CORRECTION

Choice of random rather than imprinted X inactivation in female embryonic stem cell-derived extra-embryonic cells

Kazuhiro Murakami, Kimi Araki, Satoshi Ohtsuka, Teruhiko Wakayama and Hitoshi Niwa

There were errors published in *Development* **138**, 197-202.

The authors of this article informed us of errors with the display of data in Fig. 3 and Fig. S2 in this paper. These are detailed below, and the original data provided by the authors are also included as part of this Correction. The authors apologise to the readers for these errors. The editors of the journal have examined this paper in detail and find that, while there were substantive errors in experimental layout and data presentation in Fig. 3 and Fig. S2, these do not affect the conclusions of the paper.

In Fig. 3, most samples were analysed on polyacrylamide gels, but the G6pd and Pdha1 samples were analysed on agarose gels. The difference in experimental procedure was not stated. In addition, the '−RT', 'non-cut' and 'cut' samples for these two loci were run on separate gels, although all were processed in parallel. Lanes were spliced together without showing or stating that they had come from separate gels. In these cases, it should be noted that bands are not quantitatively comparable. The corrected version of Fig. 3 and its legend are shown below. The original data for all samples in this figure are provided in Fig. A.
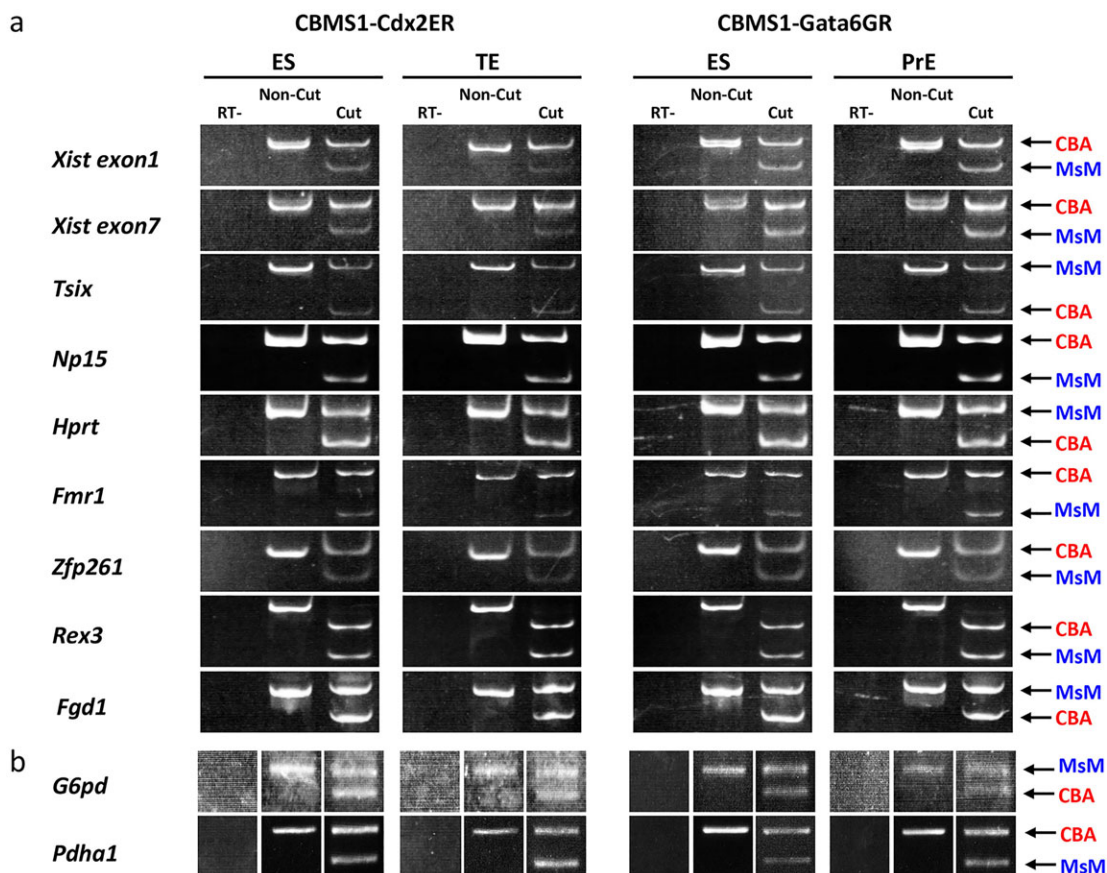

Fig. 3. PCR-RFLP analysis of the Xi pattern of CBMS1 Cdx2ER and Gata6GR cell lines. PCR fragments were cut by specific restriction enzyme sets (see Table S2 in the supplementary material). Note that two different sizes of fragments were identified in induced extra-embryonic cells, indicating the occurrence of random X inactivation in these cells. RT−, no reverse transcriptase control; TE, trophectoderm; PrE, primitive endoderm. CBA, *Mus musculus musculus*; MsM, *Mus musculus molossinus* (see Additional Materials and Methods in this Correction). The size differences between PCR fragments were analyzed in either polyacrylamide gels (a) or agarose gels (b). White lines between lanes indicate samples that were processed together but run on separate gels; band intensities are therefore not quantitatively comparable in these cases.

In Fig. S2, the 'non cut' and 'cut' samples for the CBMS1 ES lanes were run on separate gels, although processed in parallel. This was not appropriately marked on the figure. For all gels run for this figure, short and long exposure pictures were taken, and the short exposure used for assembling the original figure. Upon examination of the long-exposure pictures, it became apparent that the short exposures did not in all cases clearly show the cut bands. In the corrected version of the figure below, the long exposure has been used where appropriate. It should be noted that, since the CBMS1 ES lanes were run on separate gels, these bands are not quantitatively comparable. The authors have been unable to locate the original data for the G6pd samples, and have therefore removed these lanes from the corrected version of the figure. The original data for all other samples are provided in Fig. B. The version of Fig. S2 that appears online is the original figure without the lanes removed.
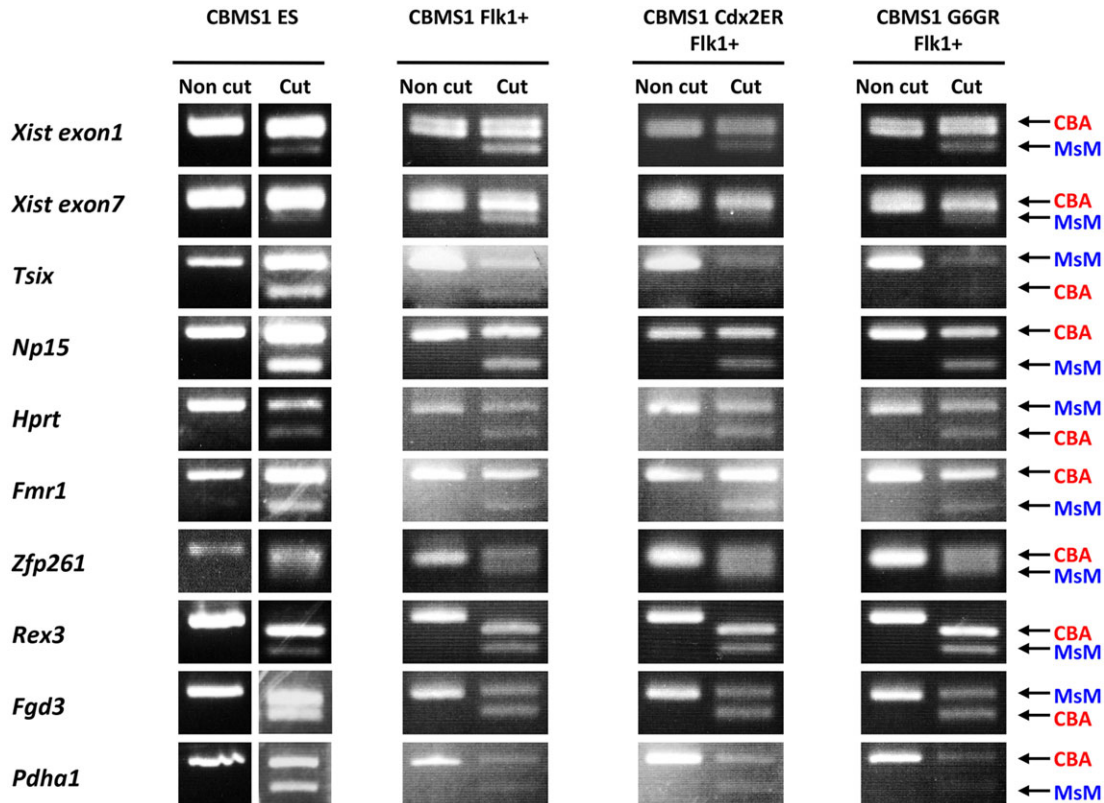

