X chromosome inactivation in nuclear transfer ES cells

著者	Shibata Shinwa, Wakayama Teruhiko, Yokota Takashi
journal or publication title	Cytogenetic and Genome Research
volume	121
number	2
page range	96-101
year	2008-06-01
URL	http://hdl.handle.net/2297/10942

doi: 10.1159/000125834

Title page:

Original Article / Epigenetics

X-chromosome inactivation in nuclear transfer ES cells

5

Shinwa Shibata^{1*}, Teruhiko Wakayama², and Takashi Yokota¹

¹⁾ Department of Stem Cell Biology, Graduate School of Medicine, Kanazawa University

13-1 Takara-machi, Kanazawa, Ishikawa 920-8640, Japan

10 ²⁾ Center for Developmental Biology, RIKEN Kobe

2-2-3 Minatojima-minamimachi, Kobe 650-0047, Japan

*Corresponding author: shinwa@med.kanazawa-u.ac.jp

phone: +81-76-265-2208, fax: +81-76-234-4238

15

Abstract

Nuclear transfer ES (ntES) cells are established from cloned blastocysts generated by somatic cell nuclear transfer and are expected to be an important resource for regenerative medicine.

5 However, cloned mammals, generated by similar methods, show various abnormalities, which suggest disordered gene regulation. Random X-chromosome inactivation (XCI) has been observed to take place in cloned female mouse embryos, but XCI does not necessarily occur according to *Xce* strength, a genetic element that determines the likelihood of each X-chromosome to be inactivated. This observation suggests incomplete reprogramming of
10 epigenetic marks related to XCI. Here, we investigated XCI in ntES cell lines, which were established using differentiated embryoid bodies that originated from a female mouse ES cell line. We examined *Xist* RNA localization, histone modifications in the *Xist* locus, and XCI choice. We did not find substantial differences between the ntES lines and their parental ES line. This suggests that the *Xist* locus and the epigenetic marks involved in XCI are reprogrammed by
15 nuclear transfer and subsequent ntES cell establishment. In contrast to skewed XCI in cloned mice, our observations indicate that normal XCI choice takes place in ntES cells, which supports the goal of safe therapeutic cloning for clinical use.

Introduction

Sex chromosome dosage compensation in mammals is achieved by inactivating either of the two female X-chromosomes in early embryogenesis. In this process, termed X-chromosome inactivation (XCI), the choice of which X-chromosome to keep active is random in embryos and in ES cells (random XCI) (Lyon, 1961; for review Heard and Disteche, 2006), while the paternal X is always inactivated in extraembryonic tissues (imprinted XCI) (Takagi and Sasaki, 1975). XCI is triggered upon differentiation by upregulation of X-linked non-coding *Xist* (*X-inactive specific transcript*) (Wutz and Jaenisch, 2000), whereas the X to remain active is protected by prolonged expression of *Tsix*, a transcript that is antisense to *Xist* and which represses *Xist* (Lee and Lu, 1999). Although it is random, the choice of which X-chromosome to inactivate (or to retain active) is biased by the X-linked genetic element named *Xce* (*X controlling element*). Hence, in hybrid embryos, XCI choice is not equal and the ratio of cells having either X rendered inactive is skewed according to the strain specific strength of *Xce* (Cattanach and Williams, 1972; Chadwick et al., 2006). For example, the XCI choice ratio in differentiated *Mus musculus musculus* (*mus*) 129 and *Mus musculus castaneus* (*cast*) hybrid cells is 85% and 15% respectively (Stavropoulos et al., 2001). Because XCI can be recapitulated *in vitro* by culturing female ES cells under differentiating conditions (Kay et al., 1993), we took advantage of using a well-defined female ES cell line carrying 129 and *cast* Xs (Lee and Lu, 1999).

