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Skewed X-inactivation in cloned mice

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Abstract

In female mammals, dosage compensation for X-linked genes is accomplished by inactivation of one of two X chromosomes. The X-inactivation ratio (a percentage of the cells with inactivated maternal X chromosomes in the whole cells) is skewed as a consequence of various genetic mutations, and has been observed in a number of X-linked disorders. We previously reported that phenotypically normal full-term cloned mouse fetuses had loci with inappropriate DNA methylation. Thus, cloned mice are excellent models to study abnormal epigenetic events in mammalian development. In the present study, we analyzed X-inactivation ratios in adult female cloned mice (B6C3F1). Kidneys of eight naturally produced controls and 11 cloned mice were analyzed. Although variations in X-inactivation ratio among the mice were observed in both groups, the distributions were significantly different (Ansary–Bradley test, $P < 0.01$). In particular, 2 of 11 cloned mice showed skewed X-inactivation ratios (19.2% and 86.8%). Similarly, in intestine, 1 of 10 cloned mice had a skewed ratio (75.7%). Skewed X-inactivation was observed to various degrees in different tissues of different individuals, suggesting that skewed X-inactivation in cloned mice is the result of secondary cell selection in combination with stochastic distortion of primary choice. The present study is the first demonstration that skewed X-inactivation occurs in cloned animals. This finding is important for understanding both nuclear transfer technology and etiology of X-linked disorders.

Keywords: Cloned mouse; X-chromosome inactivation; Skewed X-inactivation; Epigenetics; DNA methylation

In mammals, dosage compensation for X-linked genes is accomplished by inactivation of one of the two X chromosomes in female cells [1]. In mice, during the earliest stages of female embryogenesis, both X chromosomes are active [2,3]. Inactivation of the X chromosome occurs subsequently, coupled with cellular differentiation [4]. In embryonic cell lineage, the ratio of cells containing inactivated maternal X chromosome ($X_i^m X_a^p$ cells) to cells containing inactivated paternal X chromosome ($X_a^m X_i^p$ cells) (i.e., X-inactivation ratio) is approximately 1:1. However, under some circumstances, X-inactivation is 'skewed.' A number of human X-linked disorders, including several X-linked immunodeficiencies, Lesch–Nyhan disease, and incontinentia pigmenti, demonstrate skewed X-inactivation owing to secondary cell selection [5–7]. Recently, it was reported that skewed X-inactivation is relatively common in X-linked mental retardation [8]. X chromosome inactivation involves a master region on the X chromosome, the X-inactivation center (*Xic*), which carries elements for counting (sensing the number of X chromosomes in a cell), choosing, and onset of *cis*-inactivation [9]. The X-controlling element (*Xce*),

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lying distal and 3′ to *Xist* [10], also affects the choice of X chromosome to be inactivated. An X chromosome that carries a strong *Xce* allele is more likely to remain active than one that carries a weak *Xce* allele [11,12]. The X-linked loci such as *Xist* and *Tsix* are responsible for the choice of which X chromosome is inactivated [9]. Mice with genetically engineered X chromosomes show distortions of random nature of the initial choice, so-called primary nonrandom X-inactivation [13–15]. In these examples, skewed X-inactivation ratios are the consequences of genetic mutations or genetic variations. Although these studies provide information about genetic elements involved in X chromosome choice, and about X-linked mutations responsible for cell growth and survival, they do not tell us how aberrations in epigenetic modifications could affect X-inactivation.

Abnormal growth of cloned animals is associated with failure to establish tissue-specific DNA methylation patterns [16,17]. Recently we found that abnormal DNA methylation of the *Sall3* locus was associated with overgrowth of the placenta, a commonly observed phenotype in cloned mice [18]. Thus, cloned mice are potentially informative models for studying abnormal epigenetic events during mammalian development. Eggan et al. [19] determined that in cloned embryos, the X chromosome originally inactivated in the donor nucleus was preferentially inactivated in extra-embryonic tissues. To date, however, there are no studies of X-inactivation ratios in cloned animals. In the present study, the X-inactivation ratios in female adult cloned mice were examined and were found to be skewed in some animals.