Fig. S2. Random Xi in Flk1+ mesodermal cells. CBMS1 ES cells were cultured on a collagen type IV-coated dish for 5 days to induce mesoderm, and Flk1+ mesodermal cells were stained by APC-conjugated anti-Flk1 antibody and separated by FACS sorting. Xi states were analyzed using the PCR-RFLP method. Two different sizes of fragments were detected by agarose electrophoresis in both ES cells and Flk1+ mosodermal cells, indicating the transcripts from both X chromosomes were present in these samples. The two bands in ES cells indicate the state of two active X chromosomes, whereas those in Flk1+ cells indicate a state of random Xi, since the uniform Xi event is confirmed by H3K27me3 staining (see Fig. S1). White lines between lanes indicate samples that were processed together but run on separate gels; band intensities are therefore not quantitatively comparable in these cases.

Finally, the authors omitted key parts of the Materials and Methods in the published version of the paper. The missing sections are provided here.

ADDITIONAL MATERIALS AND METHODS
CBMS1 and B142bgeoEG mESC derivation and culture condition
A hybrid F1 CBMS1 female ES cell line was derived from an embryo obtained by mating female *Mus musculus musculus* (CBA) and male *Mus musculus molocimu*s (MsM) mice. A B142bgeoEG female ES cell line was derived from an embryo obtained by mating genetically modified *Mus musculus* (DBA) mice. Both female ES cell lines were cultured in GMEM (Gibco), 14% KSR (Gibco), 1% FCS (Thermo Scientific), 1× sodium pyruvate (Gibco), 1× NEAA (Gibco), 10^{-4} M 2-mercaptoethanol (Nakarai Tesque), 1000 U/ml of leukemia inhibitory factor (LIF) on a 0.1% (w/v) gelatin-coated dish.

Derivation of B142bgeoEG Cdx2ER, Gata6GR; CBMS1 Cdx2ER and Gata6GR mESC lines
A Gata6GR or Cdx2ER expression vector (50 µg) with puromycin-resistant gene (pPyCAG-Gata6GR-IP or pPyCAG-Cdx2ER-IP) (Niwa et al., 2005; Shimosato et al., 2007) was linearized and transfected into $1×10^{7}$ CBMS1 ES cells by electroporation followed by the selection with 1.5 µg/ml of puromycin (Nakarai Tesque). A Gata6GR or Cdx2ER-expression vector (50 µg) with hygromycin-resistant gene (pPyCAG-Gata6GR-IH or pPyCAG-Cdx2ER-IH) was linearized and transfected into $1×10^{7}$ B142bgeoEG ES cells by electroporation followed by selection with 100 µg/ml Hygromycin B (Invitrogen). After the selection for 7 days, the drug-resistant colonies were isolated and the cell lines that undergo differentiation to extra-embryonic lineage-like cells in the presence of 1 µg/ml 4-hydroxy tamoxifen (Tx) (Sigma) for Cdx2ER or in the presence of 100 nM dexamethasone (Dex) (Sigma) for Gata6GR were selected for further analyses.

Induction of trophectodermal (TE) and primitive endodermal (PrE) cells
$1×10^{6}$ mES cells, which express Gata6GR or Cdx2ER were seeded on a gelatin-coated dishes in GMEM, 10% FCS (Hyclone), 1× sodium pyruvate, 1× NEAA, 10^{-4} M 2-mercaptoethanol without LIF. After attaching the cells to the dish bottom, Tx or Dex were added and the cells were cultured for 5 days to obtain PrE and TE cells. Induction of PrE and TE were confirmed by the expression of lineage-specific markers.

Induction of Flk1+ mesodermal cells

Flk1-positive mesodermal cells were induced according to Nishikawa et al. (Nishikawa et al., 1998). Briefly, 1×10^4 CBMS1 and B142bgeoEG ES cells were seed onto collagen type IV-coated dishes (Nitta Gelatin) in the Flk1+ induction medium (α-MEM, 10% FCS, 10^{-4} M 2-mercaptoethanol, $1\times$ NEAA). After 5 days in culture, differentiated cells were dissociated using cell-dissociation buffer (Gibco) and stained using APC-conjugated anti-mouse Flk1 antibody (eBioscience, 17-5821-80). The population of Flk1+ mesodermal cells was analyzed and sorted by FACS Aria (BD).

PCR-RFLP analysis

Total RNA was extracted from 1×10^6 mES, TE, PrE and Flk1+ cells using RNA extraction kit (Kurabo), according to the manufacturer's instructions. Total RNA (1 μg) was used for cDNA synthesis with ReverTra ace-alpha cDNA Synthesis Kit (Toyobo) (20 μl/1 reaction). First-strand cDNA was synthesized using the random oligo primer. RT- samples were prepared at this step in the reaction without reverse transcriptase.

RT-PCR primers were designed to detect polymorphisms that were contained X-linked gene transcripts (Sugimoto and Abe, 2007; Table S2). RT-PCR was performed using 1 μl of cDNA or RT– samples by using Taq-Gold PCR polymerase (Applied Biosystems) (20 μl/1 reaction ×5). The PCR conditions were 94°C for 30 s, 52°C for 30 s, 72°C for 30 s; 35-40 cycles (for G6pd, the annealing temperature was 50°C). PCR products were purified by phenol-chloroform treatment followed by ethanol precipitation. Finally, the PCR products were dissolved in 50 μl of TE (pH 8.0). The concentration was measured using a Nanodrop ND-1000 (Thermo Fisher Scientific).