In cloned female mouse embryos, generated by somatic cell nuclear transfer (Wakayama et al., 1998), one of the two Xs is randomly selected and inactivated irrespective of their previous active-X (Xa) or inactive-X (Xi) status, while, in the placenta, the former Xi is always inactivated again (Eggan et al., 2000). This finding indicates that, in cloned mice, the epigenetic marks mediating XCI in a somatic nucleus are erased in the embryonic lineage, but are maintained in

the placenta. However, in fibroblasts derived from cloned female embryos that had been generated from *mus* x *cast* F₁ mouse nuclei, the ratio of *Xist* RNA expression from each allele demonstrated more variation than that in fibroblasts derived from conventional *mus* x *cast* embryos (Eggan et al., 2000). Similarly, the ratio of XCI choice differed between individual
5 cloned embryos that had been reconstructed with predetermined Xi^{HPRT}/Xa^{GFP} nuclei (Eggan et al., 2000). These observations suggest incomplete or aberrant reprogramming of epigenetic marks related to XCI, because complete reprogramming would result in female clones that show identical XCI choice ratios, according to *Xce* strength. An aberrant pattern of XCI was also reported in bovine clones (Xue et al., 2002). Also, in individual adult cloned mice, XCI was
10 skewed to various degrees (Senda et al., 2004) and XCI heterogeneity within cloned embryos (Nolen et al., 2005) has been reported. These observations strongly suggest insufficient XCI reprogramming in cloned embryos.

In order to estimate the reprogramming of XCI in cloned mice we examined two representative histone tail modifications, histone H3 dimethyl-lysine 4 (H3K4m2) and histone
15 H3 trimethyl-lysine 27 (H3K27m3), in the *Xist/Tsix* locus of ntES (nuclear transfer ES) cell lines. NtES cells are established from cloned blastocysts, generated by somatic cell nuclear transfer, and they are considered to mimic the embryonic lineage of cells in cloned animals upon differentiation (Wakayama et al., 2001; Yang et al., 2007). The H3K4m2 and H3K27m3 modifications in the *Xist/Tsix* locus are closely related to the regulation of XCI (Sun et al., 2006;
20 Navarro et al., 2006), therefore, these modifications would be affected if XCI reprogramming is incomplete in ntES cells. We also examined *Xist* RNA localization and XCI choice in ntES cell lines. All the results suggested that the *Xist* locus and the epigenetic marks involved in XCI were properly reprogrammed in ntES cells. Our results argue for a difference in XCI reprogramming between ntES cells and cloned embryos, which is likely to be due to the
25 prolonged time available for reprogramming that ntES cells have during their derivation.

Materials and Methods

Cell culture and establishment of ntES clones

The female mouse ES cell line, EL16.7, having heterogeneous Xs from strains 129 and *cast* was cultured, and embryoid bodies (EB) were prepared as described previously (Lee and Lu, 1999; Shibata and Lee, 2004). Briefly, ES cells were trypsinized and we removed feeder cells by incubating them for 1h on gelatinized culture dishes. Non-adherent ES cells were then split among bacterial dishes. After 4 days of suspension culture in ES media without leukemia inhibitory factor (LIF), ES cells developed to form balloon-like EB structures. EB were then replated on gelatinized dishes and grown in adherent culture for further differentiation. Five ntES cell lines were independently established from day 20 EB, consisting of completely differentiated cells, as previously described (Wakayama et al., 2006). To investigate the possibility that we accidentally obtained ntES cells from a contaminating feeder nucleus or we mistakenly derived ES cells from a parthenogenetically grown embryo in which we failed to remove the nucleus, we assayed the polymorphism of *Bsp*M I restriction enzyme sites (Shibata and Lee, 2004) between *cast* and *mus* (feeders and recipient oocytes do not have *cast* sequence, data not shown).

Immunocytochemistry and fluorescence in situ hybridization (Immuno-FISH)

EB differentiated for 3 to 4 days were trypsinized and prepared for immuno-FISH using cytopsin equipment. Immuno-FISH was carried out as described (Plath et al., 2003). Anti-H3K27m3 antibody was purchased from Upstate (#07-449).

Chromatin immunoprecipitation (ChIP) and real-time PCR

ChIP and real-time PCR protocols have been described previously (Morshead et al., 2003;

Shibata et al., submitted). Taqman probes and PCR primers have been described elsewhere (Shibata et al., submitted), or are shown in Table 1. Allele-specific analyses of ChIP products were performed as described previously (Shibata and Lee, 2004) by replacing cDNA template for immunoprecipitated chromatin sample. The PCR primers, polymorphic restriction enzymes and Southern blot probes used are documented as follows: 5'-*Tsix* and *Xist*-GB2 (Stavropoulos et al., 2001), *Xist*-GB3 (Shibata and Lee, 2004), *Xist*-P, *Tsix*-OUT (Table 1). All probe and primer sequences match with both *cast* and 129 sequences. The *Mus musculus castaneus* sequence of the *Xist* promoter is available in DDBJ/Genbank (Accession #AB241552). Quantitative assays were repeated three times with independently prepared samples.

