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X chromosome reactivation and regulation in cloned embryos

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Abstract

Somatic cell nuclear transfer embryos exhibit extensive epigenetic abnormalities, including aberrant methylation and abnormal imprinted gene expression. In this study, a thorough analysis of X chromosome inactivation (XCI) was performed in both preimplantation and postimplantation nuclear transfer embryos. Cloned blastocysts reactivated the inactive somatic X chromosome, possibly in a gradient fashion. Analysis of XCI by Xist RNA and Eed protein localization revealed heterogeneity within cloned embryos, with some cells successfully inactivating an X chromosome and others failing to do so. Additionally, a significant proportion of cells contained more than two X chromosomes, which correlated with an increased incidence of tetraploidy. Imprinted XCI, normally found in preimplantation embryos and extraembryonic tissues, was not observed in blastocysts or placentae from later stage clones, although fetuses recapitulated the Xce effect. We conclude that, although SCNT embryos can reactivate, count, and inactivate X chromosomes, they are not able to regulate XCI consistently. These results illustrate the heterogeneity of epigenetic changes found in cloned embryos.

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Introduction

Epigenetic modifications of the genome generate immense expressional diversity from a finite number of genes making it possible to create a complex organism. These modifications must be both stable through cell division and easily modified during development. During the period immediately following fertilization, extensive remodeling of the oocyte- and sperm-derived genomes occurs (Latham and Schultz, 2001). Some of the earliest events, such as the exchange of histones for protamines, are mediated by factors in the ooplasm that appear to recognize and respond to specific epigenetic information present on both gametic genomes (Chung et al., 2003; Fundele et al., 1990; Gao et al., 2004a; Latham and Solter, 1991; Surani et al., 1990).

Cloning by somatic cell nuclear transfer (SCNT) requires that ooplasmic factors that normally act on the gamete genomes reprogram somatic cell DNA instead. During this process, a fully differentiated somatic cell nucleus is thought to be transformed to an embryonic state. While the ability to generate clones by SCNT indicates that epigenetic modifications can be reprogrammed during the cloning process, successful reprogramming is a rare event. Overall cloning success rates are extremely low, with only 0.9% to 5% of nuclear transfers resulting in live births (Wilmut et al., 2002). Studies analyzing epigenetic modifications in cloned animals indicate that incorrect reprogramming may be partially responsible for the low success rate (Boiani et al., 2002; Bortvin et al., 2003; Bourc’his et al., 2001; Dean et
al., 2001; Eggan et al., 2000; Humpheries et al., 2001; Kang et al., 2001a,b; Mann et al., 2003; Ogawa et al., 2003; Xue et al., 2002). For example, DNA methylation, an epigenetic modification that undergoes dramatic changes during development, is disrupted in cloned embryos (Bourc’his et al., 2001; Dean et al., 2001; Kang et al., 2002; Mann et al., 2003). While repetitive elements become abnormally hypermethylated in bovine clones (Bourc’his et al., 2001; Kang et al., 2001b), single copy genes experience a loss of methylation in mouse and ovine SCNT clones (Mann et al., 2003; Young et al., 2003). In addition, developmentally critical genes, such as those involved in DNA methylation, stress response, and trophoblastic function are perturbed in SCNT clones (Boiani et al., 2002; Chung et al., 2003; Wrenzycki et al., 2001). Understanding the fates of somatic cell-derived epigenetic information during SCNT provides one means for elucidating the nuclear reprogramming process that operates during normal development.

One epigenetic modification that is well studied in normal embryos is X chromosome inactivation (XCI). However, it remains incompletely characterized in cloned embryos. XCI is a dosage compensation mechanism that operates in the early embryo and allows the silencing of one X chromosome in female mammals (Lyon, 1961). XCI proceeds through a complex sequence of events that are incompletely understood. Xist, a noncoding RNA that is transcribed from the silenced X chromosome, coats the chromosome in cis and initiates a cascade of epigenetic changes that generate a heritable silent state (Borsani et al., 1991; Brockdorff et al., 1991; Brown, 1991; Brown et al., 1991). These changes include modifications of histones and the recruitment of Polycomb group proteins, Eed and Ezh2, to the inactivating X chromosome (Mak et al., 2004; Okamoto et al., 2004).

Two forms of XCI, random and imprinted, occur during development. Initially, the choice of which X chromosome to inactivate is completely biased in the mouse, with the paternal X chromosome always chosen (Takagi and Sasaki, 1975; West et al., 1977). This imprinted form of inactivation occurs very early in mouse development and persists in extraembryonic tissues (Huynh and Lee, 2003; Latham and Rambhatla, 1995). Later in development, cells of the embryo proper undergo random XCI, with either X chromosome being susceptible to inactivation (Rastan, 1982; Takagi et al., 1982). Interestingly, the early imprinted inactive X chromosome lacks many of the modifications seen in somatic cells. For example, although the Xist gene is hypermethylated on the active X chromosome in somatic cells, both X chromosomes are hypomethylated in the preimplantation embryo (McDonald et al., 1998; Norris et al., 1994). Furthermore, silencing of the X chromosome in the preimplantation embryo is incomplete, with many genes that are located at a distance from the X inactivation center (Xic) biallelically expressed (Huynh and Lee, 2003; Latham and Rambhatla, 1995).

The introduction of a somatic cell nucleus containing one inactive X chromosome into oocyte cytoplasm creates an unusual epigenetic situation. Using SCNT embryos, it is possible to assess the ability of the oocyte cytoplasm to reactivate the inactive X and erase the epigenetic modifications. XCI was first studied in SCNT embryos in the mouse using an X-linked GFP transgene as an exogenous reporter. It was demonstrated that this proximally-located GFP transgene was reactivated in cloned blastocysts and that phenotypically normal day 12.5 cloned embryos had 100% skewing of X inactivation in the placenta (Eggan et al., 2000). Rather than being directed by parent-of-origin marks, the extraembryonic lineages of clones inactivated the X chromosome that was previously silenced in the donor cell, suggesting that this somatic epigenetic modification was recognized.

