X-Chromosome Inactivation in Cloned Mouse Embryos

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To study whether cloning resets the epigenetic differences between the two X chromosomes of a somatic female nucleus, we monitored X inactivation in cloned mouse embryos. Both X chromosomes were active during cleavage of cloned embryos, followed by random X inactivation in the embryo proper. In the trophectoderm (TE), X inactivation was nonrandom with the inactivated X of the somatic donor being chosen for inactivation. When female embryonic stem cells with two active X chromosomes were used as donors, random X inactivation was seen in the TE and embryo. These results demonstrate that epigenetic marks can be removed and reestablished on either X chromosome during cloning. Our results also suggest that the epigenetic marks imposed on the X chromosomes during gametogenesis, responsible for normal imprinted X inactivation in the TE, are functionally equivalent to the marks imposed on the chromosomes during somatic X inactivation.

Dosage compensation in mammals is achieved by the silencing of one X chromosome in female somatic cells, a process known as X inactivation (*1*). Before implantation, both X chromosomes in female embryos are transcriptionally active (*2–4*). Upon differentiation, one X chromosome is chosen for inactivation and silenced (*2–4*). An untranslated RNA, encoded by the *Xist* gene is both necessary and sufficient for the initiation of X-chromosome inactivation (*5–8*). The *Xist* RNA is stably transcribed from the center of inactivation (Xic) on the inactive X (X_i) and associates with the inactive chromosome over its entire length (*9–11*). *Xist* expression is involved in both the initiation of inactivation and the choice of which chromosome will be inactivated (*6, 7, 12, 13*). The X_i also differs from other chromosomes in histone content, histone H4 hypoacetylation, replication timing, and increased DNA methylation at promoter sequences (*14–18*). The maintenance of the X_i and active X (X_a) chromosomes is dependent on epigenetic marks imposed on the X chromosomes. For example, on the X_i, *Xist* is unmethylated and expressed, whereas on X_a it is methylated and silent (*19*). Induced demethylation of *Xist* on X_a leads to inappropriate *Xist* expression and silencing of X_a-linked genes (*20*).

X inactivation is random in the embryonic (epiblast) lineage of the embryo (*2–4*). In contrast, an unidentified mark, called an imprint, causes selective inactivation of the paternal X in the extraembryonic trophectoderm (TE), a tissue that contributes to the placenta and is necessary for implantation of the embryo (*6, 21–23*). An unresolved question is whether the epigenetic marks on X_a and X_i in somatic cells are functionally the same as those imposed on the X chromosomes during gametogenesis.

It has been suggested that the cloning of mammals by nuclear transfer requires epigenetic reprogramming of the differentiated state of the donor cell to a totipotent, embryonic ground state (*24, 25*). However, there is no direct molecular evidence for reprogramming. For example, it is unclear whether the epigenetic modifications of X_a and X_i in a female somatic cell are reversible during cloning (*24*). To address these questions, we used an X-linked reporter transgene (X^{GFP}), with a cytomegalovirus promoter driving expression of the green fluorescent protein (GFP). This reporter is subject to silencing by X inactivation (*26*) and allowed us to distinguish the state of X inactivation in different lineages of cloned embryos.

Control zygotes were isolated from normal females mated with hemizygous transgenic males (X^{GFP}/Y). The control embryos were cultivated in vitro and assessed for fluorescence at successive cleavage stages (Table 1). All embryos were dark at the one-, two-, four-, and early eight-cell stages, but 50%, presumably X/X^{GFP}, began to fluoresce after compaction. The other 50%, presumably X/Y, never fluoresced. These observations suggest that the paternally inherited transgene, carried in female embryos, was active during cleavage, consistent with normal paternal X-chromosome gene expression during preimplantation development (*2–4, 26*).