Allele-specific Southern blot assays were repeated twice.

Allele-specific RT-PCR

Allele-specific RT-PCR for *Xist*, *Tsix*, and *Mecp2* was performed as previously described (Stavropoulos et al., 2001). Assays were repeated three times with independently prepared samples.

Results

Derivation and morphology of ntES cell lines and XCI initiation

We cultured the female mouse ES cell line EL16.7 for 20 days without LIF and feeders, and made them differentiate to form EB. At this time point we could not find any ES-like colonies, nor rapidly growing cells in the culture, indicating the absence of undifferentiated cells (discussed later). We then established five independent ntES lines from the differentiated cells by nuclear transfer. All of the ntES lines showed well-packed colony morphology that is indistinguishable from the undifferentiated parental ES cell line (Fig. 1A). Upon differentiation *in vitro*, by withdrawing LIF and feeders, ntES lines grew by spreading over the dishes and formed EB in a similar manner to the parental ES cell line (Fig. 1B). At an early stage of differentiation, around day 3 to 4, *Xist* RNA started to accumulate in the nuclei of a portion of cells of both the ntES lines and the parental ES line, and the *Xist* signal colocalized with H3K27m3 (Fig. 1C), as was reported in normal female embryos and in ES cells (Silva et al., 2003; Plath et al., 2003). In a few ntES cells we found two *Xist* signals per nucleus (data not shown), but they were rare and thus suggested cells with abnormal karyotype rather than deregulated XCI (Nolen et al., 2005). Collectively, these findings indicate that ntES cells are very similar to conventional fertilization-derived ES cells in morphology, differentiation, and in XCI initiation.

*Histone tail modifications in the *Xist/Tsix* locus in ntES lines*

We examined the undifferentiated ntES and parental ES lines for two representative histone tail modifications, H3K27m3 and H3K4m2 in the *Xist/Tsix* locus by ChIP. The position of PCR amplicons for ChIP analyses are shown in Fig. 2A. The total amount of each modification was examined by quantitative PCR, and the ratio of the modification between 129 and *cast* alleles

was determined by Southern blot analysis, following digestion of PCR product at polymorphic restriction enzyme sites. Although the 129/*cast* ratio of H3K27m3 modification showed substantial variation among the ntES lines (see allele-specific Southern blot in Fig. 2B), the total amount of H3K27m3 modification in ntES and in the parental ES lines was remarkably low across the *Xist/Tsix* locus, which contrasted with the higher level of modification in female mouse embryonic fibroblasts (MEF) (graphs in Fig. 2B). Due to the minimal level of H3K27m3 modification in ntES and in ES cells, we speculate that the variation in H3K27m3 modification ratio does not signify a functional meaning because the absolute amount of modification in each allele is nearly zero when compared to that in female MEF. For H3K4m2 modification, both the total amount and the allelic ratio in ntES lines demonstrated only small differences compared to those of the parental ES line (Fig. 2C). These findings strongly suggest that the epigenetic marks and the chromatin structure of the *Xist/Tsix* locus are correctly reprogrammed in ntES cells.

Transcriptional activity of X-linked genes and XCI choice in ntES lines

We subsequently used allele-specific RT-PCR to investigate whether X-linked *Mecp2* and *Tsix* gene expression was properly reestablished in ntES cells. *Mecp2* expression from 129 and *cast* alleles was equal in all the ntES lines in the undifferentiated state, indicating that former Xi was completely reactivated during ntES cell derivation (Fig. 3A). Similarly, the ratios of *Tsix* expression from both alleles in all the ntES lines was very similar compared to that observed in the parental ES cell line. A slight skewing to the *cast* allele was observed, as has been previously reported (Stavropoulos et al., 2001) (Fig. 3B). Thus, this suggests that differentiated former Xa has also been reprogrammed to undifferentiated X. These observations were consistent with our ChIP results showing reprogramming of the chromatin structure at the *Xist/Tsix* locus. Finally, we tested whether the ntES lines exhibited the identical