In this study, we extended the analysis of XCI in cloned embryos by conducting a thorough investigation of X-inactivation at the embryonic and cellular level. Cloned embryos were examined for expression of a number of endogenous X-linked genes, as well as Xist RNA localization, X chromosome number and the expression and localization of chromatin-remodeling proteins. More specifically, we determined whether (1) cloned embryos can reactivate an inactivated X chromosome obtained from a differentiated somatic cell, (2) cloned embryos can count and inactivate X chromosomes, (3) inactivation in the cloned embryo is governed by epigenetic modifications that are present in the somatic cell, and (4) genetic factors controlling XCI operate appropriately.

We show that SCNT embryos can reactivate the silent X chromosome although reactivation may be incomplete. In a number of cases, including some blastomeres and later stage fetuses and placentae, this appears to be followed by normal XCI. However, a significant number of abnormalities were observed at all stages of development, including lack of an inactive X chromosome in some cells of SCNT blastocysts and absence of imprinted XCI in blastocysts and late stage placentae. Additionally, a proportion of SCNT blastomeres exhibit characteristics of two inactive X chromosomes, the frequency of which correlates to the percent of cells with 3 or 4 X chromosomes. These abnormalities could contribute to the developmental delay and large placentae that are frequently observed in cloned animals. These data provide additional insight into the stability and regulation of epigenetic modifications in clones.

Materials and methods

Mice and embryos

Control preimplantation embryos were generated by mating C57BL/6 females to males carrying a Mus musculus castaneus (CAST/Ei) X chromosome on a 129S1/SvJ background (Plenge et al., 2000) (referred to as tester in figures). Blastocysts were collected at 3.5 days post coitum (dpc), with the morning of the plug being 0.5 dpc, by flushing the uterine horns with PBS/polyvinylpyrrolidone (PVP, 3 mg/ml). In vitro cultured preimplantation embryos
were isolated at the 2-cell stage by flushing the uterine horns at 1.5 dpc then cultured in KSOM medium at 37°C as described previously (Mann et al., 2003). Control post-implantation embryos were generated using the same matings, with dissections performed at 10.5 days or 17.5 days of pregnancy. Maternal deciduae were removed cleanly from placentae recovered at 10.5 days of pregnancy.

Production of cloned embryos

Adult female mice were superovulated by sequential administration of 5 IU equine chorionic gonadotropin (Calbiochem, San Diego, CA) and human chorionic gonadotropin (Sigma-Aldrich, St. Louis, MO) 48 h later. Oocytes used for nuclear transfer were obtained from (C57BL/6 × DBA/2)F1 females [(B6D2)F1], (Taconic, Germantown, NY). Cumulus cells were obtained from ovulated cumulus cell-oocyte complexes of C57BL/6J (B6) × M. musculus castaneus (CAST) females [(B6XCAST) F1]. For DNA and RNA fluorescence in situ hybridization (FISH), cloned embryos were produced by injection of (B6D2)F1 cumulus cell nuclei into enucleated (B6D2)F1 oocytes.

Cloned embryos were produced as described (Wakayama et al., 1998) and later modified (Chung et al., 2002) using cumulus cell donor nuclei of the genotypes indicated below. Cloned constructs were cultured in minimal essential medium alpha (MEMα) supplemented with 1 mM glutamine and 5 mg/ml BSA under an atmosphere of 5% CO2 and 21% O2 in nitrogen at 37°C in a humidified modular incubator (Billups-Rothenberg, Del Mar, CA), a culture system that previously has produced a high rate of preimplantation development and typically term development at a rate of approximately 2% (Gao et al., 2004b).

To evaluate postimplantation development, morula/blastocyst stage cloned embryos produced using (B6XCAST) F1 cumulus cells were transferred to the uteri of CD1 pseudopregnant surrogate mothers that had been mated with vasectomized male mice 2.5 days earlier. Fetuses were produced as described (Plenge et al., 2000) using the High Pure PCR Bead (Amersham Biosciences UK Limited, Buckinghamshire, England), 0.5 μm of primer, and 0.175 μm of probe (TIB Berlin, Germany). The PCR program was an initial 2 min incubation at 95°C, then 32 cycles of 0 s at 95°C, 10 s 59°C, and 10 s 74°C. The melting curve was an initial 2-min incubation at 95°C, then 50°C for 30 s, followed by a 0.3°C/s transition rate to 75°C. The relative expression of the two alleles was determined by dividing the height of the CAST or B6 melting peak by the height of the combined CAST and B6 peaks.

Allele-specific expression assays using second-strand product

Real-time allele-specific PCR analysis for Xist and Pctk1 mRNAs was performed as described previously (Percec et al., 2002). Second-strand product from the cDNA beads (Mann et al., 2003) was mixed with 2× reverse primer and amplified as described. FRET probes spanning a polymorphic site in the PCR product allowed allelic analysis using Roche LightCycler Real Time PCR System (Roche Molecular Biochemicals, Indianapolis, IN). The ratio of the two alleles was determined by dividing the height of the CAST or B6 melting peak by the height of the combined CAST and B6 peaks.

