,45}, and Table 1).

What Is the Mechanism of X Chromosome Inactivation?

Our data on human embryos show a variety of *XIST*-staining patterns that are similar to mouse *Xist* embryo patterns (^{13,15,17,19,44}, Table 1, and Figure S1). Slight discrepancies between published data can be explained by differences in detection sensitivity, because double *Xist* signals were observed more frequently when larger probes and increased signal amplification were used.^{13,15,44}

Both random XCI and imprinted XCI can result in patterns of XCI that are comparable to the patterns that we have observed in human embryos: Of the different models for random XCI,⁸ the stochastic model—in which every X chromosome has a certain probability of being inactivated, resulting in a majority of cells with one Xi, as well as cells with no Xi or two Xis—⁷ best explains our results. Only this model accommodates the variety of *XIST* expression patterns such as we have observed; other random XCI models view this variation as errors of the XCI mechanism. Imprinted XCI has a preferential expression of the paternal *Xist* allele but is only manifested in 70%–90% of the cells, whereas the remainder of the cells show no *Xist* expression. These *Xist*-negative cells may at a later stage inactivate the correct number of X chromosomes, akin to random XCI.⁶⁵ The human data could thus be explained by both imprinted and random mechanisms of XCI. The majority of blastomeres from our 8-cell human female embryos showed only one pinpoint *XIST* signal, suggesting a preference for activation of a single

XIST allele that could be indicative of imprinted XCI, similar to that which is observed in mice. The obvious solution would be parental tracing of the expressed *XIST* gene. Unfortunately, this was not possible with these anonymously donated embryos. Future experiments are necessary in order to determine whether XCI in human embryos is a random process or whether imprinted XCI is fully conserved.

In summary, we find X-associated accumulation of *XIST* RNA in female cleavage-stage and blastocyst embryos, together with transcriptional silencing and Xi-specific histone modifications. These results indicate that (at least part of) the X chromosome is silenced in human preimplantation embryos. Our findings therefore suggest that X-linked dosage compensation in mammalian preimplantation embryos is evolutionary conserved.

Supplemental Data

Supplemental Data include three figures and one table and can be found with this article online at <http://www.ajhg.org/>.

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Web Resources

The URLs for data presented herein are as follows:

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/>

University of California, Santa Cruz (USSC) Genome Browser, <http://genome.ucsc.edu/>