Materials and methods

Database search and RLGS spot cloning. The *Not*I (*X-SNP-N* site)–*Pst*I genomic DNA fragment was extracted from the corresponding RLGS spot and cloned into pGEM-T easy vector (Promega, WI) as described previously [17,20]. Cloned DNA was sequenced using a Shimadzu autosequencer system (Shimadzu, Kyoto, Japan) following the manufacturer's instructions. The nucleotide sequence obtained was compared with mouse genome sequence databases at NCBI (http://www.ncbi.nlm.nih.gov) and Ensemble (http://www.ensembl.org) by the BLASTn search program. We analyzed the GC content and CpG frequency of the genomic DNA sequence around *X-SNP-N* with a program (CpG view V1.5.2) provided by the National Institute of Infectious Diseases (http://www.nih.go.jp/yoken/genbank/binaryFile/Mac/CpG-ViewV1.4.7.hqx). The CpG island was defined by a GC content of >50% and an average CpG frequency score of >0.6 [21].

Production of cloned mice. Cumulus cells of [C57BL/6 X C3H/He] (B6C3F1) adult mice were used as nucleus donors, and were individually injected into enucleated oocytes of hybrid mice [C57BL/6 X DBA/2] (B6D2F1) as described previously [22]. In cloned mouse lines B and E, cloning was repeated using cumulus cells from adult founder clones (B1-1 and E1-1) as nucleus donors to produce the next clonal generation. Second generation clones of both lines were used as donor animals for the third clonal generation. Naturally produced female B6C3F1 hybrid mice (63–807 days old) were used as controls (NM controls).

Preparation of genomic DNA. Tissues were dissected and stored at −80 °C until DNA extraction. Genomic DNA was extracted as previously described [23]. Briefly, tissue (kidney, intestine, lung, and brain) samples of cloned mice and NM controls were treated with proteinase K (Merck, Darmstadt, Germany) in lysis buffer (150 mM EDTA, 10 mM Tris–HCl, pH 8.0, and 1% SDS). Following two rounds of phenol:chloroform:isoamyl alcohol (50:49:1) extraction, the genomic DNA was precipitated in ethanol and dissolved in TE (10 mM Tris–HCl, 1 mM EDTA, pH 8.0).

Southern hybridization analysis and semiquantitation of signal intensity. Genomic DNAs (8 μg) were double digested with *Not*I and *Pst*I or with *Acc*II and *Pst*I, and separated on a 1.8% agarose gel followed by blotting onto nylon membrane. The *Not*I (*X-SNP-N* site)–*Pst*I genomic DNA fragment was used as a probe for X-inactivation ratio analysis (Figs. 2 and 4). This fragment was further digested with *Acc*II to obtain a probe sequence for *Acc*II methylation analysis (Fig. 3). Hybridization and washing were performed under stringent conditions as described elsewhere [24]. Probes were labeled with digoxigenin (DIG)-11-UTP using a DIG DNA labeling kit (Roche Diagnostics, Tokyo, Japan) and detected with a DIG luminescence detection kit (Roche Diagnostics) containing alkaline phosphatase (AP)-conjugated anti-DIG antibody and AP substrate. Signals were visualized on X-ray film (Fuji Film, Tokyo, Japan). Images on X-ray films were scanned, and intensity of each band was measured using the NIH image program (developed at the US National Institute of Health, available at http://rsb.info.nih.gov/nih-image/).