Allele-specific assay for Pgk1 mRNA

Pgk1 mRNA expression was assayed as described previously (Percec et al., 2002) with the following modifications. PCR using the primers Pgk1F (5′GGTCTGTGAATGGTGGAATCTG) and Pgk1R (5′TTGCCCCACGAGATTTGAG3′) and probes PGK1LC (5′LC Red 640-GCCTGAGTCTTTGGTTGTTATCTG-phos3′) and PGK1 Fluoro (5′CAGAAATGTGCTTGGAAACGC-fluro3′) was performed. FRET probes spanning a polymorphic site in the PCR product allowed allelic analysis using Roche LightCycler Real Time PCR System (Roche Molecular Biochemicals, Indianapolis, IN). PCR was performed in a 25-μl reaction with a Ready-To-Go PCR Bead (Amersham Biosciences UK Limited, Buckinghamshire, England), 0.5 μm of primer, and 0.175 μm of probe (TIB Berlin, Germany). The PCR program was an initial 2 min incubation at 95°C, then 32 cycles of 0 s at 95°C, 10 s 59°C, and 10 s 74°C. The melting curve was an initial 2-min incubation at 95°C, then 50°C for 30 s, followed by a 0.3°C/s transition rate to 75°C. The relative expression of the two alleles was determined by dividing the height of the CAST or B6 melting peak by the height of the combined CAST and B6 peaks.

Allele-specific assays for Mecp2 mRNA expression

Mecp2 mRNA expression was assayed by RT-PCR using the primers Mecp2F (5′ATGTTAGCTCGGATGGTTAG-3′) and Mecp2R (5′TCTGCTCTCTCCTGGAGGGG3′). For PCR amplification of postimplantation stage samples, a 25-μl reaction that contained 1 × PCR buffer II [10 mM Tris–HCl, pH 8.3, 50 mM KCl (Perkin Elmer, Boston, MA)], 2.5 mM MgCl2, 125 μm dNTP each, 0.5 μm primer each, and 1 × SYBR Green I (Molecular Probes, Eugene, OR) was amplified using the Roche LightCycler. The PCR program was an initial 2 min incubation at 95°C, then 35 cycles of 0 s at 95°C, 10 s 55°C, and 15 s 74°C. The second-strand products from single blastocysts were amplified using [α-32P]dCTP (1 μCi) and a PCR program of an initial 2 min at 95°C, then 40 cycles of 15 s at 95°C, 10 s 55°C, and 20 s 72°C. All samples were digested with DdeI and run on a 12% polyacrylamide gel. The CAST allele produced a 179 bp
Individual strands of DNA were subcloned and sequenced by rounds of PCR were performed with the primers Pr2 and Pr3.

Expression analysis of Eed, Ezh1, and Ezh2 mRNAs

Second-strand product from the cDNA libraries of individual blastocysts was used in the PCR amplification to detect Eed, Ezh1, and Ezh2 mRNAs. Amplification of Ezh1 and Ezh2 was performed as described (O’Carroll et al., 2001). Eed mRNA was amplified using the primers EedF (5’CCCAAACCTTCTCCTGTCAGTAAG3’) and EedR (5’CTTCTACTGTGGCCTCTCCAC3’). Amplification used a Ready-To-Go PCR Bead, 0.3 μM each primer and [α-32P]dCTP (1 μCi). The PCR program was an initial 2 min at 95°C, then 35 cycles of 15 s at 95°C, 10 s 54°C (Ezh1 and Ezh2), or 56°C (Eed) and 20 s 72°C. Products were resolved on a 7% polyacrylamide gel.

Immunofluorescent detection of Troma-1 and Eed

All manipulations were performed at room temperature. Individual cloned and control blastocysts were fixed in 2% paraformaldehyde (PFA) for 20 min. They were washed through 3 drops of PBS-PVP (3 mg/ml). Embryos were then permeabilized by incubation in 0.1% Triton X-100 for 15 min and then blocked for 1 h in 0.1% BSA, 0.01% Tween-20 and 0.20 μg/ml anti-mouse Fab fragment. Hybridization was performed for 1 h in block solution (0.1% BSA, 0.01% Tween-20 in PBS) at a dilution of 1:5 for Troma-1 [TROMA-1 (223 μg/ml) University of Iowa Developmental Studies Hybridoma Bank] and undiluted for the Eed antibody (Sewalt et al., 1998). Embryos were then washed three times for 15 min in block solution. The secondary antibody, donkey Cy3-conjugated anti-mouse IgG (Jackson Immunoresearch, West Grove, PA), was diluted 1:200 in block solution and incubated for 1 h. Following three more 15-min washes in block solution, embryos were mounted and analyzed. 

Methylation analysis of Xist promoter region

Bisulfite analysis was performed as described previously (Mann et al., 2003). DNA was isolated from a pool of 30 blastocysts or 1500 cumulus cells, and digested with BanHI. Region 1 of Xist, which lies over the promoter, was amplified using primers described (McDonald et al., 1998). Briefly, two rounds of PCR were performed with the primers Pr2 and Pr3. Individual strands of DNA were subcloned and sequenced by an automated sequencer. Parental-origin was determined by A/C polymorphism (B6/CAST) at nucleotide 1923 in the Xist promoter region (GenBank MMU50909).

Xist RNA FISH

FISH analysis was performed as previously described (Plath et al., 2003), with modifications. Blastocysts were treated with acid tyrode solution and then spun onto glass coverslips with a Cytospin II (Shandon, Pittsburgh, PA). Coverslips were then placed on ice and incubated with CSK (100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 10 mM PIPES, pH 6.8) for 30 s, CSK plus 0.5% Triton X-100 for 30 s and a second wash in CKS for 30 s. The coverslips were then fixed in 4% PFA/1× PBS for 10 min at room temperature and stored at 4°C in 70% ethanol. The FISH probe was in vitro transcribed Xist exon 6 RNA. Four different primer sets were used to amplify the region and introduce T3 polymerase sites into each PCR fragment. Ex6-1a, 5’GGATCTTATACGATCTCAGTTGCGGCTCTGGTTTCTG3’; Ex6-1b, 5’AGCGCGCATATATACCCCTACTAAGGAGCGGTCTGGTTTTCTCTTCTGCTA3; Ex6-2a, 5’GGATCTTATACGATCTCAGTTGCGGCTCTGGTTTCTG3’; Ex6-2b, 5’AGCGCGCATATATACCCCTACTAAGGAGCGGTCTGGTTTTCTCTTCTGCTA3.