PCR products (1 μg) were digested overnight with the restriction enzymes listed in Table S2 (20 μl/1 reaction). RT– and 1 μg of non-cut and cut samples were loaded on each well and the sizes of the DNA fragments were analyzed by electrophoresis using 2% agarose gel and 10% polyacrylamide gel.
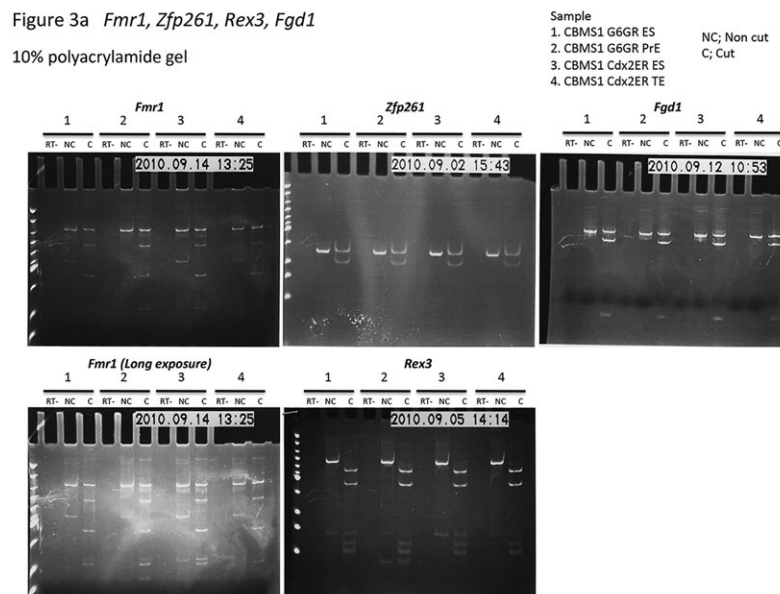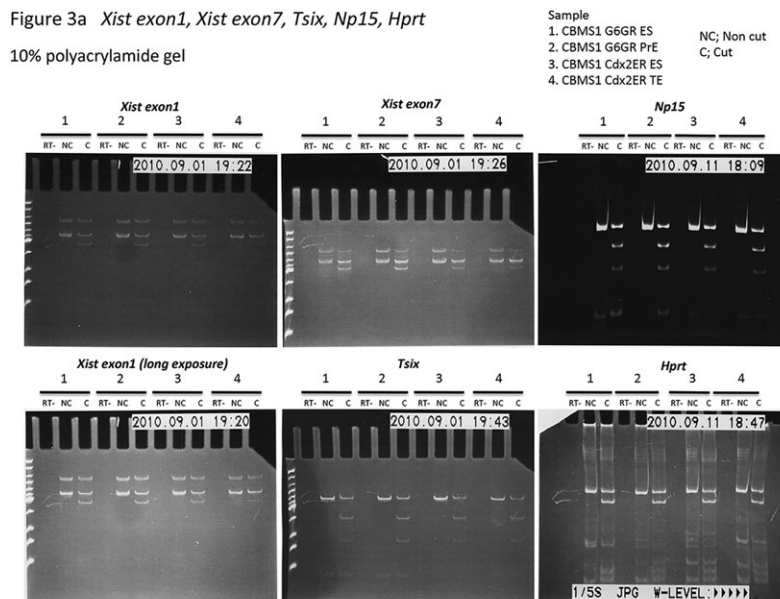

Fig. A. These data relate to Fig. 3.

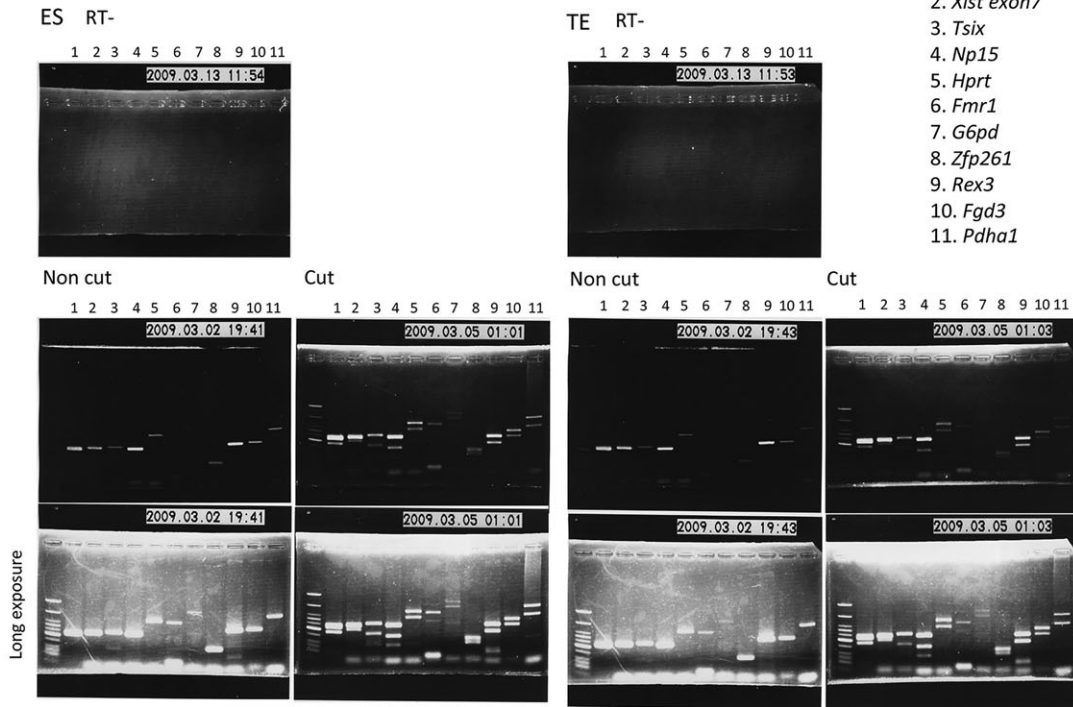## Figure 3b   *G6pd, Pdha1* for CBMS1-Cdx2ER

2% agarose gel

Sample
1. *Xist exon1*
2. *Xist exon7*
3. *Tsix*
4. *Np15*
5. *Hprt*
6. *Fmr1*
7. *G6pd*
8. *Zfp261*
9. *Rex3*
10. *Fgd3*
11. *Pdha1*

