

Initiation of epigenetic reprogramming of the X chromosome in somatic nuclei transplanted to a mouse oocyte

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The active and inactive X chromosomes have distinct epigenetic marks in somatic nuclei, which undergo reprogramming after transplantation into oocytes. We show that, despite the disappearance of *Xist* RNA coating in 30 min, the epigenetic memory of the inactive X persists with the precocious appearance of histone H3 trimethylation of lysine 27 (H3-3meK27), without the expected colocalization with Eed/Ezh2. Subsequently, *Xist* reappears on the original inactive X, and the silent *Xist* on the active X undergoes re-activation, resulting in unusual biallelic *Xist* RNA domains. Despite this abnormal *Xist* expression pattern, colocalization of H3-3meK27 and Eed is thereafter confined to a single *Xist* domain, which is presumably on the original inactive X. These epigenetic events differ markedly from the kinetics of preferential paternal X inactivation in normal embryos. All the epigenetic marks on the X are apparently erased in the epiblast, suggesting that the oocyte and epiblast may have distinct properties for stepwise programming of the genome.

Keywords: epigenetic reprogramming; histone modification; X inactivation; *Xist*

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INTRODUCTION

The reprogramming of a somatic nucleus by oocyte cytoplasm is a prime example of a change in genome function that requires appropriate epigenetic modifications. In mammals, dosage compensation of X-linked genes in females occurs through the

inactivation of one of the two X chromosomes (Lyon, 1961). Initially, the maternal X chromosome is imprinted to remain active (Xm* to denote the imprint) whereas the paternal X (Xp) is preferentially inactivated in pre-implantation embryos (Huynh & Lee, 2003; Mak *et al*, 2004; Okamoto *et al*, 2004) and this state is maintained in extraembryonic tissues (Takagi & Sasaki, 1975). By contrast, there is random inactivation of either the Xp or the Xm in the embryo proper after re-activation of the inactive paternal X chromosome (Mak *et al*, 2004; Okamoto *et al*, 2004). Thus, a female somatic donor nucleus when transplanted into an oocyte contains a randomly selected inactive X (Xi) and an active X (Xa), but no maternal imprint, which differs from the parental X chromosomes, Xp and Xm*, in a normal zygote.

Recent advances have explained in greater detail the earliest molecular events that lead to stepwise conversion of an entire X chromosome from a euchromatic to a heterochromatic conformation during pre-implantation development (Heard, 2004). The process is regulated by the expression and *cis*-localization of a large noncoding RNA, the X inactive transcript (*Xist*; Brockdorff *et al*, 1991; Brown *et al*, 1991; Panning *et al*, 1997; Sheardown *et al*, 1997). *Xist* RNA, in turn, recruits chromatin-modifying factors, including the PRC2 Polycomb group (PcG) complex Eed/Ezh2 (Enx-1), which results in chromosome-wide H3-3meK27 methylation (Plath *et al*, 2003; Silva *et al*, 2003; Kohlmaier *et al*, 2004; Rougeulle *et al*, 2004). As shown recently, the PRC1 (Mel18/Ring1B) complex also becomes enriched on the Xi in early development (de Napoles *et al*, 2004). Given these well-characterized epigenetic marks associated with X inactivation, this process is highly suited for investigations into the events underlying reprogramming of a somatic cell nucleus by oocyte cytoplasm.

In a previous study on cloned mouse embryos (Eggan *et al*, 2000), a green fluorescent protein transgene carried by a somatic-cell-derived Xi was shown to be re-activated after transfer into an enucleated oocyte, but then became preferentially inactivated in extraembryonic tissues. Conversely, in the fetus random X inactivation was observed, suggesting that the original epigenetic marks on Xi were erased on epiblast formation. However, the early crucial molecular events underlying re-activation and

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subsequent inactivation at the level of both *Xist* expression and X-linked chromatin modifications were largely unknown at that time, which precluded detailed examination of the fate of the Xi and Xa in somatic nuclei after transplantation into the oocyte. To gain insight into the early reprogramming events, we have examined the initial epigenetic changes associated with Xi and Xa after transplantation of female somatic nuclei into enucleated mouse oocytes (SCNT embryos). Our study shows that the X chromosome reprogramming initiation involves unusual events starting only 30 min after nuclear transfer. The kinetics of these events differ markedly from those in normal female embryos.