The probe was then purified by passage through a Quick Spin Column (Roche Molecular Biochemicals, Indianapolis, IN). Labeled probe (1/10 final product) was precipitated with 65% ethanol, washed in 70% ethanol and 100% ethanol, and then dried.

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with the fixed embryos were dehydrated through a series of 80%, 90%, and 100% ethanol washes. After drying coverslips on a 42°C block, probe was added and incubated overnight at 37°C. All washes the following day were done at 39°C for 5 min. Coverslips were washed with 2× SSC/50% formamide two times and in wash buffer II (0.5 M NaCl, 10 mM Tris–HCl, pH 7.5, 0.1% Tween-20) three times. Slides were then incubated with 25 μg/ml of RNase A in wash buffer II for 1 h at 37°C, then washed twice more in wash buffer II and two times in 2× SSC/50% formamide. Three washes in 2× SSC, two washes in 1× SSC and three in 4× SSC were performed. Slides were mounted with Vectashield (Vector Burlingame, CA) and viewed by confocal microscopy. Only nuclei that could be clearly delineated were scored.

DNA FISH

DXWas70 was used to identify the number of X chromosomes present in the cells of cloned embryos (Disteche et al., 1985). The DXWas70 DNA fragment was isolated and biotin-16-dUTP (Roche Molecular Biochemicals, Indianapolis, IN) was introduced by nick translation. Blastocysts were fixed after swelling and dissociation in 3:1 methanol:acetic acid and FISH was performed as described (Hodges et al., 2001). Slides were washed in 2× SSC and then denatured with the probe for 8 min at 85°C. The slides were hybridized overnight at 37°C. A 5-min wash in 50% formamide, 2× SSC followed by a wash in 2× SSC were performed at 40°C. Slides were washed in PN buffer (0.1 M NaH2PO4·H2O, 0.1 M Na2HPO4, 0.05% Nonidet P-40) for 2 min at room temperature. Hybridization with avidin-fluorescein (Roche Molecular Biochemicals, Indianapolis, IN) diluted 1:100 in PN buffer was performed at 37°C for 1 h. After a 10-min wash in PN, slides were mounted in Vectashield and viewed by epifluorescence.

For chromosome counts, SCNT and control blastocysts were cultured in 20 μM colcemid in MEM medium for 5 to 8 h. Individual blastocysts were then fixed and stained as described (Magnuson and Epstein, 1984) and viewed by light microscopy.

Results

The rate of production of various stage cloned constructs for this study is summarized in Table 1. SCNT embryos’ survival rates (~50% to blastocyst stage, 2% to term) were comparable to previous studies (Amano et al., 2001; Gao et al., 2004b; Inoue et al., 2002; Wakayama et al., 1999).

Nuclei from (B6XCAST)F1 donor cells were used in these experiments so that allelic expression patterns could be followed during the cloning procedure. In the mouse, random XCI can be biased to favor the inactivation of a given X chromosome by a genetically defined element, Xce. The donor nuclei used in the SCNT procedure were heterozygous at Xce (Xce\(^{bc}\)), with the B6 X chromosome (Xce\(^{B}\)) remaining active in 30% of cells and the CAST X chromosome (Xce\(^{C}\)) active in the remaining 70% (Cattanach and Rasberry, 1994). Individual nuclear donors could not be assessed for the identity of the inactive X prior to SCNT. However, as Xce does not play a role in XCI in preimplantation embryos, this skewed somatic pattern was used to determine whether cloned embryos could reactivate, count and inactivate a somatic cell-derived, inactive X chromosome.

**Pctk1 gene expression**

While female somatic donor cells have inactivated one, entire X chromosome, normal preimplantation female embryos exhibit a gradient of inactivation along the paternal X chromosome; genes near Xist are fully inactivated while more distal genes remain active (Huynh and Lee, 2003; Latham and Ramhatal, 1995). Consistent with these previous observations, in vivo- and in vitro-derived, control blastocysts revealed a 50% B6:50% CAST expression pattern of Pctk1, a distal gene on the X chromosome (Fig. 1A, B1). To determine if SCNT reactivated silenced genes, allelic expression of Pctk1 was assessed. If the inactive X chromosome was not reactivated by SCNT, then we would expect 30% of the embryos to exhibit expression of Pctk1 exclusively from the B6 allele and the remaining 70% to exhibit expression of Pctk1 solely from the CAST allele, due to Xce heterozygosity of the donor cells (Fig. 1A, Cu). Contrary to this prediction, all cloned embryos expressed Pctk1 biallelically (Fig. 1A; clone 9 is discussed later), suggesting that the somatic cell-derived, inactive X chromosome was reactivated during cloned embryo development.

**Xist promoter region methylation**

The possibility existed that the reactivation process may only occur for distally located X-linked genes and not extend into the region closest to X-inactivation center (Xic).