We tested for reactivation of the inactive X chromosome in cloned embryos generated by transfer of nuclei from X^{GFP} female tail tip or cumulus cells into enucleated oocytes (Table 2) (*27, 28*). Because of random X-chromosome inactivation in these somatic donor cells, 50% of the donor nuclei were expected to have an active X^{GFP} (X_a^{GFP}), and 50%, an inactive X^{GFP} (X_i^{GFP}). Green fluorescence was not seen in one-, two-, or four-cell nuclear transfer embryos but was observed in all cloned transgenic embryos by the morula and blastocyst stage (Table 1 and Fig. 1B). The fluorescence in cloned embryos was similar to that in normal female embryos carrying a paternally inherited transgene, suggesting that the GFP transgene, which was expected to be derived from a somatically inactivated X in 50% of all clones, was reactivated after nuclear transfer. However, because the X-inactivation status of the donor cells was not determined before nuclear transfer, it was possible that all morulae and blastocysts had been derived from transfer of nuclei with an X_a^{GFP}.

To confirm that X-chromosome reacti-

Table 1. Relative GFP fluorescence of preimplantation embryos. Control embryos were obtained by mating superovulated females with transgenic males then retrieving one-cell embryos. Embryos were cultured in vitro, and GFP fluorescence was monitored daily. Blastocytes derived from parthenogenetically activated nontransgenic oocytes were used as a negative control for fluorescence (*36*).

Parents or donor cell	Genotype	Relative GFP fluorescence				
		1-cell	2-cell	4- to 8-cell	Morula	Blastocyst
			Controls			
X/X × X^{GFP}/Y	X/X^{GFP}	–	–	–	++	+++
	X/Y	–	–	–	–	–
			Clones			
Tail tip cells	X^{GFP}/X	–	–	–	++	+++
Cumulus cells tail tip cells	X/X^{GFP}	–	–	–	++	+++
6TG-selected tail tip cells	X^{Hprt-}/X^{GFP}	–	–	–	++	+++

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vation occurred after nuclear transfer, we used a drug selection scheme to generate donor cells with a known X-inactivation status (Fig. 1A) (*29*). X^{GFP}/Y males were crossed with homozygous hypoxanthine-guanine phosphoribosyltransferase ($Hprt^-$) mutant females (X^{Hprt-}/X^{Hprt-}) and tail tip fibroblasts were derived from daughters carrying the transgene (X^{Hprt-}/X^{GFP}). The fibroblasts were grown in 6-thio-guanine (6TG) to select for cells with an X_i^{GFP} and in HAT (hypoxanthine, aminopterin, thymidine) medium to select for cells with an X_a^{GFP} (Fig. 1A). 6TG selection yielded a population in which >99% of cells had an X_i^{GFP}, as assayed by fluorescence-activated cell sorting (FACS) (Fig. 2A). When 6TG-selected cells were used as nuclear donors (Table 2), 100% of nuclear transfer morula and blastocysts expressed GFP in every cell. These results demonstrate that genes on X_i are reactivated after nuclear transfer (Table 1 and Fig. 1B).

The HAT-selected and 6TG-selected cells were used as donors for nuclear transfer to investigate the X-inactivation status in the TE and in the epiblast lineage. In contrast to 6TG selection of tail tip fibroblasts, which yielded a population in which >99% of cells had an X_i^{GFP}, HAT selection resulted in a population in which only 79% of cells expressed GFP (Fig. 2A) instead of the expected 100%. This result suggested either incomplete HAT selection or complete HAT selection with nonspecific silencing of the transgene in some tail tip donor cells even when present on the X_a. Consistent with effective HAT selection for cells with an X_a^{GFP}, 6TG counterselection of the previously HAT-selected cells resulted in death of all cells, indicating that every cell expressed the functional *Hprt* gene located on the same chromosome as the GFP transgene. Therefore, it is likely that the transgene, even when carried on the X_a, is subject to some nonspecific silencing in the adult cells. Nuclei from HAT- or 6TG-selected tail tip cells were injected into enucleated oocytes, and the reconstructed embryos were transferred to recipient females (Table 2). Embryos were recovered from recipient mothers at embryonic day 12.5 (E12.5), and GFP expression was assessed.