RESULTS AND DISCUSSION

We isolated tail-tip fibroblasts from adult female mice as the source of somatic nuclei. The characteristics of the Xi in these somatic cells were verified using immunofluorescence (IF) combined with *Xist* RNA fluorescence *in situ* hybridization (FISH) analysis. In these somatic nuclei, the Xi was found to be coated with *Xist* RNA in almost 100% of tail-tip cells. The H3-3meK27 is another putative epigenetic mark on Xi, which is introduced during development (Erhardt *et al*, 2003; Plath *et al*, 2003; Silva *et al*, 2003). Although we did not overtly find all the somatic cells enriched in H3-3meK27 (supplementary Fig S1 online), we detected between 20% and 40% of the cells in which the H3-3meK27 mark colocalized with *Xist* RNA. This is similar to results reported for adult-ear fibroblasts (Rougeulle *et al*, 2004), suggesting that levels of H3-3meK27 could be lower, or perhaps less well detected by antibodies, in fully differentiated adult cells than in early embryonic cells. Nevertheless, these observations show that somatic nuclei apparently have appropriate epigenetic modifications associated with Xi.

The tail-tip nuclei were transferred to enucleated oocytes by electro-fusion (Ogura *et al*, 2000) and were then examined by IF and *Xist* RNA FISH at various stages after their transfer. Previous studies have shown that during normal pre-implantation development, *Xist* from Xp is first expressed at the two-cell stage when principal embryonic gene activation occurs, followed by accumulation of the RNA in *cis* at the four-cell stage (Okamoto *et al*, 2004). The PcG proteins, Eed and Ezh2, accumulate on the Xp from the 16-cell stage onwards and this is accompanied by the appearance of H3-3meK27 and H3-K9 methylation marks on this chromosome (Okamoto *et al*, 2004). However, in embryos resulting from transplantation of tail-tip somatic nuclei, the *Xist* RNA coating that was initially associated with Xi disappeared only 30 min after SCNT to an enucleated oocyte, and it was completely absent at 2 h (Fig 1A–C; supplementary Table S1 online). This suggests that the oocyte cytoplasm contains factors that induce *Xist* RNA delocalization or destabilization.

At the two-cell stage—which is when in normal female embryos (control embryos), the paternally inherited *Xist* allele on Xp usually begins its expression—no *Xist* RNA was detectable in SCNT embryos (Fig 1D). However, there was a striking and precocious appearance of an H3-3meK27 domain that occurred within 8 h of SCNT, which was detected as a single nuclear domain on one X chromosome (Fig 2; supplementary Table S1 online). This early detection of H3-3meK27 was not associated with the Eed protein, a component of the Polycomb PRC2 complex (Fig 2A–C), as far as could be detected by IF. This is surprising, as the PRC2 complex is responsible for laying down the