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### Table 1

<table>
<thead>
<tr>
<th>Cumulus donor</th>
<th>No. of oocytes activated</th>
<th>No. of 2-cell embryos (%)</th>
<th>No. of blastocysts developed (%)</th>
<th>No. of embryos transferred</th>
<th>No. of recipient mothers</th>
<th>No. of day 10.5 fetuses (%)</th>
<th>No. of day 19.5 fetuses (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6XCAST(^a)</td>
<td>372</td>
<td>347 (93.3)</td>
<td>185 (49.7)</td>
<td>100</td>
<td>8</td>
<td>N/A</td>
<td>2 (2.0)</td>
</tr>
<tr>
<td>B6XCAST</td>
<td>269</td>
<td>245 (91.1)</td>
<td>131 (48.7)</td>
<td>131</td>
<td>9</td>
<td>6 (4.6)</td>
<td>N/A</td>
</tr>
</tbody>
</table>

\(^a\) (B6 × CAST) F1 nucleus, N/A indicates sample was not collected for analysis.
Thus, reactivation of the somatic X chromosome in SCNT embryos was also evaluated through methylation analysis of the Xist gene. In somatic cells, the Xist promoter is hypermethylated on the active X and hypomethylated on the inactive X chromosome (Norris et al., 1994). Both Xist alleles are hypomethylated in preimplantation embryos (McDonald et al., 1998). Bisulfite methylation analysis of the Xist promoter region was performed on cumulus cells, control blastocysts, and SCNT blastocysts (Fig. 2). As expected, 50% of the strands were methylated in the somatic donor cell nuclei, consistent with the presence of one active and one inactive X chromosome per cell. Control blastocysts displayed Xist promoter hypomethylation with few CpGs methylated. Cloned blastocysts also displayed Xist promoter hypomethylation, but showed slightly higher levels of methylation than in vivo-derived blastocysts, suggesting that reversal of somatic methylation occurred.

Xist expression

Because SCNT embryos appeared to reactivate the somatic cell-derived inactive X chromosome, we next determined whether these embryos could undergo an embryonic pattern of XCI. Here, it was of interest to determine whether residual epigenetic modifications on the X chromosome, either from the original paternal X or from the inactive X in donor cumulus cells, would be recognized and result in inactivation of a single X chromosome. Alternatively, XCI may be random or may fail altogether. The noncoding Xist RNA, a key regulator in XCI, exhibits a

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Fig. 1. Allelic expression of X-linked genes in cloned blastocysts. Real-time allele-specific RT-PCR was performed on single cloned blastocysts. Allelic expression is shown for Pctk1 (A), Xist (B), Pgk1 (C). Percent expression is presented as the level of the specified allele relative to the expression from both alleles. Cu represents expression in pooled cumulus cells. For controls, analysis of gene expression was performed in 5 blastocysts flushed from the oviducts of naturally mated females and in 8 blastocysts cultured in KSOM medium from the 2-cell stage. All results were identical for control blastocysts and are summarized in sample Bl. Samples 1–43 represent cloned blastocysts. Pgk1 was not assayed in samples 37–43. Pctk1 was not assayed in sample 36.

Fig. 2. Xist promoter methylation in blastocysts. Pools of cumulus cells, in vivo-derived blastocysts, and cloned blastocysts were analyzed by bisulfite mutagenesis. The fraction of strands that were methylated at each of the 19 CpGs in the Xist promoter is indicated. Methylated cytosines were sporadically distributed among individual strands.
unique developmental expression pattern that can be used to assess XCI in SCNT embryos. In normal blastocysts, imprinted XCI is evident in the trophectoderm (Takagi and Sasaki, 1975; West et al., 1977), while the inner cell mass (ICM) has not yet undergone random XCI (Rastan, 1982; Takagi et al., 1982). We observed that, due to imprinted XCI, the paternal \textit{Xist} allele was monoallelically expressed in in vivo- and in vitro-derived control blastocysts (Fig. 1B, B1). In contrast, 91\% (39/43) of cloned blastocysts expressed both \textit{Xist} alleles (Fig. 1B), indicative of a lack of imprinted XCI. A number of possibilities could explain this biallelic expression pattern. First, somatic epigenetic modifications may not be recognized after nuclear transfer, resulting in random XCI in the blastocyst. Second, the ICM may prematurely initiate random XCI resulting in biallelic \textit{Xist} expression. Finally, XCI may be deregulated in cloned embryos, with some cells failing to undergo XCI or inactivating both X chromosomes, resulting in abnormal patterns of \textit{Xist} expression. Additional experiments were performed to discriminate among these possibilities.

**Embryonic lineage markers**

The biallelic \textit{Xist} expression detected in the SCNT embryos could result from premature ICM differentiation. To determine the cell lineages present in the SCNT embryos, we used molecular markers to assay ICM (Oct4) and trophectoderm (Troma-1) (Brulet and Jacob, 1982; Chisholm and Houliston, 1987; Johnson et al., 1986). In control blastocysts, Oct4 was expressed exclusively in the ICM as expected (data not shown) while Troma-1 protein was not present until the late morula stage (Fig. 3A), when it appeared in a simple ring around the edge of each cell. By the late blastocyst stage (Fig. 3B), cells along the outside of the embryo (trophectoderm) and lining the blastocoel cavity (primitive endoderm) developed a more complex mesh-like pattern while cells within the ICM lost their Troma-1 signal. In agreement with previous results (Boiani et al., 2002), cloned embryos expressed Oct4 in cells both within and outside the ICM (data not shown). The majority of cells in all clones displayed a ring pattern of Troma-1 (Fig. 3C), similar to the pattern observed in late morula/early blastocysts. Therefore, while cloned blastocysts were morphologically normal (Fig. 3D), their Troma-1 localization pattern indicated that biallelic \textit{Xist} expression was not due to premature differentiation of the ICM.