The GFP expression in the epiblast lineage was analyzed by FACS of mouse embryonic fibroblasts (MEFs) from cloned embryos derived from 6TG- or HAT-selected donor cells. In control experiments, 45 to 65% of MEFs isolated from individual, female embryos expressed GFP, consistent with random X inactivation (Fig. 2C; compare with Fig. 1D). In the cloned embryo 6TG 1, which was derived from a 6TG-selected (X_i^{GFP}) donor cell, 35% of MEFs expressed GFP (Fig. 2B), indicating that the X_i^{GFP} was reactivated in 35% of cells and inactive in 65% of cells. Similarly, two cloned embryos, HAT 1 and HAT 2, derived from HAT-selected (X_a^{GFP}) donor cells, expressed GFP in 35 and 78% of MEFs, respectively (Fig. 2B). Fibroblasts for FACs could not be obtained from clone 6TG 2; however, visual inspection revealed a mosaic pattern of GFP expression in the embryo proper, characteristic of random X inactivation (Fig. 1D). These results indicate that either X chromosome can be chosen for inactivation, regardless of the X-inactivation status of the donor nucleus, and they strongly suggest that X inactivation in the somatic lineage of clones is random.

To confirm random X inactivation in the epiblast of clones, we generated *M. musculus* (*M. musc*)/*M. castaneus* (*M. cast*) F_1 females. These F_1 females allowed the use of strain-specific simple sequence-length polymorphisms (SSLPs) in the X-linked genes *Xist* and *Grpr* to distinguish between expression from the two X chromosomes (*30, 31*). Some skewing of X inactivation was expected in these F_1 animals, because *M. cast* carries the strong Xce^c and *M. musc*, the weak $Xce^{a/b}$ alleles (*32*). This was evident in slightly higher expression of the *M. musc Xist* allele and lower expression of the *M. musc Grpr* allele in fibroblasts isolated from control F_1 female embryos (Fig. 3A).

Midgestation clones were derived from tail tip fibroblasts of F_1 females used as donors for nuclear transfer (Table 2). Similarly to controls, strain-specific reverse