Figure 3b   *G6pd, Pdha1* for CBMS1-G6GR

2% agarose gel

Sample
1. *Xist exon1*
2. *Xist exon7*
3. *Tsix*
4. *Np15*
5. *Hprt*
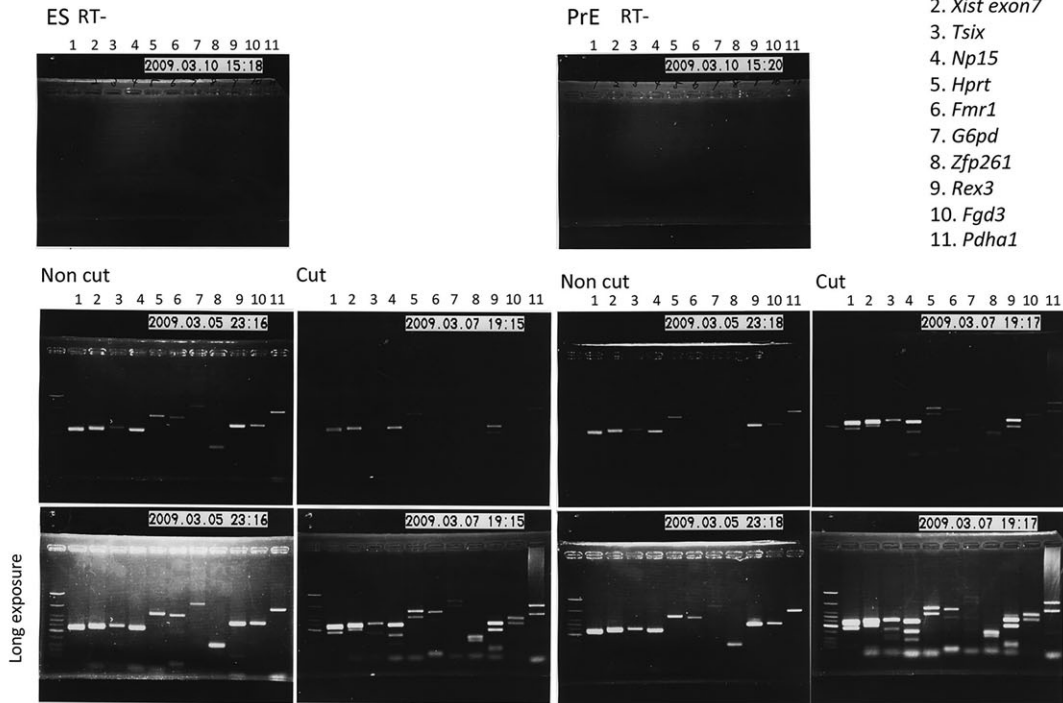6. *Fmr1*
7. *G6pd*
8. *Zfp261*
9. *Rex3*
10. *Fgd3*
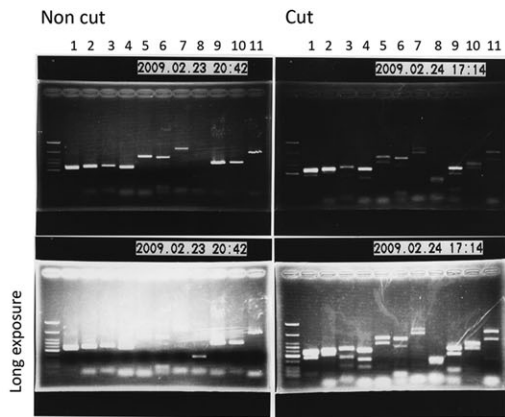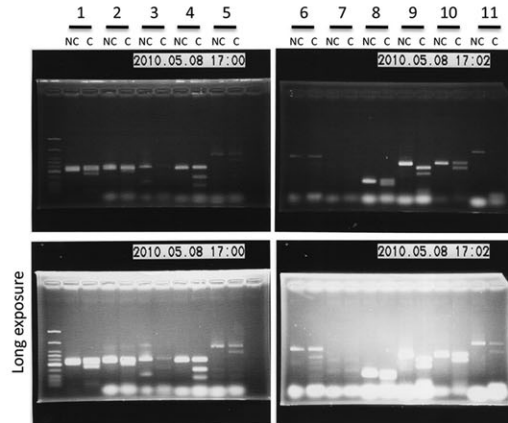11. *Pdha1*

Fig. A (continued). These data relate to Fig. 3.
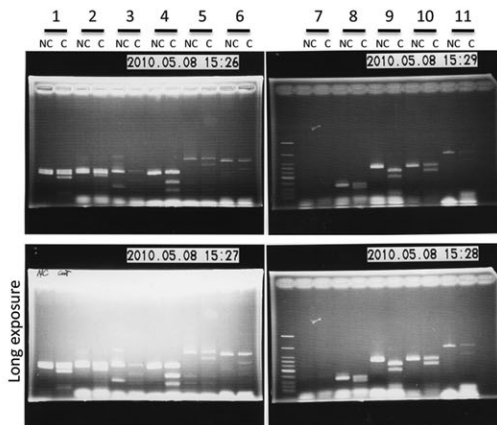
CBMS1 ES
2% agarose gel

Non cut   Cut

Sample
1. *Xist exon1*
2. *Xist exon7*
3. *Tsix*
4. *Np15*
5. *Hprt*
6. *Fmr1*
7. *G6pd*
8. *Zfp261*
9. *Rex3*
10. *Fgd3*
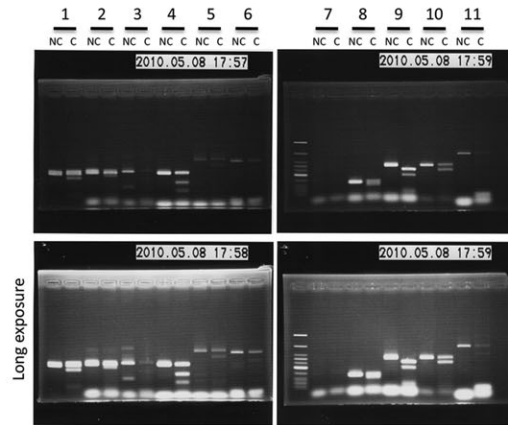11. *Pdha1*

CBMS1 Cdx2ER Flk1+
2% agarose gel

Sample
1. *Xist exon1*
2. *Xist exon7*
3. *Tsix*
4. *Np15*
5. *Hprt*
6. *Fmr1*
7. *G6pd*
8. *Zfp261*
9. *Rex3*
10. *Fgd3*
11. *Pdha1*

NC; Non cut
C; Cut

CBMS1 Flk1+
2% agarose gel

Sample
1. *Xist exon1*
2. *Xist exon7*
3. *Tsix*
4. *Np15*
5. *Hprt*
6. *Fmr1*
7. *G6pd*
8. *Zfp261*
9. *Rex3*
10. *Fgd3*
11. *Pdha1*

NC; Non cut
C; Cut

CBMS1 G6GR Flk1+
2% agarose gel

Sample
1. *Xist exon1*
2. *Xist exon7*
3. *Tsix*
4. *Np15*
5. *Hprt*
6. *Fmr1*
7. *G6pd*
8. *Zfp261*
9. *Rex3*
10. *Fgd3*
11. *Pdha1*

NC; Non cut
C; Cut

Fig. B. These data relate to Fig. S2.

Choice of random rather than imprinted X inactivation in female embryonic stem cell-derived extra-embryonic cells

Kazuhiro Murakami[1], Kimi Araki[2], Satoshi Ohtsuka[1], Teruhiko Wakayama[3] and Hitoshi Niwa[1,*]

SUMMARY

In female mammals, one of two X chromosomes is epigenetically inactivated for gene dosage compensation, known as X inactivation (Xi). Inactivation occurs randomly in either the paternal or maternal X chromosome in all embryonic cell lineages, designated as random Xi. By contrast, in extra-embryonic cell lineages, which are segregated from somatic cell lineages in pre-implantation development, the paternal X chromosome is selectively inactivated, known as imprinted Xi. Although it is speculated that erasure of the imprinted mark on either the maternal or paternal X chromosome in somatic cell lineages might change the mode of Xi from imprinted to random, it is not known when this event is completed in development. Here, we tested the mode of Xi during the differentiation of female mouse embryonic stem (ES) cells derived from the inner cell mass (ICM) of blastocyst-stage embryos toward trophectoderm (TE) and primitive endoderm (PrE) lineages induced by artificial activation of transcription factor genes *Cdx2* and *Gata6*, respectively. We found that random Xi occurs in both TE and PrE cells. Moreover, cloned embryos generated by the transfer of nuclei from the female ES cells showed random Xi in TE, suggesting the complete erasure of all X imprints for imprinted Xi in ICM-derived ES cells.