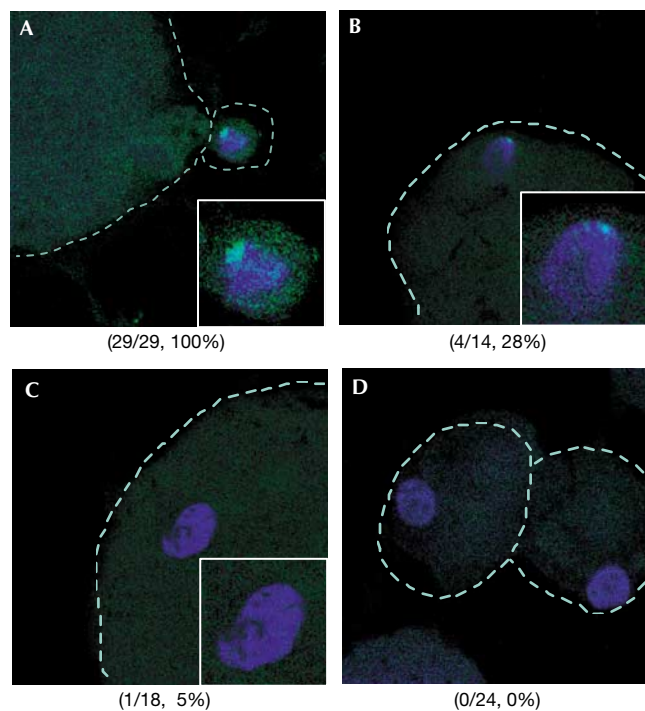


Fig 1 | Progressive loss of *Xist* RNA coating from somatic nuclei in SCNT embryos. Shown are RNA fluorescence *in situ* hybridization for *Xist* RNA coating (green) and DNA staining with TOTO3 (blue). (A) An *Xist* RNA domain was seen in somatic donor nucleus before fusion with enucleated oocyte (*Xist* RNA domain/total, 29/29). (B) At 30 min after SCNT, the *Xist* domain disappeared from a significant number of cells (*Xist* RNA domain/total, 4/14). (C) After 2 h, there was a further reduction in the number of cells with *Xist* RNA domains in SCNT embryos (*Xist* RNA domain/total, 1/18). (D) The coating of *Xist* RNA was completely lost at the two-cell stage (*Xist* RNA domain/total, 0/24).

H3-3meK27 mark in normal embryos (Silva *et al*, 2003), although at a slightly later stage of normal development. The appearance of Eed at the H3-3meK27 domain was first detected at the two-cell stage in SCNT embryos (Fig 2D) and persisted thereafter (supplementary Fig S3A,B online; supplementary Table S2 online), unlike the situation in normal female fertilized embryos in which Eed first accumulates on the paternal X chromosome at the morula stage (Fig 2E; supplementary Fig S3C,D online). Therefore, we reasoned that the early appearance of the H3-3meK27 mark in SCNT embryos may represent an enduring mark on the original Xi in donor somatic nuclei, which we detected at least in a proportion of the donor cells before transfer to the oocyte. Its precocious appearance may be the result of an ‘unmasking’ or exposure of the epitope, resulting from the marked changes that affect the chromatin of the somatic-cell nucleus in the oocyte cytoplasm after activation. Consistent with this, we observed a marked overall loss in the somatic-cell nucleus of histone H3 methylation at the amino-acid residue immediately adjacent to the H3 Lys-27, namely H3-R26, within 30 min of SCNT (supplementary Fig S2 online; supplementary Table S1 online). These dynamic changes in histone modifications are likely to be symptomatic of significant reprogramming of the somatic nucleus.