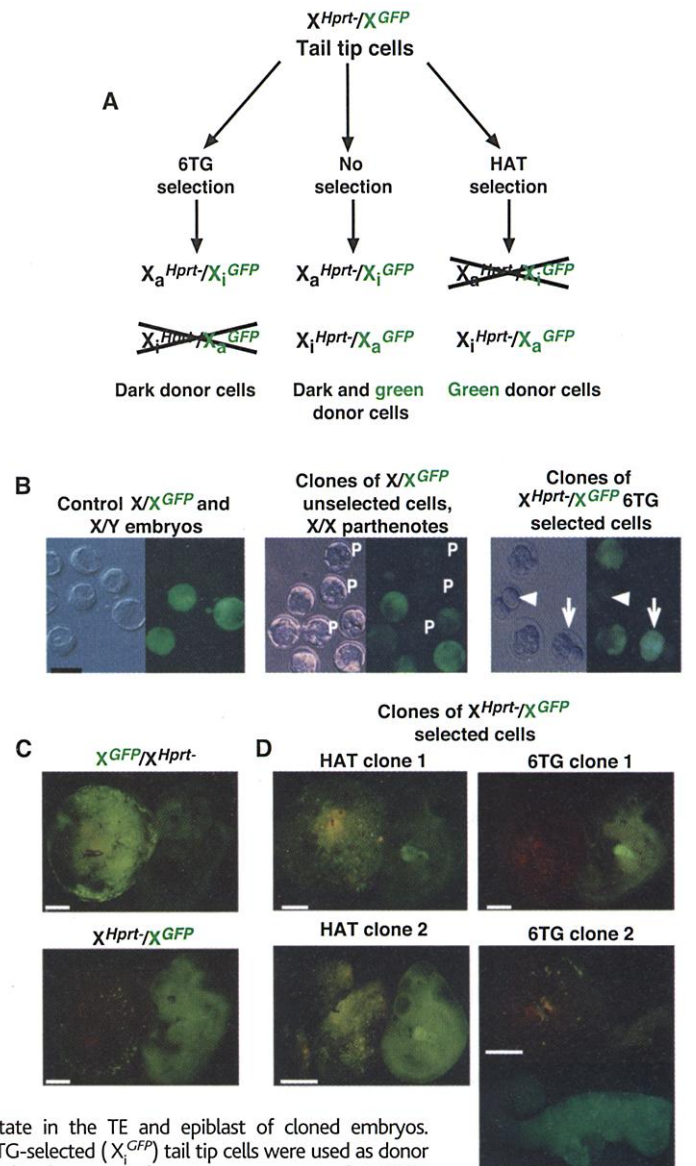

Fig. 1. X inactivation in cloned embryos observed with an X-linked GFP transgene. (**A**) Drug selection scheme for obtaining populations of tail tip cells with a predetermined X-inactivation state (*38*). (**B**) X-linked GFP transgene is reactivated after nuclear transfer. Bright- and dark-field photomicrographs of control and nuclear transfer blastocysts (Table 1). All transgenic control and nuclear transfer blastocysts clearly express GFP. P denotes parthenogenetic control blastocyst. Arrowhead points to a nuclear transfer embryo arrested at the two-cell stage; note lack of fluorescence. Arrow marks nuclear transfer embryo in which one blastomere of the two-cell embryo has arrested and does not express GFP, while the other blastomere continued to divide, and all cells are GFP-positive. Bar = 100 μm. (**C**) X-linked GFP transgene was expressed in the placentas of female E12.5 control embryos (X^{GFP}/X^{Hprt-}) with the maternally inherited transgene but was not expressed if the transgene was paternally inherited (X^{Hprt-}/X^{GFP}) (*39*). (**D**) X-inactivation state in the TE and epiblast of cloned embryos. HAT-selected (X_a^{GFP}) or 6TG-selected (X_i^{GFP}) tail tip cells were used as donor cells for nuclear transfer, and embryos and placentas were recovered at E12.5. Both embryos and placentas derived from the transfer of HAT-selected (X_a^{GFP}) donor were fluorescent. In contrast, 6TG-selected (X_i^{GFP}) nuclei gave rise to GFP-expressing embryos with placentas that did not express GFP. Bars = 1 mm.