The X-inactivation ratios were determined using the following formula: 200 × [intensity (361 bp band)/{intensity(361 bp band + 655 bp band)}]. The difference in distribution of X-inactivation ratios between cloned and NM control groups was evaluated by the Ansary–Bradley test. The 95% confidence interval of the NM control group was calculated as a mean ± t (df; 0.05) × standard deviation. In *Acc*II methylation analysis (Fig. 3), digestion with *Acc*II in each sample was calculated using the following formula: 100 × [intensity (246 bp band + 366 bp band)/intensity (655 bp band + 246 bp band + 366 bp band)].

Results

Determination of X-inactivation ratio in B6C3F1 female

In humans and mice, the majority of CpG islands on the inactive X chromosome are methylated, whereas those on the active X chromosome are unmethylated [25,26]. Therefore, we can determine the X-inactivation ratio by identifying a heterozygous locus in a CpG island on the X chromosome, allowing maternal and paternal X chromosomes to be distinguished, and by using a methylation-sensitive restriction enzyme to study the locus. Using *Not*I as the landmark enzyme in restriction landmark genomic scanning (RLGS), CpG islands on the X chromosome were searched to find a polymorphism between C57BL/6 and C3H/He mouse strains. We found a single nucleotide polymorphism (SNP) in a 760 bp long CpG island, covering the first exon of a gene (Accession No. BC031748) located approximately 30 Mb from *Xic* (Figs. 1A and B). The SNP (named *X-SNP-N*) creates a *Not*I recognition site (5′-GCGGC CGC-3′) in C3H/He, but not in C57BL/6 (5′-GCGGCC AC-3′) (Fig. 1B). Since *Not*I is methylation-sensitive, it

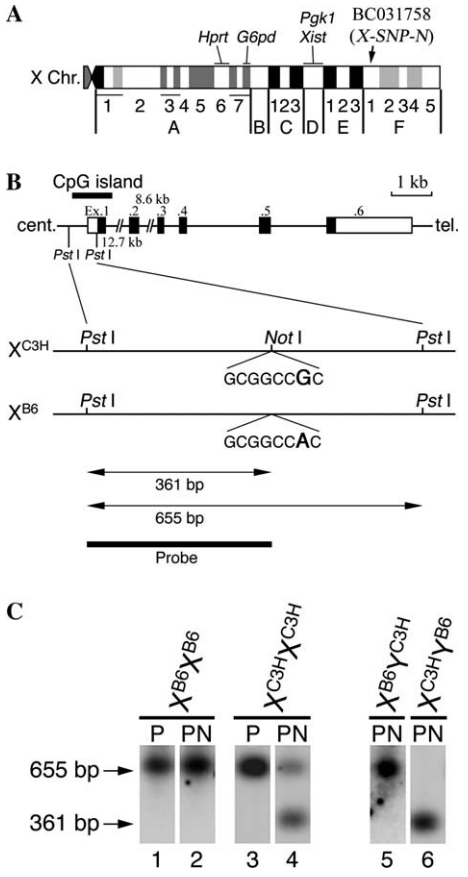

Fig. 1. (A) Schematic representation of location of *X-SNP-N* and other X-linked loci (*Hprt*, *G6pd*, *Xic*, and *Pgk1*). (B) (Top) Genomic structure of an X-linked gene (Accession No. BC031748) immediately downstream of *X-SNP-N*. Boxes represent exons 1–6. The CpG island (horizontal bar) was defined by a GC content of >50% and an average CpG frequency score of >0.6. cent., centromere side; tel., telomere side. (Bottom) *Pst*I–*Pst*I fragment containing *X-SNP-N* showing the eight nucleotide *Not*I recognition sequence, with the G/A polymorphism in bold letters. In C3H/He mice, the site is cut only if unmethylated. In C57BL/6 mice, the substitution of A for G abolishes the recognition sequence. Double-headed arrows (655 and 361 bp) indicate fragments possibly resulting from Southern hybridization, utilizing treatment with *Not*I and *Pst*I followed by detection with the probe (horizontal bar, below the double-headed arrows). (C) Genomic DNAs from kidneys of C3H/He females ($X^{C3H}X^{C3H}$), C57BL/6 females ($X^{B6}X^{B6}$), [C3H/He X C57BL/6] F1 males ($X^{C3H}Y^{B6}$), and [C57BL/6 X C3H/He] F1 males ($X^{B6}Y^{C3H}$) were treated with *Not*I and *Pst*I, and hybridized with the probe indicated in (B). P, *Pst*I digestion; PN, *Pst*I–*Not*I digestion.

can probe the methylation status of the X chromosome at this site.