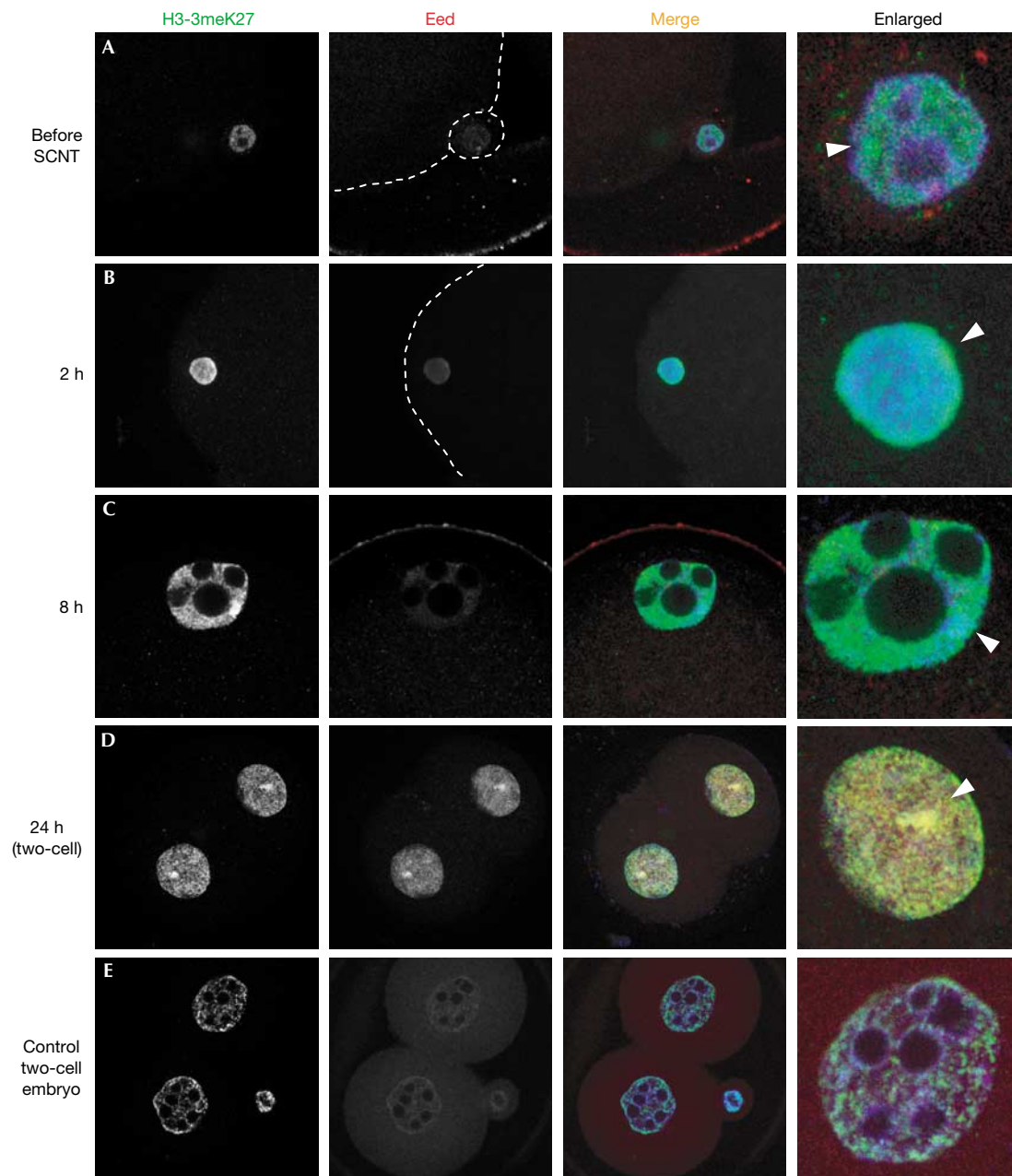


Fig 2 | Detection of H3-3meK27 and Eed in one- to two-cell SCNT embryos. Embryos were immunostained at 0, 2, 8 and 24 h after SCNT with H3-3meK27 (green) and Eed (red) antibodies. The white arrowheads indicate a domain of H3-3meK27. (A–C) A weak signal for H3-3meK27 was detected without Eed in somatic nucleus before transfer to oocytes, and also at 8 h after nuclear transfer. (D) At 24 h after SCNT (two-cell stage), both H3-3meK27 and Eed accumulated to a single domain in each blastomere. (E) There was no similar detectable signal or colocalization of H3-3meK27 and Eed in the two-cell stage control embryo.

An additional unexpected observation in SCNT embryos was the appearance of a single domain of dimethylation of histone H3 at lysine 9 (H3-2meK9) after 2 h, which colocalized with histone methyltransferase, G9a, as well as with H3-3meK27 (supplementary Figs S4,S5 online; supplementary Table S1 online). Both H3-2meK9 and G9a were not initially detectable in donor somatic nuclei (supplementary Fig S4A online). However, the accumulation of both H3-2meK9 and G9a rapidly disappeared at the two-cell stage (supplementary Fig S4D online; supplementary

Table S1 online), which occurred coincidentally with the accumulation of Eed on the X chromosome (Fig 2D). Similar sequences of early epigenetic modifications are not observed in control embryos (not shown), although H3-2meK9 modification on the X chromosome is observed at the late morula stage (Heard *et al*, 2001; Rougeulle *et al*, 2004). The G9a enzyme has been reported to have a predominantly H3-K9 methylation activity although biochemical analyses show a weak H3-K27 methylation activity (Peters *et al*, 2003). The biological significance of this