XCI choice preference as the parental line, according to *Xce* strength. The ntES cells were cultured under differentiating conditions for 11 days, and *Xist* expression was analyzed. Because all the ntES lines should have been derived from single nuclei of differentiated EB, which carry an Xi of either 129 or *cast* origin, resultant XCI choice ratios in ntES lines would display a bipartite tendency if XCI reprogramming is affected by the previous active/inactive condition of each X allele. Incomplete or aberrant XCI reprogramming is likely to result in unusual skewing of XCI choice in ntES cells. However, this was not the case. The ratio of *Xist* expression from 129 and *cast* alleles was the same among all the ntES and the parental ES lines (Fig. 3C), indicating that the XCI choice was also restored in ntES lines. Therefore, these results demonstrate that reprogramming of XCI is properly achieved in ntES cells during their derivation.

Discussion

All the observations in this report indicate that there are only small differences in XCI properties between ntES cells and conventional fertilization-derived ES cells. These results are inconsistent with previous reports showing irregular XCI choice in cloned mice and embryos (Eggan et al., 2000; Senda et al., 2004; Nolen et al., 2005). One possibility is that we have actually established ntES cells from nuclei of undifferentiated cells which were still present within the day 20 EB culture used for nuclear donation. However, we think this is unlikely because EB have been shown to reach the egg-cylinder embryo stage by day 8 of differentiation. After 11 days cystic structures resembling 10-day yolk sacs are apparent, and after 3 weeks most developmental processes, as well as growth, have ceased (Doetschman et al., 1985). Incidentally, more than 95% of cells in day 10.5 mouse embryos show strong *Xist* RNA signal, which is indicative of Xi (Sugimoto and Abe, 2007). Therefore, culture of EB for 20 days should be enough to complete XCI processes and differentiation. One may still argue that ES-like undifferentiated cells are maintained by surrounding cells for prolonged periods, however, we did not find any rapidly growing cells in the day 20 EB culture, and vigorous self-renewal is one of the fundamental features of undifferentiated ES cells (Niwa, 2007). We do not exclude the possibility of the presence of cells resembling adult stem cells, represented by hematopoietic stem cells and neural stem cells, in the EB culture. Nevertheless, such adult stem cells are inefficient nuclear donors for generating cloned mice, as well as cloned blastocysts, when compared to other types of adult cell (Inoue et al., 2006; Mizutani et al., 2006; Inoue et al., 2007). These reports not only indicate that adult stem cells are epigenetically distinct from pluripotent ES cells with reprogrammed epigenome, but also that there is less chance that ntES cells would be derived from nuclei of adult stem cells. Collectively, it is reasonable to consider that most, if not necessarily all, of the five ntES lines

described in this report originated from well-differentiated cells.

We suggest ntES cells reestablish their epigenome to a greater degree than cells in cloned embryos because of the prolonged incubation time during which reprogramming can occur. In cloning, the transferred nucleus must complete its reprogramming within a few days, during the earliest embryonic development. By contrast, ntES cells are allowed to reprogram for more than 10 days during their derivation (Wakayama et al., 2006). In addition, cloned embryos might include cells with insufficient reprogramming, while such cells would grow slower than reestablished ES cells and be easily eliminated from the ntES cell culture. In fact, ntES cells were reported to be transcriptionally and functionally indistinguishable from fertilization-derived ES cells (Brambrink et al., 2006; Wakayama et al., 2006), whereas many genes showed abnormal expression in cloned mice (Humpherys et al., 2002; Kohda et al., 2005). Epigenetic reprogramming of X-chromosomes in female somatic nuclei transplanted into oocytes has been described by Bao et al (Bao et al., 2005). The transplanted nuclei immediately become subject to reprogramming, where biallelic *Xist* expression from both former Xi and Xa is observed from the 8-cell stage, but some epigenetic marks seem to persist, according to the localization of H3K27m3 and Eed. All the epigenetic marks are erased in the epiblast, but cells in the extra-embryonic lineage continue to develop with aberrant epigenetic marks, which is likely to cause placental dysfunction. Actually, placental overgrowth is common in cloned mice (Wakayama et al., 1999). The placental disorder may cause unusual XCI skewing in the embryo, even if reprogramming in the epiblast is complete.