References

1. Brockdorff, N., Ashworth, A., Kay, G.F., Cooper, P., Smith, S., McCabe, V.M., Norris, D.P., Penny, G.D., Patel, D., and Rastan, S. (1991). Conservation of position and exclusive expression of mouse *Xist* from the inactive X chromosome. *Nature* 351, 329–331.
2. Brown, C.J., Ballabio, A., Rupert, J.L., Lafreniere, R.G., Grompe, M., Tonlorenzi, R., and Willard, H.F. (1991). A gene from the region of the human X inactivation centre is expressed exclusively from the inactive X chromosome. *Nature* 349, 38–44.
3. Penny, G.D., Kay, G.F., Sheardown, S.A., Rastan, S., and Brockdorff, N. (1996). Requirement for *Xist* in X chromosome inactivation. *Nature* 379, 131–137.
4. Heard, E., and Disteché, C.M. (2006). Dosage compensation in mammals: Fine-tuning the expression of the X chromosome. *Genes Dev.* 20, 1848–1867.
5. Lyon, M.F. (1961). Gene action in the X-chromosome of the mouse (*Mus musculus* L.). *Nature* 190, 372–373.
6. Avner, P., and Heard, E. (2001). X-chromosome inactivation: Counting, choice and initiation. *Nat. Rev. Genet.* 2, 59–67.
7. Monkhorst, K., Jonkers, I., Rentmeester, E., Grosveld, F., and Gribnau, J. (2008). X inactivation counting and choice is a stochastic process: Evidence for involvement of an X-linked activator. *Cell* 132, 410–421.
8. Starmer, J., and Magnuson, T. (2009). A new model for random X chromosome inactivation. *Development* 136, 1–10.
9. Graves, J.A. (1996). Mammals that break the rules: Genetics of marsupials and monotremes. *Annu. Rev. Genet.* 30, 233–260.
10. Takagi, N., and Sasaki, M. (1975). Preferential inactivation of the paternally derived X chromosome in the extraembryonic membranes of the mouse. *Nature* 256, 640–642.
11. West, J.D., Frels, W.I., Papaioannou, V.E., Karr, J.P., and Chapman, V.M. (1977). Development of interspecific hybrids of *Mus*. *J. Embryol. Exp. Morphol.* 41, 233–243.
12. Kay, G.F., Barton, S.C., Surani, M.A., and Rastan, S. (1994). Imprinting and X chromosome counting mechanisms determine *Xist* expression in early mouse development. *Cell* 77, 639–650.
13. Nesterova, T.B., Barton, S.C., Surani, M.A., and Brockdorff, N. (2001). Loss of *Xist* imprinting in diploid parthenogenetic preimplantation embryos. *Dev. Biol.* 235, 343–350.
14. Zuccotti, M., Boiani, M., Ponce, R., Guizzardi, S., Scandroglio, R., Garagna, S., and Redi, C.A. (2002). Mouse *Xist* expression begins at zygotic genome activation and is timed by a zygotic clock. *Mol. Reprod. Dev.* 61, 14–20.
15. Huynh, K.D., and Lee, J.T. (2003). Inheritance of a pre-inactivated paternal X chromosome in early mouse embryos. *Nature* 426, 857–862.
16. Mak, W., Nesterova, T.B., de Napoles, M., Appanah, R., Yamana, S., Otte, A.P., and Brockdorff, N. (2004). Reactivation of the paternal X chromosome in early mouse embryos. *Science* 303, 666–669.
17. Okamoto, I., Otte, A.P., Allis, C.D., Reinberg, D., and Heard, E. (2004). Epigenetic dynamics of imprinted X inactivation during early mouse development. *Science* 303, 644–649.
18. Sugawara, O., Takagi, N., and Sasaki, M. (1985). Correlation between X-chromosome inactivation and cell differentiation in female preimplantation mouse embryos. *Cytogenet. Cell Genet.* 39, 210–219.
19. Matsui, J., Goto, Y., and Takagi, N. (2001). Control of *Xist* expression for imprinted and random X chromosome inactivation in mice. *Hum. Mol. Genet.* 10, 1393–1401.
20. Lyon, M.F., and Rastan, S. (1984). Parental source of chromosome imprinting and its relevance for X chromosome inactivation. *Differentiation* 26, 63–67.
21. Tada, T., Obata, Y., Tada, M., Goto, Y., Nakatsuji, N., Tan, S., Kono, T., and Takagi, N. (2000). Imprint switching for non-random X-chromosome inactivation during mouse oocyte growth. *Development* 127, 3101–3105.
22. Okamoto, I., Arnaud, D., Le Baccon, P., Otte, A.P., Disteché, C.M., Avner, P., and Heard, E. (2005). Evidence for de novo imprinted X-chromosome inactivation independent of meiotic inactivation in mice. *Nature* 438, 369–373.
23. Dhara, S.K., and Benvenisty, N. (2004). Gene trap as a tool for genome annotation and analysis of X chromosome