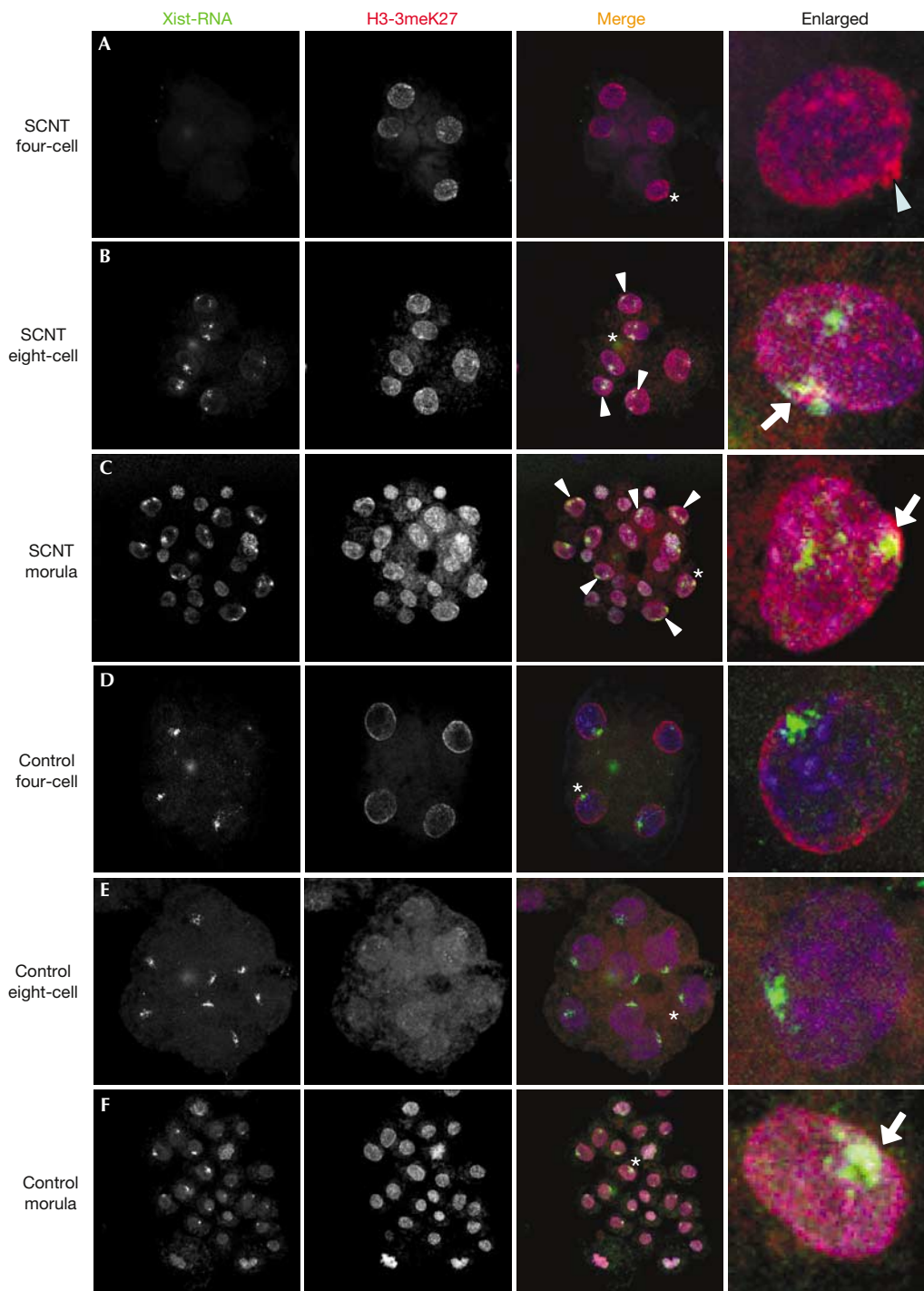


Fig 3 | *Xist* RNA coating in relation to H3-3meK27 from four-cell stages to morula stages. (A–C) SCNT embryos and (D–F) control embryos were immunostained with H3-3meK27 (red) combined with *Xist* RNA fluorescence *in situ* hybridization (green). DNA was stained with TOTO3 (shown in blue). White arrowheads indicate the blastomeres that showed two domains of *Xist* RNA coating. The magnified field is indicated in the figure with asterisks and white arrows indicate colocalization of *Xist* RNA and H3-3meK27. (A) *Xist* RNA was not detectable in most of the SCNT embryos at the four-cell stage. The light-blue arrowhead indicates a domain of H3-3meK27 on the X chromosome. (B) Two domains of *Xist* RNA coating were observed from six- to eight-cell embryos, but only one of the two domains colocalized with H3-3meK27 in more than half the embryos examined. (C) The two domains of *Xist* RNA coating continued in morula, and in early blastocyst (not shown). (D) Control *in vitro*-fertilized embryos show a single domain of *Xist* RNA coating at the four-cell stages, which continued to (E) eight-cell stages and (F) morula stages, which do not colocalize with H3-3meK27 except in some blastomeres of morula (white arrow).