In conclusion, we could not find significant aberration of XCI in female ntES cells, according to H3K27m3 and H3K4m2 modifications in the *Xist/Tsix* locus, reestablishment of X-linked *Mecp2* and *Tsix* gene expression, *Xist* RNA localization and H3K27m3 distribution during the initiation of XCI, and XCI choice preference upon differentiation. Generally, X-chromosome aneuploidy in humans, known as Turner syndrome (45,X) and Klinefelter syndrome (47,XXY),

results in less severe phenotypes compared to autosomal aneuploidy disorders because X-chromosome dosage is compensated. Hence, it is speculated that XCI dysfunction would cause serious cellular and developmental aberrations. In fact a paternally derived *Xist*-deficient allele (Mararahrens et al., 1997) and a maternally derived *Tsix*-deficient allele (Lee, 2000; Sado et al., 2001) in female mice result in embryonic lethality due to the presence of two transcriptionally active Xs and the inactivation of both Xs in their extraembryonic tissues, respectively. A recent paper reporting the production of primate ntES cells (Byrne et al., 2007) increases the likelihood of being able to generate human ntES cells. However, the present rate of successful primate ntES cell establishment is low, but the refinement of culture conditions and the development of cloning techniques, such as trichostatin A treatment (Kishigami et al., 2006), may increase the rate sufficiently to enable clinical application in humans. Our observations, presented here, that normal XCI takes place in female ntES cells, supports the goal of safe therapeutic cloning for clinical use.

Acknowledgement

This work was supported by a grant-in-aid from the Ministry of Education, Science, Sports and Culture of Japan (18780250 to S.S. and 15080211 to T.W.).

References

- Bao S, Miyoshi N, Okamoto I, Jenuwein T, Heard E, Azim Surani M: Initiation of epigenetic reprogramming of the X chromosome in somatic nuclei transplanted to a mouse oocyte. EMBO Rep 6:748-754 (2005).
- 5 Brambrink T, Hochedlinger K, Bell G, Jaenisch R: ES cells derived from cloned and fertilized blastocysts are transcriptionally and functionally indistinguishable. Proc Natl Acad Sci U S A 103:933-938 (2006).
- Byrne JA, Pedersen DA, Clepper LL, Nelson M, Sanger WG, Gokhale S, Wolf DP, Mitalipov SM: Producing primate embryonic stem cells by somatic cell nuclear transfer. Nature 450:497-502 (2007).
- 10 Cattanach BM, Williams CE: Evidence of non-random X chromosome activity in the mouse. Genet Res 19:229-240 (1972).
- Chadwick LH, Pertz LM, Broman KW, Bartolomei MS, Willard HF: Genetic control of X chromosome inactivation in mice: definition of the Xce candidate interval. Genetics 173:2103-2110 (2006).
- 15 Doetschman TC, Eistetter H, Katz M, Schmidt W, Kemler R: The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. J Embryol Exp Morphol 87:27-45 (1985).
- 20 Eggan K, Akutsu H, Hochedlinger K, Rideout W, 3rd, Yanagimachi R, Jaenisch R: X-Chromosome inactivation in cloned mouse embryos. Science 290:1578-1581 (2000).
- Heard E, Disteche CM: Dosage compensation in mammals: fine-tuning the expression of the X chromosome. Genes Dev 20:1848-1867 (2006).
- 25 Humpherys D, Eggan K, Akutsu H, Friedman A, Hochedlinger K, Yanagimachi R, Lander ES, Golub TR, Jaenisch R: Abnormal gene expression in cloned mice derived from embryonic stem cell and cumulus cell nuclei. Proc Natl Acad Sci U S A 99:12889-12894 (2002).
- Inoue K, Ogonuki N, Miki H, Hirose M, Noda S, Kim JM, Aoki F, Miyoshi H, Ogura A: Inefficient reprogramming of the hematopoietic stem cell genome following nuclear transfer. J Cell Sci 119:1985-1991 (2006).
- 30 Inoue K, Noda S, Ogonuki N, Miki H, Inoue S, Katayama K, Mekada K, Miyoshi H, Ogura A: Differential developmental ability of embryos cloned from tissue-specific stem cells. Stem Cells

25:1279-1285 (2007).

Kay GF, Penny GD, Patel D, Ashworth A, Brockdorff N, Rastan S: Expression of Xist during mouse development suggests a role in the initiation of X chromosome inactivation. *Cell* 72:171-182 (1993).