Southern blot analysis (Fig. 1C) detected only a 361 bp band in the [C3H/He X C57BL/6] F1 male (X^{C3H}/Y^{B6}) (lane 6), reflecting the unmethylated status at the *X-SNP-N* site on active X chromosome (X_a). In C3H/He females (X^{C3H}/X^{C3H}) (lane 4), a 655 bp band appeared as well as the 361 bp band, indicating the methylated *X-SNP-N* site on inactive X chromosome (X_i). The 361 bp band was not evident in the [C57BL/6 X C3H/He] F1 male (X^{B6}/Y^{C3H}) or the C57BL/6 female (X^{B6}/X^{B6}) (lanes 5 and 2, respectively), indicating the absence of a restriction site at *X-SNP-N* in X^{B6}. These results ensure that in [C57BL/6 X C3H/He] (B6C3F1) females, cutting at the *X-SNP-N* site with *Not*I enables us to determine a percentage of (X_i^{B6}/X_a^{C3H}) cells in each sample. In this report, hereafter, the "X-inactivation ratio" refers to this percentage.

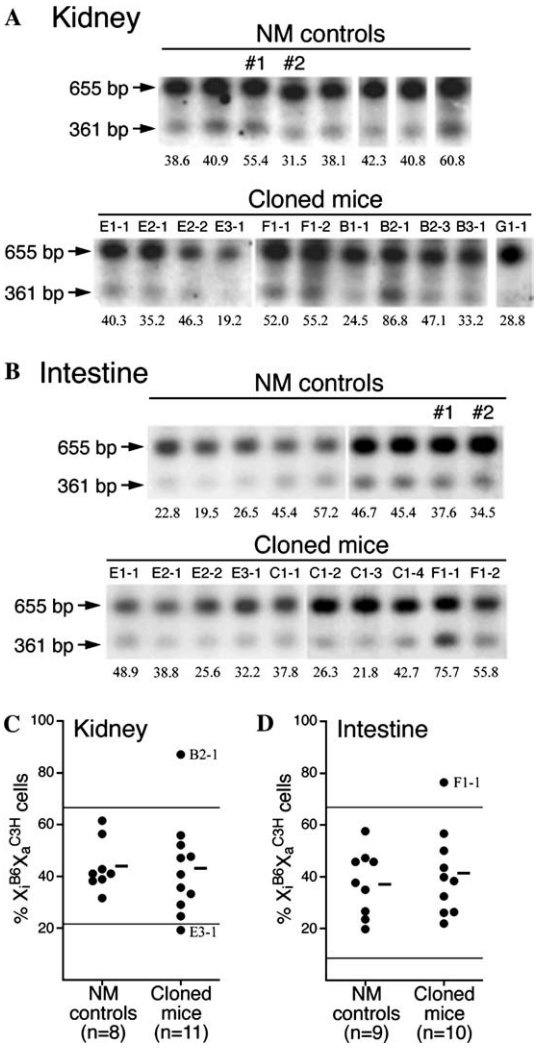

Fig. 2. (A,B) Southern blot analysis of X-inactivation ratio of cloned mice kidneys and intestines, respectively. DNAs from naturally produced BCF1 females (NM controls, 63–807 days old) and cloned mice were digested with *Not*I and *Pst*I, and then hybridized with the probe shown in Fig. 1B. The X-inactivation ratios were determined using the following formula: 200 × [intensity (361 bp band)/{intensity (361 bp band + 655 bp band)}]. Results are shown beneath each lane. The names of clones and two NM controls (#1 and #2, further analyzed in Fig. 4) are shown above each lane. (C,D) Distributions of X-inactivation ratios in kidney and intestine, respectively, of NM controls and cloned mice. The 95% confidence interval of the NM control group (mean ± 2.365 × SD in kidney and mean ± 2.306 × SD in intestine) is shown as two lateral lines. The horizontal bar represents the mean X-inactivation ratio of each group.