KEY WORDS: Embryonic stem cells, Extra-embryonic lineages, Imprinted X inactivation, Mouse

INTRODUCTION

In eutherian mammals, female cells have two X chromosomes, derived from oocyte and sperm. In adult females, one of the two X chromosomes is inactivated for gene dosage compensation in almost all cell types, with a few notable exceptions. This phenomenon is known as X inactivation (Xi) (Lyon, 1961). Xi is initiated by the expression of the *Xist* non-coding RNA from one X chromosome, which then coats that X chromosome. The *Xist*-coated X chromosome recruits polycomb-related complex 2 (PRC2) for the accumulation of tri-methylation of histone H3 lysine 27 (H3K27me3), resulting in the formation of a heritable repressive chromatin state (Silva et al., 2003).

Xi first occurs in the morula stage embryo and selectively inactivates the X chromosome of paternal origin, designated as imprinted Xi (Takagi and Sasaki, 1975). Imprinted Xi is maintained in the trophectoderm (TE), which emerges in 16- to 32-cell stage embryos as the outer layer cells. In early blastocyst stage embryos, the inner cell mass (ICM) initially retains imprinted Xi. However, after the segregation of primitive endoderm (PrE) from epiblast, imprinted Xi is transmitted to PrE but erased in the epiblast, resulting in two active X chromosomes in the pluripotent cell population (Mak et al., 2004). Immediately after implantation, all epiblast cells undergo Xi by the formation of primitive ectoderm in egg-cylinder stage embryos, in which either the maternal or paternal X chromosome is randomly chosen for inactivation,

known as random Xi. It is clear that this imprinted Xi mechanism is necessary for extra-embryonic development because paternal *Xist* knockout embryos cannot induce imprinted Xi in pre-implantation embryos and show severe defects in extra-embryonic tissue development from E6.5, with two active X chromosomes (Marahrens et al., 1997).

The molecular mechanism of imprinted Xi is still obscure. One report has suggested that the selective inactivation of the paternal X chromosome is achieved by epigenetic marks on the maternal X chromosome that are imprinted in the maturing oocyte and allow selective escape of the maternal X chromosome from Xi in TE and PrE (Tada et al., 2000). Others have shown that the epigenetic marks on the paternal X chromosome, similar to the silencing of repetitive sequences, contribute to its selective inactivation (Namekawa et al., 2010). Even if both paternal and maternal X chromosomes carry specific marks to direct imprinted Xi, they should be erased in the epiblast of blastocyst stage embryos and undergo random Xi at later developmental stages.

Embryonic stem (ES) cells are pluripotent cell lines derived from epiblast. They retain the same epigenetic state of the X chromosome as the epiblast, as the female ES cells carry two active X chromosomes and it has been reported that random Xi occurs after the induction of differentiation. Interestingly, ES cells retain the ability to differentiate into extra-embryonic endoderm cells when they form embryoid bodies in suspension culture, in which random Xi is chosen rather than the imprinted Xi that normally occurs in extra-embryonic endoderm in vivo, suggesting that the imprinting marks on X chromosomes are erased in ES cells (Sado et al., 1996). However, it remains ambiguous whether all epigenetic marks for imprinted Xi are erased completely in any context and which type of Xi occurs if ES cells differentiate into TE in conventional culture conditions.

The ability of ES cells to give rise to TE had been opposed by evidence that ES cells never contribute to the TE lineage after injection into pre-implantation embryos (Beddington and

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DEVELOPMENT

Robertson, 1989). However, we previously showed that the forced expression of the homeobox transcription factor *Cdx2* triggers the differentiation of ES cells toward TE (Niwa et al., 2005). It is also possible to induce trophoblast stem (TS) cells from ES cells that contribute to all placental lineages after injection into blastocysts, as found in the case of embryo-derived TS cells (Tanaka et al., 1998), indicating proper differentiation. We also confirmed that the forced expression of *Gata4* or *Gata6* induces the differentiation of ES cells to PrE cells that mimic the character of embryo-derived extra-embryonic endoderm cells (XEN cells), which contribute to parietal endoderm after injection into blastocysts (Kunath et al., 2005; Shimosato et al., 2007). Here, we applied these strategies to female ES cells in which the paternal and maternal X chromosomes can be distinguished by genetic manipulation or polymorphic markers to address which types of Xi occur in these functional extra-embryonic cell types derived from ES cells. We also applied somatic cell nuclear transfer to female ES cells to test their choice of Xi in TE of cloned embryos.

MATERIALS AND METHODS
CBMS1 and B142bgeoEG ES cell derivation and culture conditions
A hybrid F1 CBMS1 female ES cell line was derived from an embryo obtained by mating female *Mus musculus musculus* (CBA) and male *Mus musculus molocimus* (MsM) mice. A B142bgeoEG female ES cell line was derived from an embryo obtained by mating genetically modified *Mus musculus* (C57BL/6 and CBA) mice. Both female ES cell lines were cultured in GMEM (Gibco) containing 14% knockout serum replacement (KSR; Gibco), 1% fetal calf serum (FCS; Thermo Scientific), 1× sodium pyruvate (1 mM; Gibco), 1× non-essential amino acids (NEAA; Gibco), 0.1 mM 2-mercaptoethanol (Nakarai Tesque) and 1000 U/ml leukemia inhibitory factor (LIF) on a 0.1% (w/v) gelatin-coated dish.

Derivation of B142bgeoEG Cdx2ER, Gata6GR and CBMS1 Cdx2ER, Gata6GR ES cell lines
Gata6GR or *Cdx2ER*-IRES-*puromycin acetyltransferase* vectors (50 μg) (Niwa et al., 2005; Shimosato et al., 2007) were transfected into 1×10^7 CBMS1 ES cells and 50 μg *Gata6GR* or *Cdx2ER*-IRES-*hygromycin phosphotransferase* vectors were transfected into 1×10^7 B142bgeoEG ES cells by electroporation. *Cdx2ER* or *Gata6GR* stably expressing clones were selected by 1.5 μg/ml puromycin (Nakarai Tesque) or 100 μg/ml hygromycin B (InvivoGen), respectively. Then, the cell lines that could be induced to extra-embryonic lineage-like cells were selected under 1 μg/ml 4-hydroxytamoxifen (Tx) (Sigma) or 100 nM dexamethasone (Dex) (Sigma).