- 5 Kishigami S, Mizutani E, Ohta H, Hikichi T, Thuan NV, Wakayama S, Bui HT, Wakayama T: Significant improvement of mouse cloning technique by treatment with trichostatin A after somatic nuclear transfer. *Biochem Biophys Res Commun* 340:183-189 (2006).

Kohda T, Inoue K, Ogonuki N, Miki H, Naruse M, Kaneko-Ishino T, Ogura A, Ishino F: Variation in gene expression and aberrantly regulated chromosome regions in cloned mice.

- 10 *Biol Reprod* 73:1302-1311 (2005).

Lee JT, Lu N: Targeted mutagenesis of Tsix leads to nonrandom X inactivation. *Cell* 99:47-57 (1999).

Lee JT: Disruption of imprinted X inactivation by parent-of-origin effects at Tsix. *Cell* 103:17-27 (2000).

- 15 Lyon MF: Gene action in the X-chromosome of the mouse (*Mus musculus* L.). *Nature* 190:372-373 (1961).

Marahrens Y, Panning B, Dausman J, Strauss W, Jaenisch R: Xist-deficient mice are defective in dosage compensation but not spermatogenesis. *Genes Dev* 11:156-166 (1997).

Mizutani E, Ohta H, Kishigami S, Van Thuan N, Hikichi T, Wakayama S, Kosaka M, Sato E, 20 Wakayama T: Developmental ability of cloned embryos from neural stem cells. *Reproduction* 132:849-857 (2006).

Morshead KB, Ciccone DN, Taverna SD, Allis CD, Oettinger MA: Antigen receptor loci poised for V(D)J rearrangement are broadly associated with BRG1 and flanked by peaks of histone H3 dimethylated at lysine 4. *Proc Natl Acad Sci USA* 100:11577-11582 (2003).

- 25 Navarro P, Page DR, Avner P, Rougeulle C: Tsix-mediated epigenetic switch of a CTCF-flanked region of the Xist promoter determines the Xist transcription program. *Genes Dev* 20:2787-2792 (2006).

Niwa H: How is pluripotency determined and maintained? *Development* 134:635-646 (2007).

- Nolen LD, Gao S, Han Z, Mann MR, Gie Chung Y, Otte AP, Bartolomei MS, Latham KE: X 30 chromosome reactivation and regulation in cloned embryos. *Dev Biol* 279:525-540 (2005).

Plath K, Fang J, Mlynarczyk-Evans SK, Cao R, Worringer KA, Wang H, de la Cruz CC, Otte AP, Panning B, Zhang Y: Role of histone H3 lysine 27 methylation in X inactivation. *Science* 300:131-135 (2003).

5 Sado T, Wang Z, Sasaki H, Li E: Regulation of imprinted X-chromosome inactivation in mice by Tsix. *Development (Cambridge, UK)* 128:1275-1286. (2001).

Senda S, Wakayama T, Yamazaki Y, Ohgane J, Hattori N, Tanaka S, Yanagimachi R, Shiota K: Skewed X-inactivation in cloned mice. *Biochem Biophys Res Commun* 321:38-44 (2004).

Shibata S, Lee JT: Tsix transcription- versus RNA-based mechanisms in Xist repression and epigenetic choice. *Curr Biol* 14:1747-1754 (2004).

10 Silva J, Mak W, Zvetkova I, Appanah R, Nesterova TB, Webster Z, Peters AH, Jenuwein T, Otte AP, Brockdorff N: Establishment of histone h3 methylation on the inactive X chromosome requires transient recruitment of Eed-Enx1 polycomb group complexes. *Dev Cell* 4:481-495 (2003).

15 Stavropoulos N, Lu N, Lee JT: A functional role for Tsix transcription in blocking Xist RNA accumulation but not in X-chromosome choice. *Proc Natl Acad Sci USA* 98:10232-10237 (2001).

Sugimoto M, Abe K: X chromosome reactivation initiates in nascent primordial germ cells in mice. *PLoS Genet* 3:1309-1317 (2007).

20 Sun BK, Deaton AM, Lee JT: A transient heterochromatic state in Xist preempts X inactivation choice without RNA stabilization. *Mol Cell* 21:617-628 (2006).