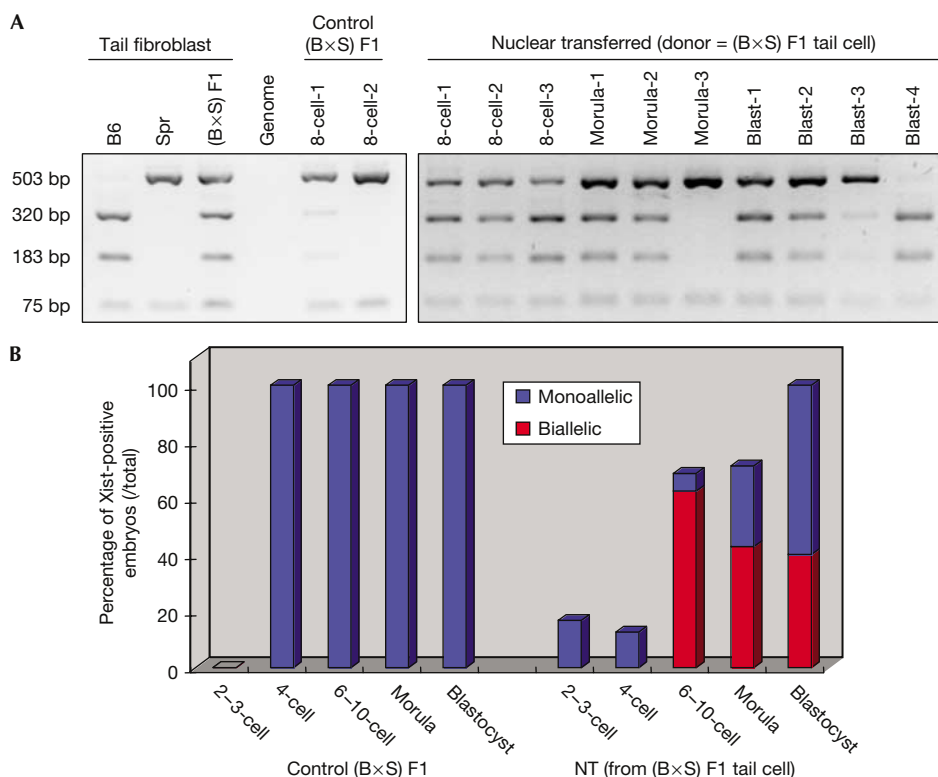


Fig 4 | Verification of allelic expression of *Xist* transcript in SCNT embryos. (A) Complementary DNA from individual SCNT embryo was PCR amplified using *Xist* transcript-specific primer sets, and the PCR products were digested with *HindIII*. Tail fibroblast cDNAs from C57BL/6 (B6), *Mus spretus* (Spr) and (B6 × Spr) F1 and cDNA from (B6 × Spr) F1 *in vitro*-fertilized (IVF) embryos were used as positive controls. (B) Summary of allelic expression of *Xist* transcript. In the control IVF embryos ((B6 × Spr) F1), monoallelic *Xist* expression (blue bar) was detected from the four-cell stage onwards. In contrast, *Xist* expression in the SCNT embryo was delayed and mainly detected from the eight-cell stage onwards (68% of total analysed embryos), and the proportion of cells with *Xist* increased progressively at the morula (71%) and the blastocyst stage (100%). Among the cells showing *Xist* RNA domains, 91% at the eight-cell stage, 60% at the morula stage and 40% at the blastocyst stage showed biallelic *Xist* RNA domains in SCNT embryos (denoted by red bars).

observation is unknown at present, but it serves to illustrate the epigenetic perturbations that occur during the early stages of development of SCNT embryos.

Xist RNA expression and histone modifications of the X chromosome were also investigated at subsequent stages of development. Here, we made another unexpected finding, as two domains of *Xist* RNA could be found in most of the cells from the eight-cell stage onwards in SCNT embryos (Fig 3B,C; supplementary Table S2 online). This suggests that both the Xi that originally expressed *Xist* and the Xa on which *Xist* gene was silent are capable of activating *Xist* expression at this time. This type of *Xist* expression pattern is never observed in control female embryos generated by *in vitro* fertilization of oocytes (Fig 3D–F), where a single *Xist* domain is seen, in pre-implantation embryos, to be associated with Xp (see below).