- inactivation in human embryonic stem cells. *Nucleic Acids Res.* 32, 3995–4002.
24. Silva, S.S., Rowntree, R.K., Mekhoubad, S., and Lee, J.T. (2008). X-chromosome inactivation and epigenetic fluidity in human embryonic stem cells. *Proc. Natl. Acad. Sci. USA* 105, 4820–4825.
 25. Hall, L.L., Byron, M., Butler, J., Becker, K.A., Nelson, A., Amit, M., Itskovitz-Eldor, J., Stein, J., Stein, G., Ware, C., et al. (2008). X-inactivation reveals epigenetic anomalies in most hESC but identifies sublines that initiate as expected. *J. Cell. Physiol.* 216, 445–452.
 26. Hoffman, L.M., Hall, L., Batten, J.L., Young, H., Pardasani, D., Baetge, E.E., Lawrence, J., and Carpenter, M.K. (2005). X-inactivation status varies in human embryonic stem cell lines. *Stem Cells* 23, 1468–1478.
 27. Shen, Y., Matsuno, Y., Fouse, S.D., Rao, N., Root, S., Xu, R., Pellegrini, M., Riggs, A.D., and Fan, G. (2008). X-inactivation in female human embryonic stem cells is in a nonrandom pattern and prone to epigenetic alterations. *Proc. Natl. Acad. Sci. USA* 105, 4709–4714.
 28. Harrison, K.B., and Warburton, D. (1986). Preferential X-chromosome activity in human female placental tissues. *Cytogenet. Cell Genet.* 41, 163–168.
 29. Ropers, H.H., Wolff, G., and Hitzeroth, H.W. (1978). Preferential X inactivation in human placenta membranes: Is the paternal X inactive in early embryonic development of female mammals? *Hum. Genet.* 43, 265–273.
 30. Harrison, K.B. (1989). X-chromosome inactivation in the human cytotrophoblast. *Cytogenet. Cell Genet.* 52, 37–41.
 31. Goto, T., Wright, E., and Monk, M. (1997). Paternal X-chromosome inactivation in human trophoblastic cells. *Mol. Hum. Reprod.* 3, 77–80.
 32. Migeon, B.R., Wolf, S.F., Axelman, J., Kaslow, D.C., and Schmidt, M. (1985). Incomplete X chromosome dosage compensation in chorionic villi of human placenta. *Proc. Natl. Acad. Sci. USA* 82, 3390–3394.
 33. Looijenga, L.H., Gillis, A.J., Verkerk, A.J., van Putten, W.L., and Oosterhuis, J.W. (1999). Heterogeneous X inactivation in trophoblastic cells of human full-term female placentas. *Am. J. Hum. Genet.* 64, 1445–1452.
 34. Bamforth, F., Machin, G., and Innes, M. (1996). X-chromosome inactivation is mostly random in placental tissues of female monozygotic twins and triplets. *Am. J. Med. Genet.* 61, 209–215.
 35. Uehara, S., Tamura, M., Nata, M., Ji, G., Yaegashi, N., Okamura, K., and Yajima, A. (2000). X-chromosome inactivation in the human trophoblast of early pregnancy. *J. Hum. Genet.* 45, 119–126.
 36. Zeng, S.M., and Yankowitz, J. (2003). X-inactivation patterns in human embryonic and extra-embryonic tissues. *Placenta* 24, 270–275.
 37. Migeon, B.R., Schmidt, M., Axelman, J., and Cullen, C.R. (1986). Complete reactivation of X chromosomes from human chorionic villi with a switch to early DNA replication. *Proc. Natl. Acad. Sci. USA* 83, 2182–2186.
 38. Migeon, B.R., Axelman, J., and Jeppesen, P. (2005). Differential X reactivation in human placental cells: Implications for reversal of X inactivation. *Am. J. Hum. Genet.* 77, 355–364.
 39. Shao, C., and Takagi, N. (1990). An extra maternally derived X chromosome is deleterious to early mouse development. *Development* 110, 969–975.
 40. Lee, J.T. (2000). Disruption of imprinted X inactivation by parent-of-origin effects at Tsix. *Cell* 103, 17–27.
 41. Takagi, N. (2003). Imprinted X-chromosome inactivation: Enlightenment from embryos in vivo. *Semin. Cell Dev. Biol.* 14, 319–329.
 42. Daniels, R., Zuccotti, M., Kinis, T., Serhal, P., and Monk, M. (1997). XIST expression in human oocytes and preimplantation embryos. *Am. J. Hum. Genet.* 61, 33–39.
 43. Ray, P.F., Winston, R.M., and Handyside, A.H. (1997). XIST expression from the maternal X chromosome in human male preimplantation embryos at the blastocyst stage. *Hum. Mol. Genet.* 6, 1323–1327.
 44. Sheardown, S.A., Duthie, S.M., Johnston, C.M., Newall, A.E., Formstone, E.J., Arkell, R.M., Nesterova, T.B., Alghisi, G.C., Rastan, S., and Brockdorff, N. (1997). Stabilization of Xist RNA mediates initiation of X chromosome inactivation. *Cell* 91, 99–107.
 45. Latham, K.E., Patel, B., Bautista, F.D., and Hawes, S.M. (2000). Effects of X chromosome number and parental origin on X-linked gene expression in preimplantation mouse embryos. *Biol. Reprod.* 63, 64–73.
 46. Panning, B., Dausman, J., and Jaenisch, R. (1997). X chromosome inactivation is mediated by Xist RNA stabilization. *Cell* 90, 907–916.
 47. Hohmann, F.P., Macklon, N.S., and Fauser, B.C. (2003). A randomized comparison of two ovarian stimulation protocols with gonadotropin-releasing hormone (GnRH) antagonist cotreatment for in vitro fertilization commencing recombinant follicle-stimulating hormone on cycle day 2 or 5 with the standard long GnRH agonist protocol. *J. Clin. Endocrinol. Metab.* 88, 166–173.
 48. Plath, K., Fang, J., Mlynarczyk-Evans, S.K., Cao, R., Worringer, K.A., Wang, H., de la Cruz, C.C., Otte, A.P., Panning, B., and Zhang, Y. (2003). Role of histone H3 lysine 27 methylation in X inactivation. *Science* 300, 131–135.
 49. Repping, S., van Daalen, S.K., Brown, L.G., Korver, C.M., Lange, J., Marszalek, J.D., Pyntikova, T., van der Veen, F., Skaletsky, H., Page, D.C., et al. (2006). High mutation rates have driven extensive structural polymorphism among human Y chromosomes. *Nat. Genet.* 38, 463–467.
 50. Hartshorn, C., Rice, J.E., and Wangh, L.J. (2003). Differential pattern of Xist RNA accumulation in single blastomeres isolated from 8 cell stage mouse embryos following laser zona drilling. *Mol. Reprod. Dev.* 64, 41–51.
 51. Hartshorn, C., Rice, J.E., and Wangh, L.J. (2002). Developmentally-regulated changes of Xist RNA levels in single preimplantation mouse embryos, as revealed by quantitative real-time PCR. *Mol. Reprod. Dev.* 61, 425–436.
 52. Costanzi, C., and Pehrson, J.R. (1998). Histone macroH2A1 is concentrated in the inactive X chromosome of female mammals. *Nature* 393, 599–601.
 53. Chadwick, B.P., and Willard, H.F. (2002). Cell cycle-dependent localization of macroH2A in chromatin of the inactive X chromosome. *J. Cell Biol.* 157, 1113–1123.
 54. Chaumeil, J., Okamoto, I., Guggiari, M., and Heard, E. (2002). Integrated kinetics of X chromosome inactivation in differentiating embryonic stem cells. *Cytogenet. Genome Res.* 99, 75–84.
 55. Chow, J.C., Yen, Z., Ziesche, S.M., and Brown, C.J. (2005). Silencing of the mammalian X chromosome. *Annu. Rev. Genomics Hum. Genet.* 6, 69–92.
 56. Latham, K.E. (2005). X chromosome imprinting and inactivation in preimplantation mammalian embryos. *Trends Genet.* 21, 120–127.