Takagi N, Sasaki M: Preferential inactivation of the paternally derived X chromosome in the extraembryonic membranes of the mouse. *Nature* 256:640-642 (1975).

25 Wakayama S, Jakt ML, Suzuki M, Araki R, Hikichi T, Kishigami S, Ohta H, Van Thuan N, Mizutani E, Sakaide Y et al.: Equivalency of nuclear transfer-derived embryonic stem cells to those derived from fertilized mouse blastocysts. *Stem Cells* 24:2023-2033 (2006).

Wakayama T, Perry AC, Zuccotti M, Johnson KR, Yanagimachi R: Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature* 394:369-374 (1998).

Wakayama T, Yanagimachi R: Cloning of male mice from adult tail-tip cells. *Nat Genet* 22:127-128 (1999).

30 Wakayama T, Tabar V, Rodriguez I, Perry AC, Studer L, Mombaerts P: Differentiation of

embryonic stem cell lines generated from adult somatic cells by nuclear transfer. *Science* 292:740-743 (2001).

Wutz A, Jaenisch R: A shift from reversible to irreversible X inactivation is triggered during ES cell differentiation. *Mol Cell* 5:695-705 (2000).

- 5 Xue F, Tian XC, Du F, Kubota C, Taneja M, Dinnyes A, Dai Y, Levine H, Pereira LV, Yang X: Aberrant patterns of X chromosome inactivation in bovine clones. *Nat Genet* 31:216-220 (2002).

Yang X, Smith SL, Tian XC, Lewin HA, Renard JP, Wakayama T: Nuclear reprogramming of cloned embryos and its implications for therapeutic cloning. *Nat Genet* 39:295-302 (2007).

Figure legends

Fig. 1. Morphology and immuno-FISH in a representative ntES cell line and the parental female ES cell line. (A) Morphology of undifferentiated colonies and (B) EB differentiated for 11 days. (C) Immuno-FISH for H3K27m3 (green) and *Xist* RNA (red) in day 3 EB. Right panels are images of ntES cells and left panels are those of parental ES cells.

Fig. 2. Quantitative and allele-specific ChIP. (A) Position of PCR amplicons. Upper open rectangles represent *Xist* exons and lower grey rectangles represent *Tsix* exons. (B) ChIP for H3K27m3. Graphs illustrate total amount of the modification and images below show representative allele-specific Southern blot results. Numbers under the lanes indicate the ratio (%) of the modification at the 129 allele per total. Error bars demonstrate SD. ND: not done. (C) ChIP for H3K4m2.

Fig. 3. X-linked gene expression in ntES lines. (A) Allele-specific RT-PCR for *Mecp2* from cells in undifferentiated condition. The graph illustrates percentage of *Mecp2* mRNA expression from 129 allele per total and the image below shows a representative Southern blot result. Error bars demonstrate SD. (B) Allele-specific RT-PCR for *Tsix* from cells in undifferentiated condition. (C) Allele-specific RT-PCR for *Xist* in EB differentiated for 11 days.

Table 1. Primers and probes used in this study.

Amplicon	Application	PCR primers	Reference*	Probe sequence	Reference*	Polymorphic restriction enzyme
<i>Xist</i> -GB3	Taqman PCR	cta tac gtg gcg gtg caa ac aga ggc caa ggt gta agt aga cta g	L04961.1 @2021-2040 L04961.1 @2105-2081	ccactgaacaatacaaaggaatggaacgggctgagttta	L04961.1 @2080-2041	N/A
<i>Tsix</i> -OUT	Allele-specific Southern blot	cag gca taa gct act tac cat gtc gaa atg gtc ttc caa tgt gca gag	AJ421479.1 @103610-103633 AJ421479.1 @104198-104175	gagactggatgcctgatctacctatagtgtggctccaac	AJ421479.1 @103834-103873	<i>Hha</i> I
<i>Xist</i> -P	Allele-specific Southern blot	ttt gca tag aca ggt gtg tga cct aag ccg ttg cac gcc ttt aac tga	AJ421479.1 @106008-106031 AJ421479.1 @106301-106278	aatctttgtggccactcctctctgtctctccgcctca	AJ421479.1 @106228-106267	<i>Tsp</i> 509I

*The reference is shown as the nucleotide position in the indicated Genbank sequence. N/A, not applicable

A

B

C

A

B

C