**\textit{Xist} RNA localization**

Another possible explanation for biallelic \textit{Xist} expression in cloned blastocysts is the inactivation of two X chromosomes in individual cells. To analyze XCI on a cell-by-cell basis, \textit{Xist} RNA FISH was performed. Normally, \textit{Xist} RNA coats the inactive X chromosome generating one distinct focus of localization in cells that have silenced a single X chromosome (Panning et al., 1997; Sheardown et al., 1997). To assess the number of \textit{Xist} signals within each blastomere nucleus, sequential confocal images were acquired for embryos that were subjected to \textit{Xist} RNA FISH. Analysis of in vivo-derived blastocysts consistently revealed a single \textit{Xist} RNA signal in the majority of cells of half of the embryos (presumably female) (Fig. 4A). In contrast, cloned blastocysts had variable \textit{Xist} RNA signals (Figs. 4B–D). Some blastocysts appeared to have many cells with normal signals, while others had a significant fraction of cells that were abnormal (~37\%, Table 2); cells without any signals or cells with two large signals. Additionally, a number of cells within cloned blastocysts showed multiple small foci of \textit{Xist} staining throughout the nucleus, consistent with mislocalization or overexpression of \textit{Xist} RNA (H. Cohen, B. Panning, personal communication). The multiple small and large \textit{Xist} signals likely account for the biallelic \textit{Xist} expression we observed in individual cloned blastocysts by
RT-PCR. As Xist localization was highly variable among SCNT blastocysts and among cells of any given cloned blastocyst, it indicates that XCI was disrupted.

Polycomb group protein expression and localization

The Polycomb group genes *Eed* (Wang et al., 2002) and *Ezh2* (Erhardt et al., 2003) are important for silencing the inactive X chromosome in both ICM and trophectoderm (Plath et al., 2003; Silva et al., 2003). We examined whether the proteins necessary for silencing the X chromosome were correctly expressed in SCNT embryos. RT-PCR was performed on single cloned and control blastocysts to determine the expression of *Eed* and *Ezh2* as well as another Polycomb family member, *Ezh1* (Laible et al., 1997). All three genes were expressed in normal blastocysts and cumulus cells (data not shown). All SCNT blastocysts expressed *Eed*. Only one clone expressed *Ezh1* at a detectable level, while 12 out of 18 blastocysts expressed *Ezh2* (data not shown).
To determine if improper Eed localization could contribute to abnormal XCI in cloned embryos, we examined Eed protein distribution. During the initial stages of XCI, Eed protein localizes to the inactive X chromosome (Mak et al., 2002, 2004; Silva et al., 2003). Staining revealed a single point of localization in each cell in half (presumably female) of the control blastocysts (Fig. 5A). Similar to the pattern observed for Xist RNA-FISH, one Eed focus was apparent in many cells of SCNT embryos (Figs. 5B and C); however, a number of cells had abnormal signals. These cells exhibited either zero or two foci of Eed localization. These abnormal signals were observed at a greater frequency in cloned embryos than in control blastocysts (~45%, Table 3). Thus, while the presence of discrete foci of Eed staining indicated that Xist was able to recruit Eed, and that this component of XCI was occurring in many cells in the clones, abnormal Eed and Xist signals argue that XCI was aberrant in many cells.

**X chromosome number**

The greater than expected number of Eed and Xist foci observed in SCNT embryos could be due to abnormal X chromosome number. To investigate this possibility, X chromosome DNA FISH was performed. A probe that detects repetitive DNA adjacent to the centromere on the X chromosome was used on dissociated cells from individual in vivo-derived or SCNT blastocysts. The majority of cells in both types of embryos had two X chromosomes (81.2% SCNT, 88.5% controls, Fig. 6). For control blastocysts, this correlated with the percent of cells with one Xist (91.4%) and one Eed (91.4%) localization signal. In contrast, only 62.9% and 58.6% of SCNT cells had one Xist and one Eed signal, respectively. Furthermore, the percent of SCNT cells with only one X chromosome (9.8%) was significantly less than the percent of cells that lacked an Xist or Eed signal.

![Fig. 5. Eed localization in blastocysts. Two representative image layers of confocal stack from immunohistochemistry staining of Eed in (A) in vivo-derived blastocysts and (B and C) cloned blastocysts. Examples of abnormal cells with two foci of Eed accumulation are indicated by arrows.](image)

*Fig. 6. X chromosome number in cells of blastocysts. Percent of cells with 1–4 X chromosomes as determined by DNA FISH. Only female in vivo-derived embryos were included in the control sample. Significant t-test results (two-tailed) were $P = 0.005$ for cells with 3 signals (*), and $P = 0.004$ for cells with greater than 2 signals.*