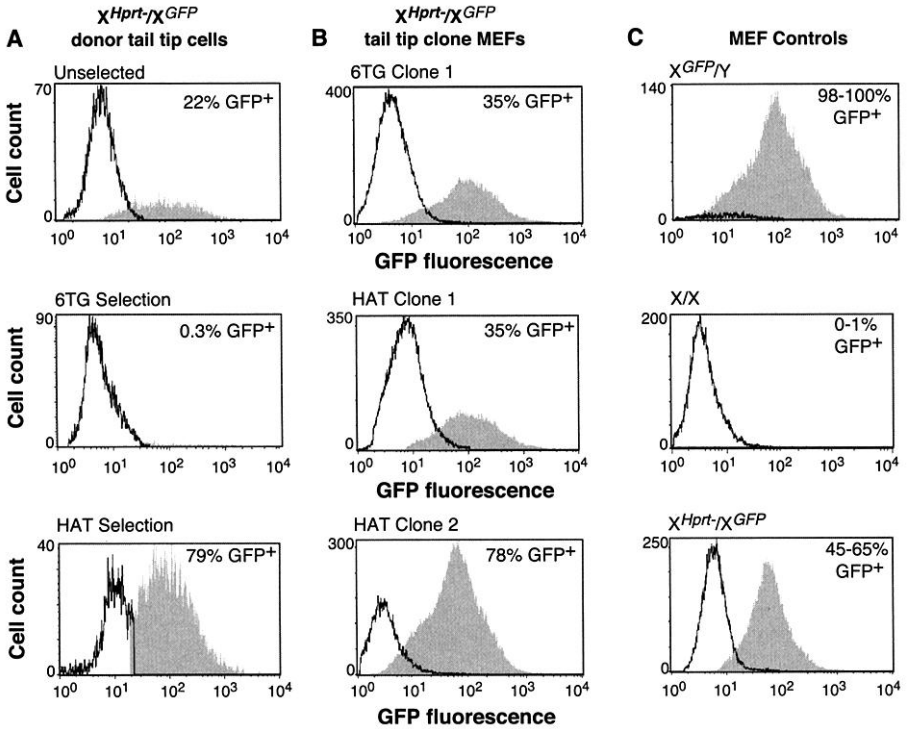

Fig. 2. Analysis of GFP expression by FACS (*40*). The Fl-1 "high" population contained GFP$^+$ cells and is displayed in gray on the histogram; the FL-1 "low" population was GFP$^-$ and is represented by the black line overlay. (**A**) GFP expression in unselected and selected tail tip donor cells prepared as in Fig. 1A. (**B**) GFP expression in mouse embryonic fibroblasts (MEFs) derived from E12.5 cloned embryos (Table 2 and Fig. 1D). (**C**) GFP expression in MEFs from control E12.5 embryos. For controls %GFP$^+$ is displayed as the range observed in the number of cells expressing GFP from $n >$ 10 individual embryos.

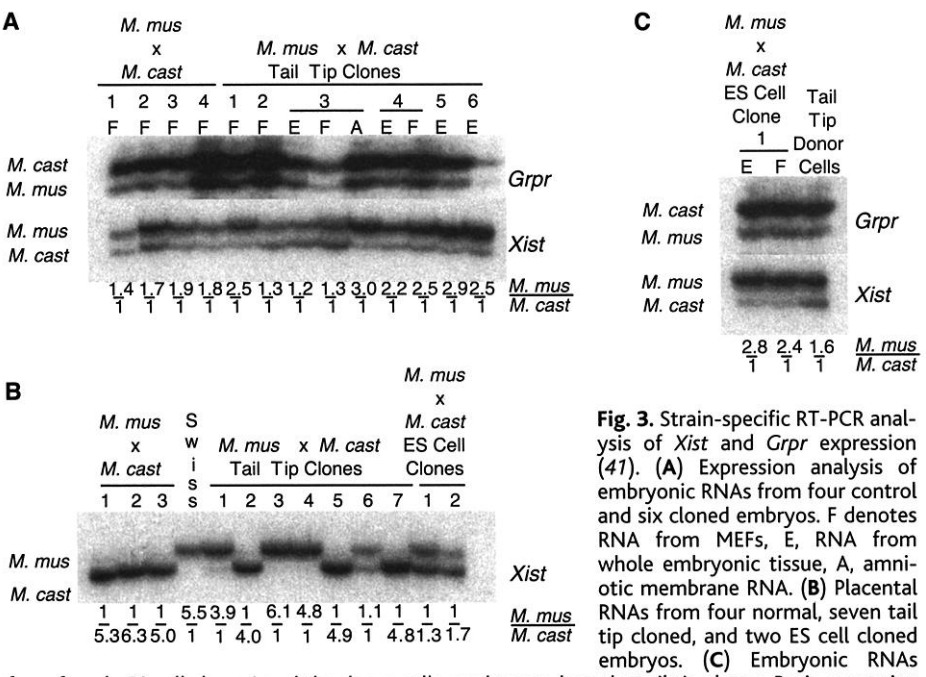

Fig. 3. Strain-specific RT-PCR analysis of *Xist* and *Grpr* expression (*41*). (**A**) Expression analysis of embryonic RNAs from four control and six cloned embryos. F denotes RNA from MEFs, E, RNA from whole embryonic tissue, A, amniotic membrane RNA. (**B**) Placental RNAs from four normal, seven tail tip cloned, and two ES cell cloned embryos. (**C**) Embryonic RNAs from female ES cell clone 1 and the donor cells used to produce the tail tip clones. Ratio appearing beneath each lane represents quantification of the ratio of *M. musc* to *M. cast Xist* expression as measured by phosphoimaging.

transcription–polymerase chain reaction (RT-PCR) showed that both *M. musc* and *M. cast Xist* and *Grpr* transcripts were expressed in the clones, with the *M. cast* X more active than the *M. musc* X. These results confirm that X inactivation is random in the somatic lineage of cloned female embryos (Fig. 3A).