RESULTS AND DISCUSSION
Female ES cell lines capable of distinguishing paternal and maternal Xi
To distinguish random and imprinted Xi events in differentiated cells from ES cells, we used female ES cell lines in which the activities of the paternal and maternal X chromosomes are distinguishable. One line we chose was the hybrid F1 CBMS1 female ES cell derived from CBA and MsM mice. Since there are many genomic polymorphisms available between these different subspecies, the activities of two X chromosomes can be easily confirmed by PCR-restriction fragment length polymorphism (RFLP) analysis (Sugimoto and Abe, 2007). Another line we used was the B142bgeoEG female ES cell line, which carries *EGFP* and *puromycin acetyltransferase* on the paternal X chromosome and a fusion of *lacZ* and *neomycin phosphotransferase* on the maternal X chromosome. These transgenes on the X chromosomes are inactivated by Xi. ES cells carrying two active X chromosomes can be maintained under puromycin and G418 selection, although it is known that female ES cells tend to lose one X chromosome in
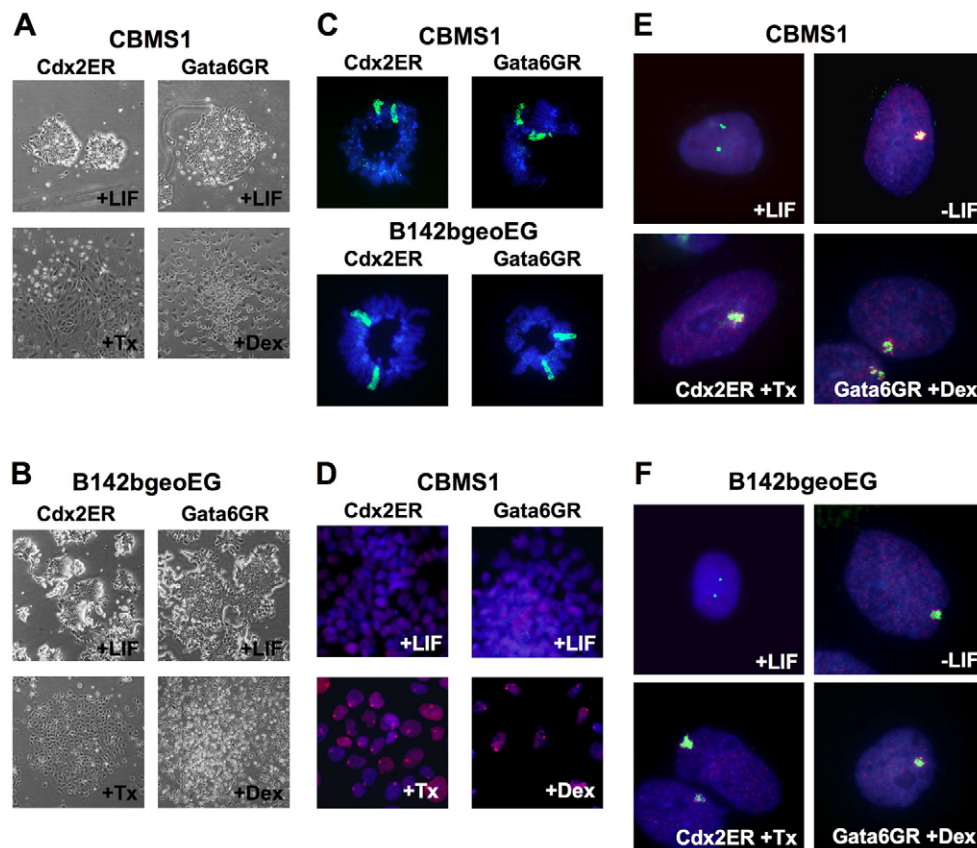

Fig. 1. Establishment of CBMS1 Cdx2ER, Gata6GR and B142bgeoEG Cdx2ER, Gata6GR mouse ES cell lines. (**A**) Phase-contrast images of CBMS1 Cdx2ER and Gata6GR ES cells (top) and corresponding induced extra-embryonic lineage cells (bottom). (**B**) Phase-contrast images of B142bgeoEG Cdx2ER and Gata6GR ES cells (top) and corresponding induced extra-embryonic cells (bottom). (**C**) X painting of the established ES cell lines. Two X chromosomes hybridized with an X-specific probe (Alexa 488, green). Chromosomes were counterstained with Hoechst 33342 (blue). (**D**) Immunostaining of H3K27me3 (Alexa 594, red), an X inactivation (Xi) marker, in induced extra-embryonic cells. (**E,F**) *Xist* RNA/H3K27me3 immuno-FISH in induced extra-embryonic cells. The spreading of *Xist* RNAs, an early marker of Xi, was detected by *Xist* exon probes (Alexa 488, green). The H3K27me3 signal was detected by Alexa 594 (red). Dex, dexamethasone; Tx, 4-hydroxytamoxifen; LIF, leukemia inhibitory factor.

long-term culture, and the inactivation of the paternal X chromosome can be monitored by the loss of EGFP fluorescence as detected by microscopy or FACS in living cells.

These two female ES cell lines were transduced with either *Cdx2ER* or *Gata6GR* to allow their inducible differentiation toward TE and PrE by Tx and Dex, respectively. To avoid overexpression effects, these extra-embryonic lineage-like cells were induced by transient addition of Tx and Dex, for 3 days and 2 days, respectively. Proper differentiation of these transgenic female ES cell lines was confirmed by following their morphological differentiation (Fig. 1A,B) as well as by monitoring marker gene expression as detected by quantitative (q) PCR or immunostaining (Fig. 2). The loss of pluripotency markers [*Oct3/4* (*Pou5f1*), *Nanog*, *Rex1* (*Zfp42*)] and upregulation of either TE markers [*Dlx3*, *Elf5*, *Psx1* (*Rhox6*) and Hand1] or PrE markers (*Gata4*, *Sox7*, *Sox17* and Dab2) confirmed their exclusive differentiation toward

the extra-embryonic cell lineages (Fig. 2). The maintenance of two X chromosomes in these transgenic female ES cell lines was confirmed by X chromosome painting analysis (Fig. 1C), indicating that they were ready to undergo Xi after induction of differentiation.