To confirm biallelic *Xist* expression in SCNT embryos, we exploited the presence of polymorphisms between the two *Xist* alleles in the donor nucleus from an interspecific cross between C57BL/6 and *Mus spretus* mice (Kay et al, 1993). In the resulting SCNT embryos, allele-specific reverse transcription–PCR (RT–PCR)/restriction fragment length polymorphism (RFLP) was performed at different embryonic stages. Biallelic *Xist* expression was

detectable in most of the SCNT embryos at the eight-cell stage, which confirms the appearance of two *Xist* RNA domains that were detected by IF (Fig 4). In control *in vitro*-fertilized (IVF) embryos, there was exclusive monoallelic expression of the paternally derived *Xist* locus, consistent with the preferential inactivation of the paternal X chromosome in pre-implantation embryos. Given that the SCNT embryos are derived from a single somatic cell nucleus in which either one or the other *Xist* allele was expressed, this result shows that both the previously active and inactive *Xist* alleles are expressed at this stage.

Although biallelic *Xist* expression was observed from the eight-cell stage, H3-3meK27 remained restricted to only one X chromosome (Fig 3B,C; supplementary Table S2 online). This is presumably the previously inactive X chromosome, as the histone modification mark is present before and after SCNT of female donor cell nuclei. Thus, H3-3meK27 does not seem to be solely dependent on *Xist* expression, as there are two *Xist* RNA domains in the cell, but only one domain with accumulated H3-3meK27. More importantly, the H3-3meK27 mark could not be detected when either a male somatic donor nucleus or a donor nucleus from a pluripotent female embryonic stem cell was used (supplementary Fig S6 online). Donor nuclei from both these

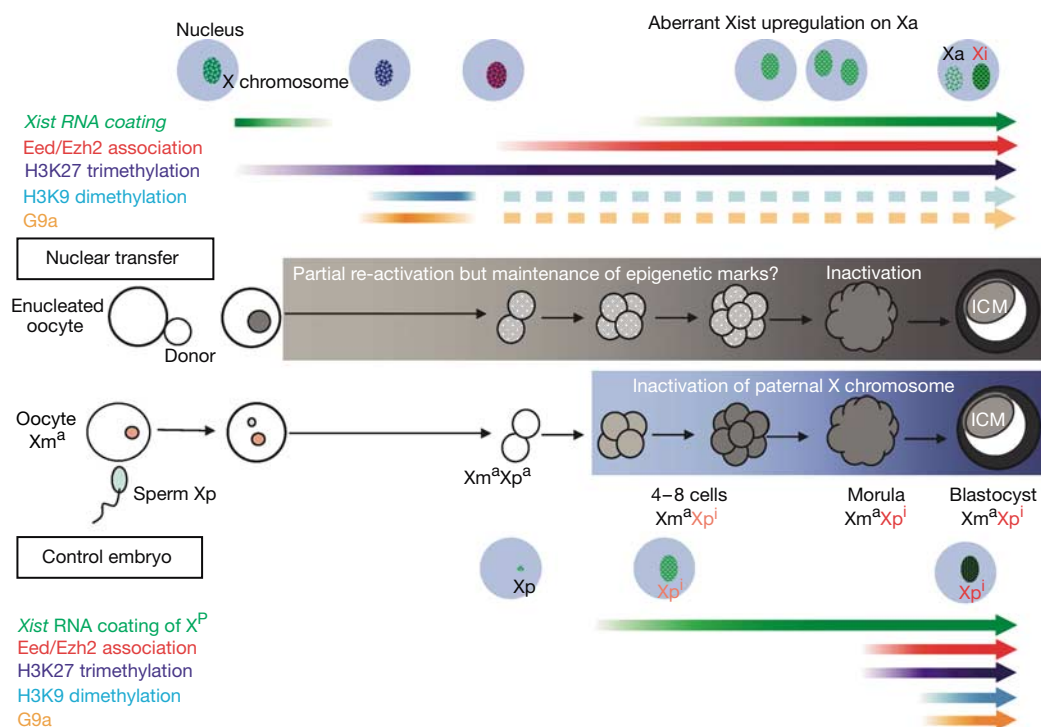


Fig 5 | Comparative kinetics of epigenetic modifications of X chromosome in SCNT (top) and control embryos (bottom). There is rapid disappearance of *Xist* RNA coating from Xi in SCNT embryos commencing within 30 min after activation of the reconstructed embryo. *Xist* RNA re-appeared in these embryos from the four- to eight-cell stages, and colocalized with the H3-3meK27 and Eed/Ezh2 protein complex. Colocalization of G9a histone methyltransferase with H3-3meK27 and H3-2meK9 was also observed 30 min after activation, but disappeared at the two-cell stage. H3-3meK27 appeared precociously and initially in the absence of the Eed/Ezh2 complex at 8 h in SCNT embryos. Biallelic expression of *Xist* RNA was observed from the four- to eight-cell stages onwards in SCNT embryos, but eventually declined in blastocysts. The H3-3meK27 was associated with and persisted on only one of the two *Xist* domains associated with the original Xi. Compare the kinetics of the epigenetic events in SCNT with control embryos, which differ markedly. See text for detailed explanations.