To assess X inactivation in the TE lineage, we investigated GFP expression in the placentas of clones derived from HAT- or 6TG-selected donor tail tip cells. Control hemizygous female embryos inheriting the transgene from their mother (X^{GFP}/X^{Hprt-}) were expected to express GFP in all TE cells because of imprinted X inactivation, giving rise to fluorescent placentas (Fig. 1C) (*26*). Conversely, normal hemizygous female embryos inheriting the transgene from their fathers (X^{Hprt-}/X^{GFP}) did not express GFP in the TE and had dark placentas because of imprinted X inactivation (Fig. 1C) (*26*). Two clones recovered at E12.5 from HAT (X_a^{GFP})-selected donor cells had fluorescent placentas and embryos (Table 2 and Fig. 1D). In contrast, two cloned embryos derived from 6TG (X_i^{GFP})-selected donor cells had nonfluorescent placentas but showed fluorescence in the embryo proper (Table 2 and Fig. 1D). Two additional implants containing only placentas were also recovered (Table 2). One of these placentas, derived from a HAT-selected donor, was fluorescent, the other from a 6TG-selected donor was not. These observations suggest that X inactivation in the TE lineage of cloned embryos is nonrandom and that the status of X inactivation in the donor nucleus determines which X chromosome is active or inactive.

We also analyzed allele-specific expression in placentas of cloned female embryos derived from F_1 *M. musc*/*M. cast* tail tip cells. RT-PCR was performed on seven placentas whose embryos were all cloned from cells derived from a single F_1 female (Fig. 3B). Three of the seven clones expressed only the *M. musc Xist* allele (clones 1, 3, and 4) while three expressed only the *M. cast Xist* allele (clones 2, 5, and 7). As expected, all control placentas (n = 3) expressed only the paternal *M. cast Xist* allele. The placenta from one tail tip clone showed expression of both *Xist* alleles (clone 6). This embryo had the smallest placenta and was severely malformed, suggesting that perhaps inappropriate reprogramming of the genome had occurred. These results confirm that X-chromosome inactivation in the TE lineage of cloned embryos is nonrandom.

Early work has shown that both X chromosomes are active in female ES cells and that one X is chosen for inactivation only after induction of differentiation (*5, 33, 34*). It is therefore presumed that neither X chromosome in undifferentiated ES cells carries a distinguishable epigenetic mark. This hypothesis predicts that transfer of ES cell nuclei into enucleated oocytes might produce clones with random X inactivation in both the TE and epiblast lineage. To test this, we produced clones from a female F_1

Table 2. Development of nuclear transfer embryos from enucleated oocytes injected with various donor cells. Preparation of donor cells and nuclear transfer into enucleated oocytes was performed as previously described (*27*, *28*, *37*). All midgestation tail tip and ES cell clones were derived from two-cell embryos transferred to the oviduct of recipient foster mothers. Morulae and blastocysts produced for in vitro observation of GFP expression were discarded. NA, not applicable.

Female donor cells	Oocytes reconstructed	Pronuclear formation (% reconstructed)	2-cell (% pronuclear)	Morula blastocyst (% pronuclear)	Embryos transferred (% pronuclear)	Midgestation embryos recovered (% transferred)
Cumulus cells						
X/X^{GFP}	76	56 (74)	38 (68)	17 (30)	N/A	N/A
Tail tip cells						
X^{GFP}/X	26	12 (46)	10 (83)	8 (67)	N/A	N/A
X/X^{GFP}	131	63 (48)	56 (89)	31 (49)	N/A	N/A
Tail tip cells X^{Hprt-}/X^{GFP}						
HAT	162	82 (51)	64 (78)	N/A	61 (74)	3 (5)
6TG	233	187 (80)	154 (82)	N/A	135 (72)	3 (2.2)
6TG	81	34 (42)	30 (88)	9 (30)	N/A	N/A
Tail tip cells						
C57/*M. cast*	704	218 (31)	191 (88)	N/A	189 (87)	7 (3.7)
ES cells						
129/*M. cast*	142	36 (25)	35 (97)	N/A	34 (94)	2 (6)

M. musc/*M. cast* ES cell line (Table 2). Two live implants were recovered at E13.5, one containing a normal embryo with placenta, and the other containing a normal placenta with a growth-retarded fetus. Allele-specific RT-PCR on RNAs from these normal appearing placentas revealed expression of both *Xist* alleles, suggesting that random X inactivation had occurred (Fig. 3B). These results imply that clones derived from donor cells that have not yet undergone X inactivation do not carry an epigenetic mark on either X that would predispose one of them to be inactivated in the TE lineage. Expression analysis of both *Xist* and *Grpr* in the epiblast of the first female ES cell clone showed, as expected, that random X inactivation also occurred in the embryo proper (Fig. 3C).