Skewed X-inactivation in cloned mice

We determined the X-inactivation ratios in kidneys of cloned mice and control B6C3F1 females produced by natural matings (NM controls) by Southern blot analysis of *X-SNP-N* (Figs. 2A and C). Individual cloned mice are identified in Table 1. In NM controls, there was a variation in X-inactivation ratios among individuals and the mean X-inactivation ratio was 43.6 ± 9.7% (±SD, $n = 8$). The X^{C3H} and X^{B6} carry the Xce^a allele and the Xce^b allele, respectively [11]. The ratio in strength of Xce^a to Xce^c is approximately 2:8, and that of Xce^b to Xce^c is approximately 3:7 [27]. Therefore, the relative strength (Xce^a:Xce^b) can be estimated as 4:6. Alternatively, in B6C3F1 females the X-inactivation ratio is considered to be about 40%. Therefore, the value (43.6%) is close to the theoretical one. In the cloned mice (lines E, F, B, and G), the mean X-inactivation ratio was 42.6 ± 18.6% (±SD, $n = 11$). Most cloned mice had X-inactivation ratios within the range of the NM controls. Interestingly, however, the distributions are significantly different (Ansary–Bradley test, $P < 0.01$). In cloned mice, the highest and lowest X-inactivation ratios were 86.8% (mouse B2-1) and 19.2% (mouse E3-1), respectively, which fell outside the 95% confidence interval of the NM control group, suggesting extremely distorted, or skewed, X-inactivation in kidney.

Table 1
The clarification of cloned mice used in this study

Clone identification	Cloned with a cumulus cell of	Clone generation	Age (month) of clone when examined
B-line			
B1-1	B6C3F1	1	27
B2-1	B1-1	2	25
B2-2	B1-1	2	25
B3-1	B2-2	3	23
C-line			
C1-1	B6C3F1	1	11
C1-2	B6C3F1	1	11
C1-3	B6C3F1	1	11
C1-4	B6C3F1	1	11
E-line			
E1-1	B6C3F1	1	11
E2-1	E1-1	2	8
E2-2	E1-1	2	8
E3-1	E2-1 or E2-2	3	5
F-line			
F1-1	B6C3F1	1	11
F1-2	B6C3F1	1	11
G-line			
G1-1	B6C3F1	1	16

In mouse lines B and E, cloned mice were produced by sequential cloning procedure. All of the clones of lines C, F, and G and the founder clones of lines B and E (B1-1 and E1-1, respectively) were generated from their respective donor animals, naturally produced B6C3F1 adult females. B1-1 and E1-1 were used as donor animals for subsequent clonal generations. All the cloning manipulations were performed as described previously [21], using cumulus cells as nucleus donors.

In intestine, mean X-inactivation ratios were 37.3 ± 12.7% (±SD, $n = 9$) in the NM controls and 40.5 ± 13.6% (±SD, $n = 10$) in the cloned mice (lines E, C and F; Figs. 2B and D). In cloned mouse F1-1, the X-inactivation ratio was 75.7%, which fell outside the 95% confidence interval of the NM control group. Thus, skewed X-inactivation was not limited to the kidney.