<table>
<thead>
<tr>
<th>Eed foci</th>
<th>In vitro</th>
<th>In vitro</th>
<th>In vitro</th>
<th>In vitro</th>
<th>Clone 1</th>
<th>Clone 2</th>
<th>Clone 3</th>
<th>Clone 4</th>
<th>Clone 5</th>
<th>Clone 6</th>
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<td>1 Large*</td>
<td>113</td>
<td>130</td>
<td>111</td>
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<td>22</td>
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<td>23</td>
<td>15</td>
<td>56</td>
</tr>
<tr>
<td>2 Large</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
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<td>1</td>
<td>0</td>
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<td>3</td>
<td>0</td>
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<tr>
<td>No localized signal</td>
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<td>7</td>
<td>5</td>
<td>5</td>
<td>6</td>
<td>13</td>
<td>15</td>
<td>15</td>
<td>11</td>
<td>20</td>
<td>6</td>
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<td>5.8</td>
<td>5.9</td>
<td>5.9</td>
<td>10.6</td>
<td>27.0</td>
<td>71.4</td>
<td>51.1</td>
<td>55.0</td>
<td>55.8</td>
<td>40.0</td>
<td>34.9</td>
</tr>
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*Includes all cells considered normal; 1 large focus or 1 pinpoint.
(17.3% and 30.4%), indicating that many of the cells in the clones had two X chromosomes but did not undergo proper XCI. Control blastocysts displayed similar numbers of cells with one X chromosome (8.8%), and no Xist (7.7%) or no Eed (7.5%) foci. Additionally, significantly more ($P = 0.004$) cells in cloned blastocysts had 3 or 4 X chromosomes. The percent of cells with excess X chromosomes (9.0%) was similar to the percent of cells with two Xist or Eed foci (11.0% and 8.5%, respectively), suggesting that the additional Eed and Xist signals seen in cells of the cloned embryos were a result of supernumerary X chromosomes. Control blastocysts possessed few cells with extra X chromosomes (2.8% 3–4 Xs, 0.9% two Xist foci, 0.2% two Eed foci). Preliminary karyotype analysis demonstrated that a significant number of cells in SCNT blastocysts were tetraploid (5%), which correlated with the number of cells having two Xist and two Eed signals (data not shown).

**Pgk1 and MeCP2 expression in blastocysts**

Because XCI in the early embryo exhibits a gradient of inactivation with genes located close to the Xic more effectively silenced (Huynh and Lee, 2003; Latham, 1996; Latham and Rambhatla, 1995; Williams et al., 2002), it was necessary to examine the expression of additional X-linked genes to assess re-inactivation in the cloned embryo. *Pgk1* (3 cM from Xist) was expressed from the maternal allele in the control embryos, in agreement with previous data (Fig. 1C, B1) (Huynh and Lee, 2003; Latham and Rambhatla, 1995). Only 16% of the SCNT embryos exhibited biallelic

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![Fig. 7](image_url) Analysis of cloned fetuses at 19.5 days of gestation. (A) Day 19.5 wild type fetus and placenta. (B) Day 19.5 SCNT fetus and placenta that was dissected from the uterus and survived for 2 h before death. Real-time RT-PCR amplification results for two X-linked genes in the fetuses (C) and placenta (D) recovered at day 19.5 of pregnancy. All results for in vivo-derived animals at 17.5 days of pregnancy are summarized in sample WT. All wild type placentae showed a variable amount of maternal cell contamination. Samples 1 and 2 are cloned fetuses. Fetus 1 is shown in (B).
expression that correlated with biallelic $Xist$ expression (for example, Clone 15 has 83% CAST $Xist$ and 85% B6 $Pgk1$ expression, Fig. 1C), suggesting that these embryos were undergoing random XCI (i.e., did not recognize any imprinting mark). A large proportion of the clones (72%) had monoallelic (less than 10% expression from other allele) $Pgk1$ expression. The vast majority of these SCNT embryos expressed $Xist$ biallelically, indicating faulty reactivation of the $Pgk1$ gene. In the remaining SCNT embryos, the levels of $Xist$ and $Pgk1$ expression did not correlate, suggesting that a problem occurred with reactivation and/or inactivation. While $Mecp2$ (12.4 cM from $Xist$) imprinting was variable in control embryos as previously observed (Huynh and Lee, 2003), a number of the clones exhibited monoallelic $Mecp2$ expression (data not shown, monoallelic expression in clones numbered 2, 4, 16, 24, 29 in Fig. 1). Significantly, embryos that exhibited monoallelic $Mecp2$ expression also expressed $Pgk1$ monoallelically, with the expression of both genes originating from the same chromosome, suggesting that in these embryos the somatic cell-derived inactive X chromosome failed to reactivate.

One cloned embryo (Fig. 1, clone 24) exhibited the developmentally appropriate XCI pattern, with 100% CAST $Xist$ expression, 100% B6 $Pgk1$ and $Mecp2$ expression and biallelic $Pctk1$ expression. Clone 9 appeared to have lost one X chromosome during the SCNT procedure and subsequent development, with expression of all genes emanating from the B6 X chromosome.

**X-linked gene expression in postimplantation clones**

To determine the status of XCI in postimplantation cloned fetuses, reconstructed blastocysts were transferred to foster mothers (Fig. 7). Two clones were recovered at 19.5 days of pregnancy (Fig. 7B) and allelic expression patterns for $Xist$ and $Pctk1$ were analyzed in each fetus and placenta. The SCNT fetuses displayed XCI appropriate for their $Xce$ genotype (30% of cells inactivated the CAST X chromosome), as indicated by both $Xist$ and $Pctk1$ expression (Fig. 7C). That is, approximately 30% of $Xist$ expression was from the CAST allele and 30% of the $Pctk1$ expression was from the B6 allele in both SCNT fetuses. Thus, the $Xce$ effect was appropriately established by day 19.5 in SCNT clones.

We also examined XCI in placental tissues. One day 19.5 placenta exhibited a pattern consistent with continued silence of the somatic cell-derived inactive X chromosome, with 100% expression of $Xist$ from the B6 X chromosome and >80% of $Pctk1$ expression from the CAST allele (Fig. 7D). The other day 19.5 placenta expressed the X-linked genes biallelically. Because maternal contamination of day 19.5 placentae was a potential concern, we extended our analysis to include day 10.5 conceptuses, the placentae of which could be isolated without maternal contamination. As seen in day 19.5 fetuses, the day 10.5 fetuses exhibited the predicted $Xce$ pattern of X inactivation (Fig. 8A). A different pattern of allelic expression was observed for