In summary, we have demonstrated that X inactivation is random in the epiblast lineage of cloned mice. This random inactivation indicates that the epigenetic marks that distinguish X_a and X_i in somatic cells can be removed and reestablished on either X during the cloning process, resulting in random X inactivation in the cloned animal. In contrast, the epigenetic marks on X_a and X_i in the donor cell are not removed in the TE lineage of the clone and predispose the X_a of the donor cell to be active and the donor X_i to be inactive. However, this somatically acquired mark is ignored during early cleavage stages of development as both X chromosomes are expressed. This expression is consistent with that in normal cleavage development, where both *Xist* alleles are expressed and both X chromosomes are active (*34*). Similarly, the gametic imprint in normal development is removed only after allocation of the TE lineage, but before allocation of the epiblast lineage, leading to imprinted X inactivation in extraembryonic tissue, and random inactivation in the embryo proper (*23*).

The nature of the marks responsible for imprinted X inactivation in the TE lineage is not known (*35*). However, our results imply that the epigenetic marks acquired during random X inactivation in the somatic lineage are functionally equivalent to the marks acquired during gametogenesis, as they both can determine which X will be active and which will be inactive in the TE lineage. Finally, clones derived from ES cells that contain two active and as yet unmarked X chromosomes showed random X inactivation in both the TE and epiblast lineages. This observation indicates that, in the TE, random instead of imprinted X inactivation occurs in the absence of somatic, or gametic, epigenetic modification.

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36. Parthenogenetic blastocysts were obtained by activating unmanipulated B6D2 F_1 recipient oocytes for 5.5 hours in Ca^{2+}-free medium containing 10 mM Sr^{2+} and 5 μg/ml Cytochalasin B.
37. Two-cell embryos (n = 10 to 15) were transferred to each oviduct of Swiss Webster recipients mated with vasectomized males the previous evening. After 11 to 12 days, recipient females were killed and analyzed for the presence of living implants.
38. A tail tip biopsy was prepared from a 4-week-old X^{Hprt-}/X^{GFP} female, freed of skin, minced into small pieces and placed in culture. Tail tip cells were cultured as previously described (*27*) or in the presence of HAT (Stratagene, La Jolla, CA) or 2 μg/ml 6TG (*27*).
39. One-cell zygotes were obtained from superovulated X^{GFP}/X^{GFP} or X^{Hprt-}/X^{Hprt-} females mated with X^{Hprt-}/Y or X^{GFP}/Y males and cultured overnight. Embryos were transferred to recipient females (*37*). In the case of paternal transmission of the transgene, all green embryos were assumed to be female, and placental fluorescence was observed. In the case of maternal transgene transmission, embryos were photographed and embryonic fibroblasts were prepared as described (Fig. 2C). FACS profile of GFP expression was then used to identify the sex of the recovered embryo.
40. Briefly, cells were trypsinized, washed once in Dulbecco's modified Eagle's medium (DMEM) with 15% fetal calf serum and resuspended in DMEM with 10 μg/ml propidium iodide (PI). Live cells were gated on the basis of size and PI exclusion. When Fl-1 (GFP fluorescence) and Fl-2 (autofluorescence) were compared, two populations of cells were observed and gated.
41. Strain-specific RT-PCR was performed with primers previously described (*30*, *31*). Briefly, first strand cDNA was primed from deoxyribonuclease-treated RNA using random hexamer primers. We then carried out 27 rounds of amplification with end-labeled forward primer and cold reverse primer (linear range) for *Xist* and *Grpr* on both RT^+ and RT^- samples. PCR products were never observed in RT^- controls. PCR products were purified and either applied directly to a 6% sequencing gel (*Grpr*) or first digested with Taq I (*Xist*) and then applied.

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