There were also *Acc*II recognition sites near *X-SNP-N* in the CpG island of both C3H/He and C57BL/6 mice (Fig. 3A). Since *Acc*II is methylation sensitive, *Acc*II recognition sites in X_a should be cut but not in X_i, regardless of the strain (digestion rate with *Acc*II should be 50%), if X-inactivation occurs correctly in each cell. In contrast, when methylation on X_a or unmethylation on X_i occurs in the *X-SNP-N* site, the digestion rate with *Acc*II should fluctuate. DNAs from kidneys of E3-1 and B2-1, and from the intestine of F1-1 were subjected to Southern blot using *Acc*II. Digestion rates were almost 50% in NM control and cloned mice (Fig. 3B). Based on these results, methylation on X_a or unmethylation on X_i does not contribute the extreme degrees of digestion rate with *Not*I observed in cloned mice. In other

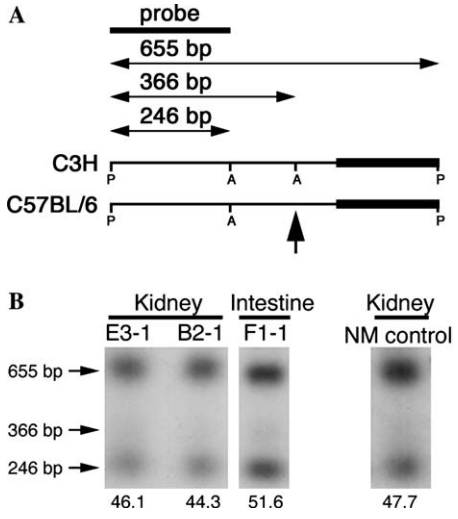

Fig. 3. Southern blot analysis using *Acc*II, a methylation-sensitive restriction enzyme. (A) Schematic representation of the *Pst*I–*Pst*I genomic DNA fragment containing *X-SNP-N*, showing the *Pst*I site (P), the *Acc*II site (A), and the first exon of the gene (Accession No. BC031748; black box). The location of *X-SNP-N* is indicated by an arrow. On X^{C3H}, *X-SNP-N* creates an additional *Acc*II site (C57BL/6, 5′-CACG-3′; C3H/He, 5′-CGCG-3′). Double-headed arrows (655, 366, and 246 bp) indicate fragments possibly resulting from Southern hybridization. (B) DNAs from kidneys of cloned mice E3-1 and B2-1, and from intestine of F1-1 were digested with *Acc*II and *Pst*I and then hybridized with the probe indicated in (A). Digestion with *Acc*II in each sample was calculated from the following formula: 100 × [intensity (246 bp band + 366 bp band)/intensity (655 bp band + 246 bp band + 366 bp band)]. Percentages are shown beneath each lane. The ratios were nearly 50% in cloned mice, and there was no correlation between the ratio from *Not*I and that from *Acc*II in each sample, excluding the possibility that methylation on X_a or un-methylation on X_i at this CpG island contributes to the lower or higher *Not*I ratio observed.

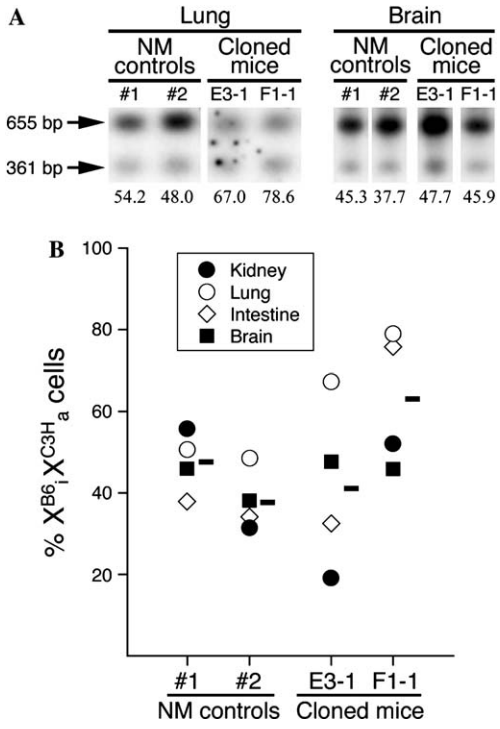

Fig. 4. (A) X-inactivation ratios were further analyzed in brain and lung of cloned mice, E3-1 and F1-1, and NM controls, #1 and #2, as in Fig. 2. Results are shown beneath each lane. (B) Distribution of the X-inactivation ratios of four tissues in each mouse, showing data from Fig. 2 and (A) of this figure. The horizontal bar represents the mean X-inactivation ratio of each mouse.

words, X-inactivation occurred correctly in each cell, but the X-inactivation ratios were skewed in the tissues of cloned mice examined.