sources do not possess an Xi and showed no H3-3meK27 accumulation at the two-cell stage of SCNT. By contrast, at this time, we detected the striking accumulation of both H3-3meK27 and Eed in donor nuclei from adult females in SCNT embryos.

With further development to the morula and blastocyst stages however, both RNA FISH analysis and allele-specific RT-PCR showed a shift back to monoallelic *Xist* RNA expression patterns (Fig 4B; supplementary Table S2 online). The fact that the *Xist* RNA coating of one of the two chromosomes eventually declines in some blastomeres suggests that even in SCNT embryos some form of counting or recognition of the number of X chromosomes must occur to ensure proper X-linked gene dosage for survival. A similar phenomenon has been described in both XpO embryos (Papaioannou & West, 1981) and in XpXp androgenotes (Okamoto et al, 2000). In the latter, both Xp chromosomes are initially coated in *Xist* RNA but one of these subsequently reverses at later stages. However, this loss of *Xist* RNA is not complete, as in some cells, biallelic *Xist* expression persists (Fig 4) to the blastocyst stage (data not shown). Presumably, such cells will die through nullisomy for the X chromosome if X inactivation is triggered.

Our study shows that reprogramming events after SCNT of donor nuclei have different effects on the active and inactive X chromosomes (Fig 5). The silent *Xist* locus on the Xa in somatic nuclei can be clearly activated after SCNT, which suggests that its

mechanism of repression must differ from that of the *Xist* locus on the maternal Xm^{*}, because the latter is not activated during pre-implantation development. A previous study suggests that a maternal imprint on Xm^{*} is established during oocyte growth and serves to render the Xm^{*} resistant to inactivation in pre-implantation embryos (Tada et al, 2000). The precise nature of the imprint on the maternal X that maintains repression of the *Xist* locus on Xm^{*} is unknown (Takagi, 2003). A comparative analysis of Xa and Xm^{*} may provide further insight into the mechanisms that regulate repression of the *Xist* locus.

For the Xi, after a rapid loss of *Xist* RNA coating, there is precocious appearance of the H3-3meK27 mark without association with the Polycomb Eed/Ezh2 complex, which may indicate that some epigenetic marks on Xi are not erased in the oocyte. *Xist* RNA on the Xi is, however, detected subsequently in SCNT embryos, but later than at the two-cell stage, which is the case on Xp in control embryos. This study suggests a possible mechanism for previous observations on preferential inactivation of the Xi in the extraembryonic tissues in cloned embryos (Eggan et al, 2000; Nolen et al, 2005), which could be due to the persistence of chromatin marks such as H3-3meK27 on the Xi, presumably because they are not erased by the oocyte. In control embryos, the epigenetic mark on Xp is likely to be at the level of the *Xist* gene itself, as we could not detect H3-3meK27 methylation and other

chromatin marks before the 16-cell stages (Okamoto *et al*, 2004). The epigenetic marks present on the Xi and Xa in the somatic nucleus are thus different from those on Xp and Xm, although both Xi and Xp undergo preferential inactivation in pre-implantation embryos but involve a different sequence of epigenetic events.

We propose that some of the epigenetic marks on the Xi endure through the initial stages of genomic reprogramming, and result in an unusual series of epigenetic changes in SCNT embryos. It is probable that the persistent epigenetic marks on the Xi and Xa are subsequently fully erased in the epiblast of the blastocysts, judging from recent evidence showing re-activation of Xp in the epiblast (Mak *et al*, 2004; Okamoto *et al*, 2004). This is consistent with random X inactivation in the fetuses of both normal and cloned embryos. Epigenetic reprogramming during SCNT is thus a stepwise process that is initiated in the oocyte but continues in the epiblast, and may involve diverse mechanisms.

METHODS

See the supplementary information online for details.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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