Random Xi in ES cell-derived TE and PrE

In the culture without LIF, all morphologically differentiated cells showed an H3K27me3 focus, a marker of Xi (Heard and Disteche, 2006), in each nucleus (see Fig. S1 in the supplementary material). The homogeneous acquisition of an H3K27me3 focus was also observed in Flk1 (Kdr)-positive mesoderm precursors induced by culture without LIF on a collagen IV-coated surface (Nishikawa et al., 1998). We attempted to confirm Xi in TE and PrE cells derived from these female ES cells carrying *Cdx2ER* or *Gata6GR* by *Xist* RNA fluorescence in situ hybridization (FISH) combined with
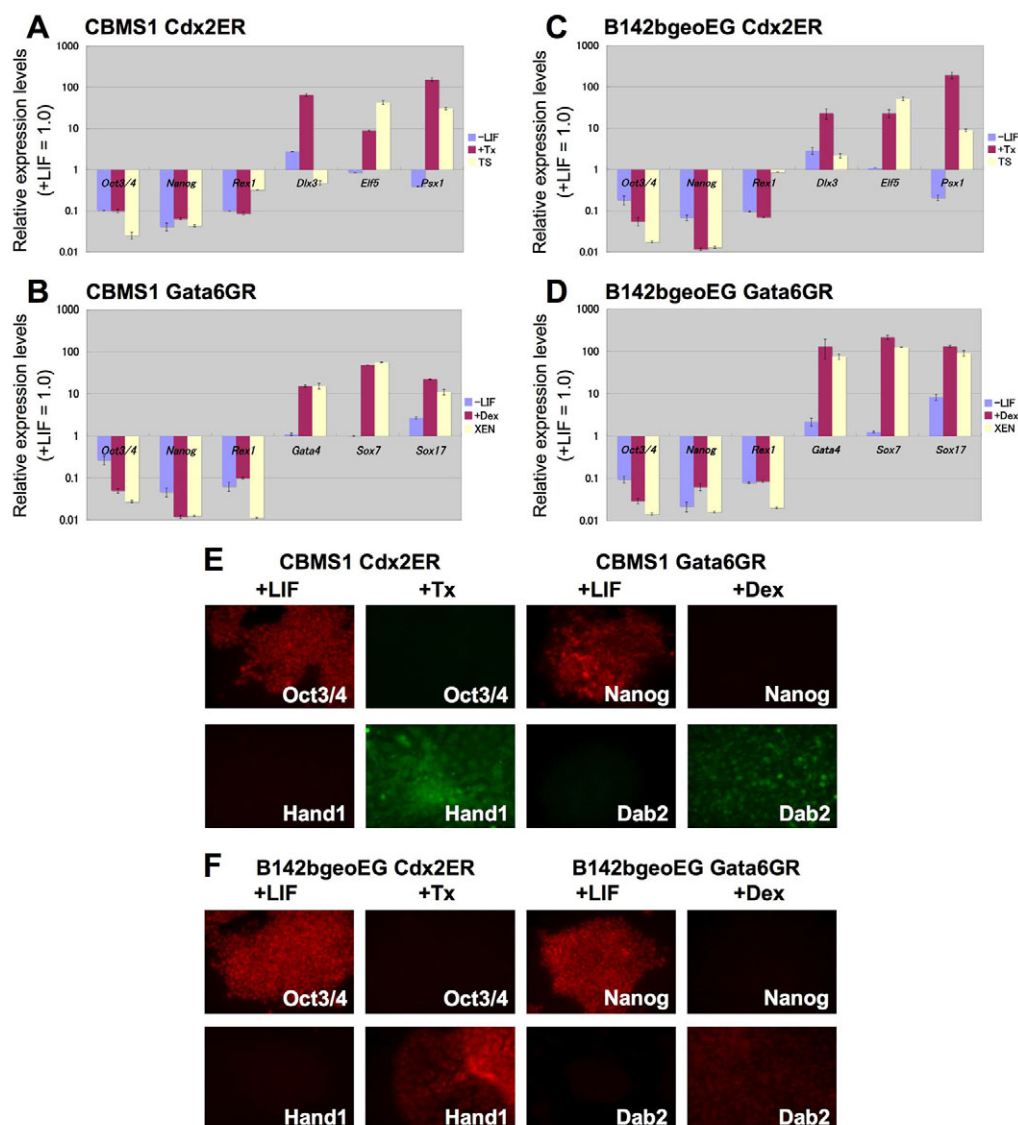

Fig. 2. Confirmation of extra-embryonic lineage induction. (A-D) qPCR analysis of marker gene expression in induced extra-embryonic lineage cells. The expression levels of each gene, normalized to *Gapdh*, are shown on a log scale. Embryo-derived extra-embryonic endoderm (XEN) and trophoblast stem (TS) cells were used as positive controls. Gene expression levels in +LIF are set at 1.0. For primers, see Table S1 in the supplementary material. (E,F) Immunostaining of lineage markers. Oct3/4 and Nanog were detected by Alexa 594 (red). Hand1 and Dab2 were detected by Alexa 488 (green) or Alexa 594.
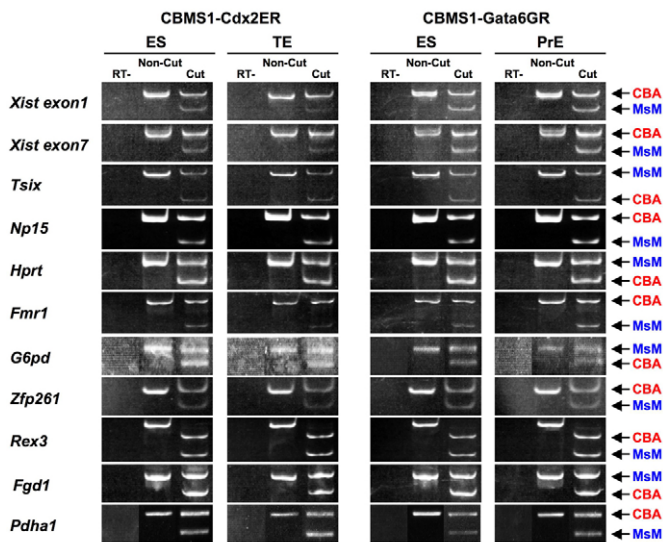
Fig. 3. PCR-RFLP analysis of the Xi pattern of CBMS1 Cdx2ER and Gata6GR cell lines. PCR fragments were cut by specific restriction enzyme sets (see Table S2 in the supplementary material). Note that two different sizes of fragments were identified in induced extra-embryonic cells, indicating the occurrence of random Xi in these cells. RT–, no reverse transcriptase control; TE, trophectoderm; PrE, primitive endoderm. CBA, *Mus musculus musculus*; MsM, *Mus musculus molocimus* (see Materials and methods).

immunostaining for H3K27me3 (Fig. 1E,F). In undifferentiated ES cells, because both X chromosomes are active, two pinpoint *Xist* RNA signals that do not merge with H3K27me3 immunostaining were observed. By contrast, in TE and PrE cells, a single, large *Xist* RNA focus that merged with the H3K27me3 focus was observed,

indicating the Xi event. As shown in Fig. 1D, large foci were observed in all nuclei of differentiated extra-embryonic cells by immunostaining for H3K27me3, indicating that all TE and PrE cells carry one inactive X chromosome.

Next, we tested which type of Xi occurred in these cells. For TE and PrE cells derived from CBMS1 ES cells, we performed the PCR-RFLP assay to detect transcripts from paternal and maternal X chromosomes separately. The reverse-transcribed transcripts from genes on the X chromosome were amplified by PCR (for primers, see Table S2 in the supplementary material), followed by digestion with appropriate restriction enzymes that cut either CBA (maternal) or MsM (paternal) derived PCR fragments, resulting in fragments of different sizes as sized on agarose or acrylamide gels. First, we tested Xi in CBMS1 differentiated into embryonic cell lineages. When CBMS1 ES cells differentiated into mesoderm on a collagen IV-coated dish were examined by PCR-RFLP (see Fig. S2 in the supplementary material), since both X chromosomes are active in ES cells, the analysis gave two fragments for each transcript (Fig. 3 and see Fig. S2 in the supplementary material). Both paternal and maternal transcripts were also detected in purified Flk1$^+$ mesodermal cells derived from either parental CBMS1 ES cells or the derivatives carrying *Cdx2ER* or *Gata6GR*. Since these mesodermal cells exhibited the H3K27me3 focus as a sign of Xi, as shown above (see Fig. S1 in the supplementary material), these data indicated that they had undergone random Xi as in normal development (see Fig. S2 in the supplementary material). Then, we tested TE or PrE cells derived from CBMS1 ES cells induced by activation of Cdx2ER or Gata6GR, respectively. If they underwent imprinted Xi, then only maternal X-linked transcripts would be expressed. However, in both cases, both paternal and maternal transcripts were detected for all X chromosome-linked genes tested, as in the case of the mesodermal cells (Fig. 3). The same results were confirmed in three independently established clones. Since the Xi event was confirmed, as shown above, these
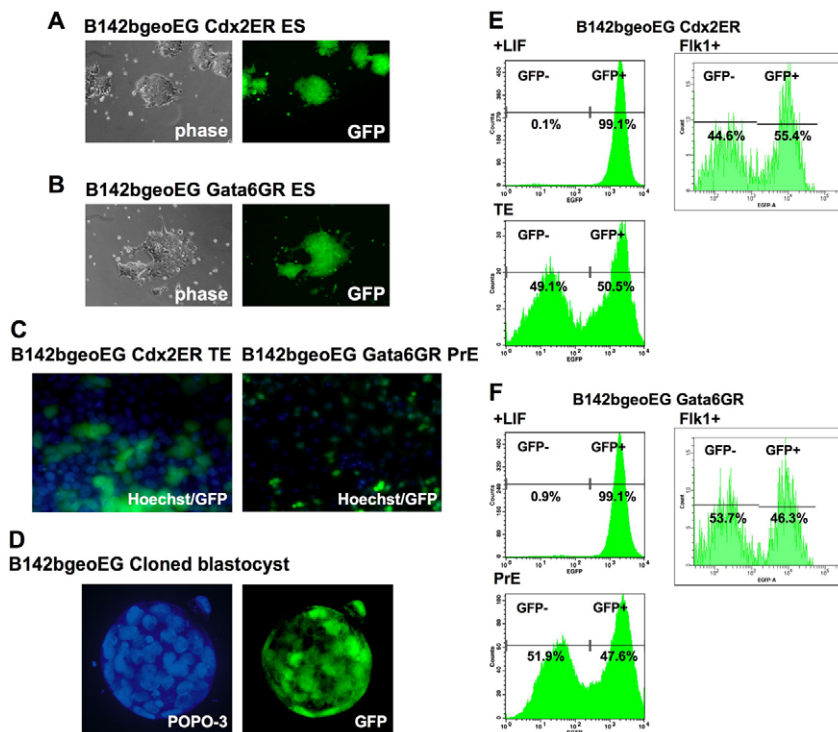