Tissue-dependent variation of skewed X-inactivation

Ratios of X-inactivation were analyzed further in the brains and lungs of cloned mice, E3-1 and F1-1 (Fig. 4). The X-inactivation ratios in E3-1 were 67.0% (lung) and 47.7% (brain). Those in F1-1 were 78.6% (lung) and 45.9% (brain) (Fig. 4A). In NM controls, variations in the X-inactivation ratio were 37.6–55.4% (NM control #1, Fig. 4B) and 31.5–48.0% (NM control #2, Fig. 4B). In cloned mouse E3-1, the X-inactivation ratio varied from 19.2% (kidney) to 67.0% (lung) (Fig. 4B). Similarly, cloned mouse F1-1, which showed skewed X-inactivation in intestine (75.7%, Figs. 2B, D and 4B), also showed skewed X-inactivation in lung (78.6%, Figs. 4A and B) but not in brain (45.9%, Figs. 4A and B) and kidney (52.0%, Figs. 2A, C and 4B). Thus, it is evident that skewed X-inactivation occurs in certain tissues of a given individual, but not necessarily in the same tissue of a different individual.

Discussion

Eggan et al. [19] reported that an inactive X chromosome in the donor nucleus was preferentially inactivated in the extra-embryonic tissues of cloned embryos. Xue et al. [28] found biallelic repression of X-linked genes in cloned female cattle that died within 24 h after birth. In the present study, we demonstrated for the first time skewed ratios of X-inactivation in adult cloned animals. This suggests that epiblast-derived cell lineage can have aberrant epigenetic modifications in cloned female embryos, and that these can persist until adulthood.

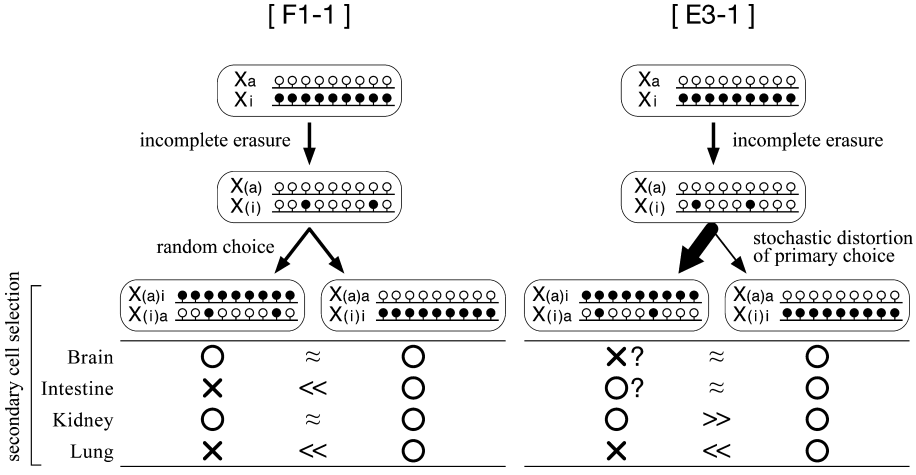

Fig. 5. Schematic representation of the hypothetical mechanism leading to skewed X-inactivation in cloned animals. Incomplete erasure of epigenetic modifications, which had been imposed on the inactive X chromosome in the donor nucleus (X_i), would confer ectopic silent state on some part of the former inactive X chromosome ($X_{(i)}$). Then, after random (left) or stochastically distorted (right) choice of inactivation, cells silencing the formerly active X chromosome ($X_{(a)}$) would have biallelically silenced some X-linked loci, leading to secondary cell selection against such cells (crosses). Filled circles and open circles with short vertical bars represent the inactive and active epigenetic status at each X-linked locus, respectively. X_a, active X chromosome; X_i, inactive X chromosome.