57. Brown, C.J., and Robinson, W.P. (1997). XIST expression and X-chromosome inactivation in human preimplantation embryos. *Am. J. Hum. Genet.* *61*, 5–8.
58. Schultz, G.A., and Heyner, S. (1992). Gene expression in preimplantation mammalian embryos. *Mutat. Res.* *296*, 17–31.
59. Dobson, A.T., Raja, R., Abeyta, M.J., Taylor, T., Shen, S., Haqq, C., and Pera, R.A. (2004). The unique transcriptome through day 3 of human preimplantation development. *Hum. Mol. Genet.* *13*, 1461–1470.
60. Wells, D., Bermudez, M.G., Steuerwald, N., Thornhill, A.R., Walker, D.L., Malter, H., Delhanty, J.D., and Cohen, J. (2005). Expression of genes regulating chromosome segregation, the cell cycle and apoptosis during human preimplantation development. *Hum. Reprod.* *20*, 1339–1348.
61. Baart, E.B., Martini, E., van den Berg, I., Macklon, N.S., Galjaard, R.J., Fauser, B.C., and Van Opstal, D. (2006). Preimplantation genetic screening reveals a high incidence of aneuploidy and mosaicism in embryos from young women undergoing IVF. *Hum. Reprod.* *21*, 223–233.
62. Bretherick, K., Gair, J., and Robinson, W.P. (2005). The association of skewed X chromosome inactivation with aneuploidy in humans. *Cytogenet. Genome Res.* *111*, 260–265.
63. Okamoto, I., and Heard, E. (2006). The dynamics of imprinted X inactivation during preimplantation development in mice. *Cytogenet. Genome Res.* *113*, 318–324.
64. Migeon, B.R., Lee, C.H., Chowdhury, A.K., and Carpenter, H. (2002). Species differences in TSIX/Tsix reveal the roles of these genes in X-chromosome inactivation. *Am. J. Hum. Genet.* *71*, 286–293.
65. Huynh, K.D., and Lee, J.T. (2001). Imprinted X inactivation in eutherians: A model of gametic execution and zygotic relaxation. *Curr. Opin. Cell Biol.* *13*, 690–697.