Fig. 4. Analysis of the Xi pattern in extra-embryonic lineages induced from B142bgeoEG Cdx2ER or Gata6GR cell lines. (**A**,**B**) Phase-contrast and GFP fluorescence images of B142bgeoEG Cdx2ER and Gata6GR ES cell lines. Because both X chromosomes are reactivated in ES cells, EGFP fluorescence from the paternal X chromosome is visualized in all cells. (**C**) Fluorescent images of induced extra-embryonic cells. The active state of the paternal X chromosome was detected by GFP fluorescence. Nuclei were counterstained by Hoechst 33342 (blue). (**D**) Fluorescence image of a cloned blastocyst carrying nuclei derived from a B142bgeoEG ES cell. Nuclei were counterstained with POPO-3 (blue). (**E**,**F**) FACS analysis of EGFP fluorescence. The ratio of EGFP-positive (paternal X active) to EGFP-negative (paternal X inactive) cells was estimated by FACS analysis of each cell lineage.

data clearly showed that random Xi had occurred in these TE and PrE cells. By contrast, two autosomal imprinted genes, *H19* and *Igf2r*, showed exclusive expression from the maternal chromosome in TE and PrE cells, indicating the proper maintenance of imprinted marks on CBMS1 ES cell chromosomes (see Fig. S3 in the supplementary material).

We then examined the Xi events in B142bgeoEG ES cell-derived TE and PrE cells by FACS analysis to monitor *EGFP* expression from the paternal X chromosome. This analysis confirmed their homogeneous EGFP expression, indicating that the paternal X chromosome is active in all cells. After culture without LIF on collagen IV-coated dishes, ~50% of the Flk1^{+} mesodermal cells showed EGFP expression, indicating the occurrence of random Xi (Fig. 4E,F and see Fig. S4 in the supplementary material). The B142bgeoEG derivatives carrying either *Cdx2ER* or *Gata6GR* also showed homogeneous EGFP expression, confirming the activity of the paternal X chromosome (Fig. 4A,B). If imprinted Xi occurs in TE and PrE cells derived from these ES cells, then they would lose EGFP fluorescence completely, whereas half of them would retain the EGFP signal after random Xi. The data shown in Fig. 4C,E,F unequivocally show the latter to be the case in both TE and PrE cells, indicating that they chose random Xi (Fig. 4C,E,F). The same results were confirmed in three independently established clones. These data indicated that extra-embryonic lineage cells induced from ES cells chose random Xi, as in Flk1^{+} mesodermal cells. Therefore, differentiation toward the extra-embryonic cell lineages is not coupled with the choice of imprinted Xi in this context.

Erasure of imprinting marks on X chromosomes of ES cells

The data obtained using the ES cell in vitro differentiation system indicated that female ES cells carrying two active X chromosomes lost all the imprinted marks on the paternal and maternal X chromosomes that might otherwise direct imprinted Xi in TE and PrE. To confirm the complete erasure of X-linked imprinting, we asked whether random Xi occurs in TE cells in cloned embryos generated by the transfer of B142bgeoEG nuclei into enucleated oocytes. We found that 30-40% of nuclear-transferred oocytes developed to blastocysts, which is comparable to that observed for male ES cell-derived nuclear transfer (Wakayama et al., 1999). Homogeneous EGFP expression was detected in the ICM, indicating activation of the paternal X chromosome (Fig. 4D and see Fig. S5 in the supplementary material). By contrast, a mosaic pattern of EGFP expression was observed in TE, although these cells carried the H3K27me3 focus in each nucleus, suggesting that they chose random rather than imprinted Xi, as in the ES cell in vitro differentiation system. Therefore, the female ES cell nuclei chose random Xi during differentiation to TE, even in the context of embryonic development.

It has been reported that the inactive X chromosome in female somatic cell nuclei is reactivated after nuclear transfer but is then selectively inactivated in extra-embryonic cell lineages, in which imprinted Xi occurs in normal embryos (Eggan et al., 2000), suggesting that the reactivation of the X chromosome is imperfect in cloned oocytes and that the residual marks on randomly inactivated X chromosomes are traced for the initiation of the first Xi in morula as the imprinted Xi based on the parental imprinted marks. By contrast, the female ES cell nuclei undergo random Xi in cloned embryos, indicating that all marks that trigger selective Xi are completely erased from both maternal and paternal X chromosomes.

Recent reports applied the ES cell system to analyze the molecular mechanisms governing the initiation of Xi and revealed that pluripotency-associated transcription factors, such as Oct3/4, Sox2 and Nanog, are involved in the repression of *Xist* expression in the undifferentiated state (Donohoe et al., 2009; Navarro et al., 2008). Such regulation might confer the initiation of Xi after induction of cell differentiation. However, in pre-implantation embryos, the paternal X chromosome is activated in 2-cell embryos and then starts to be inactivated at the 4-cell stage, although such cells express *Oct3/4* and *Sox2* from both maternal transcripts and by zygotic gene expression (Avilion et al., 2003; Pesce et al., 1998). By contrast, zygotic expression of *Nanog* starts in 16- to 32-cell embryos and is retained in the ICM of blastocysts. In late blastocysts, paternal X reactivation occurs only in the *Nanog*-expressing ICM cells. Since the reactivation of the paternal X chromosome does not occur in the ICM of *Nanog*-null embryos, Nanog is evidently required for X reactivation, although it remains unclear whether the reactivation is achieved by the repression of *Xist* by Nanog in cooperation with Oct3/4 and Sox2 (Silva et al., 2009). The molecular mechanisms of the complete erasure of the imprinted marks that accompanies reactivation of the paternal X chromosome as triggered by Nanog will be the next question to address in the future.

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