The causes of skewed X-inactivation are not only distortions of the random initial choice of which X chromosome to inactivate (so-called primary nonrandom X-inactivation), but also 'secondary cell selections.' For primary nonrandom X-inactivation, the skewed ratios must be the same direction in all tissues. Secondary cell selection occurs after random choice, on cells that keep an active X chromosome bearing a malfunctioned locus. Skewed X-inactivation is thought to take place in specific cell lineages in which the malfunctioned X-linked gene is required for proliferation or survival. This leads to a variety of X-inactivation ratios among tissues in the same individual [5–7,29]. In cloned mouse F1-1, the X-inactivation ratios skewed in intestine and lung toward the same direction, but not in brain and liver (Fig. 4). Thus, the skewed X-inactivation ratios observed in cloned mice F1-1 are probably consequences of secondary cell selection, rather than primary nonrandom X-inactivation (Fig. 5, left panel). In the case of cloned mouse E3-1, it is more complicated. In E3-1, the X-inactivation ratio skewed toward the decrease in ($X_i^{B6}X_a^{C3H}$) cells in kidney but toward the opposite direction in lung (Fig. 4). We assume that stochastic distortion of primary choice had occurred in this animal. It is not clear if this distortion was caused by somatic cell cloning. As mentioned above, however, distortion of primary choice alone cannot account for a tissue-dependent, skewed X-inactivation ratio toward opposite directions unless it occurred late in the embryogenesis. We therefore suppose that the secondary cell selection also took place in the cloned mouse E3-1, resulting in a tissue-dependent variation of the X-inactivation ratios (Fig. 5, right panel).

The most likely mechanism for skewed X-inactivation ratios by secondary cell selection in cloned mice is variable and incomplete erasure of the inactive state of X_i from the donor nucleus. The X_i in the donor nucleus bears epigenetic marks. When the embryo starts to form, those marks should be completely erased, but in some clones the erasure is incomplete, leaving parts of the chromosome still silenced. As the embryo develops, cells inactivate one or the other X chromosome at random, and this leads to formation of two types of cells: those in which the chromosome inactivated is the former X_i, leaving the cell with a full set of active genes from its former X_a; and those in which the chromosome inactivated is the former X_a, leaving the cell with a partial set of active genes on the former X_i. Since the second type of cells will be unable to express some genes, they will be selected against. This is perhaps what causes the skewed ratios seen in adult animals (Fig. 5).

The incomplete erasure model described above may contribute partially to the phenomenon observed by Xue et al. [28]. However, under this model, biallelic repression must occur only when the former X_a is chosen to become inactive. To explain their result, in addition to the incomplete erasure, ectopic de novo inactivation of the former X_a is required. Therefore, during development of the cloned cattle which died immediately after birth, a combination of factors may result in aberrant inactivation of X-linked genes. In their report, the live clones did not show biallelic repression of X-linked genes. It is not known whether the surviving calves had skewed ratios of X-inactivation.

X-linked disorders are due to genetic mutations, as well as demonstrate skewed X-inactivation attributed to secondary cell selection [5–7]. Our study suggests that aberrant epigenetic modifications on the X chromosome could also produce the same outcome.

In conclusion, we found that some cloned mice showed skewed X-inactivation. Furthermore, our results suggest that skewed X-inactivation in cloned mice is the result of secondary cell selection in combination with stochastically distorted primary choice. Our finding is important for understanding reprogramming mechanisms, X-inactivation mechanisms, and the etiology of X-linked disorders.

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