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**Fig. 8.** Analysis of cloned fetuses recovered at day 10.5 of pregnancy. (A) Percent CAST $Xist$ gene expression and B6 $Pctk1$ gene expression in day 10.5 cloned fetuses. (B) Percent CAST gene expression of $Xist$ and B6 gene expression of $Pgk1$, $Mecp2$, and $Pctk1$ in day 10.5 placentae. WT is representative of five 10.5 day in vivo-derived fetuses that exhibited identical expression patterns. Samples 1–6 are independent SCNT fetuses. Clone 5 had no detectable $Pctk1$ expression in the placenta.
almost every day. 10.5 SCNT placentas (Fig. 8B). One placenta had a gene expression pattern characteristic of a single X chromosome undergoing inactivation (Fig. 8B, clone 2); Xist expression originated from the B6 chromosome, and Pgk1, MeCP2, and Pck1 were expressed from the CAST chromosome. Therefore, this clone was undergoing 100% skewed X inactivation in its extraembryonic tissues, possibly indicating that epigenetic modifications carried by somatic cell-derived inactive X chromosome were retained and recognized in the trophodermect of this clone. The placentae from three clones expressed only the CAST Xist allele (Fig. 8B, clones 4–6); however, the other X-linked genes were not monoallelically expressed. One placenta (clone 4) displayed monoallelic expression for all genes except Pck1, reminiscent of the gradient of spreading of XCI seen in very early embryos (Huynh and Lee, 2003; Latham and Rambhatla, 1995). The other two placentae with monoallelic Xist expression did not exhibit such a gradient. Finally, the last two SCNT embryos had extraembryonic tissues that showed biallelic expression of all genes analyzed (Fig. 8B, clones 1 and 3).

Discussion

SCNT embryos reactivate an inactivated, somatic X chromosome in a gradient fashion

Epigenetic changes are stable during development. Random XCI occurs in the cells of the peri-implantation embryo and is stably propagated for the remainder of the female mammal’s life (McMahon et al., 1983). SCNT embryos represent an unusual situation where an inactive X chromosome is reactivated, a process usually restricted to gametogenesis (Monk and McLaren, 1981; Tada et al., 1997, 1998; Tam et al., 1994). By examining an endogenous X-linked gene that is subjected to XCI in somatic cells but is expressed from both X chromosomes in preimplantation embryos (Pck1), it was possible to determine if the somatic X chromosome was reactivated. In addition, reactivation could be assessed by analysis of the Xist promoter, which is methylated on one X chromosome in the somatic cell but not in the preimplantation embryo (McDonald et al., 1998). We observed biallelic Pck1 expression and hypomethylation of the Xist promoter in SCNT blastocysts, indicating that reactivation occurred and that factors within the oocyte cytoplasm have the ability to reverse epigenetic changes that silence the somatic X chromosome. These results agree with a previous study that demonstrated reactivation of an X-linked transgene (Eggen et al., 2000).

Interestingly, we observed a large proportion of SCNT blastocysts (72%) with monoallelic Pgk1 expression but biallelic Xist expression. The most parsimonious explanation for this expression pattern is that reactivation only occurs for part of the X chromosome. Similar to XCI in the preimplantation embryo, a gradient of reactivation may form along the X chromosome, with genes farther from the Xic reactivated (Pck1, X-linked transgene), and those closer to the Xic remaining silent (Pgk1, MeCP2). Demethylation at the Xist promoter may reflect aberrant general demethylation in cloned embryos rather than a specific action to reactivate the somatic inactive X chromosome. Generalized demethylation has been observed at both single copy genes and on a genome-wide level (Bourc’his et al., 2001; Dean et al., 2001; Kang et al., 2001b; Mann et al., 2003; Young et al., 2003). In support of the gradient reactivation hypothesis is the response of cloned blastocysts to the somatic Xce effect. The donor cell population is mixed, with 30% of cells possessing an active B6 chromosome and 70% with an active X CAST chromosome. This ratio is preserved in SCNT blastocysts; of the cloned embryos that had monoallelic Pgk1 expression, 37% expressed the B6 allele while 63% expressed the CAST allele. As Xce is not recognized in normal preimplantation embryos, this suggests that the somatic cell-derived inactive X chromosome has not been completely reactivated. Biallelic Xist expression argues against preferential inactivation of the previously silenced somatic cell-derived X chromosome. Little is known about X chromosome reactivation, including whether it is an active or passive process, or whether molecules involved in remodeling the paternal pronucleus can actively remodel the somatic nucleus following SCNT. As XCI is more stable in somatic cells compared to preimplantation embryos, this stability may be retained in the SCNT embryo, where it impedes X chromosome reactivation.

Cloned embryos exhibit characteristics of an inactive X chromosome

Erasure of somatic epigenetic marks is only one hurdle that SCNT embryos must overcome. Cloned embryos must also recapitulate epigenetic changes that occur during normal embryogenesis. Ordinarily, DNA methylation and XCI patterns are altered during preimplantation development (Monk and Harper, 1979; Monk et al., 1987; Santos et al., 2003). In order for a clone to develop properly, the somatic nucleus must be remodeled following SCNT.

We observed that cloned blastocysts displayed characteristics of an inactive X chromosome. However, a heterogeneous pattern of Xist localization was exhibited in all cloned embryos. Early mislocalization of factors involved in establishment and maintenance of epigenetic marks, including Dnmt1 (Chung et al., 2003), could generate an embryo that is mosaic, with each cell carrying different epigenetic modifications, similar to what we observed. We conclude that SCNT embryos are unable to regulate XCI consistently, and thus contain a mixture of cells with varying degrees of XCI. Viable clones may be those that most closely approximate a normal XCI pattern. Furthermore, during early development, an incomplete form of imprinted XCI occurs in all cells (Huynh and Lee, 2003). Xist, located in...
the Xic, has completely imprinted expression in normal blastocysts. In comparison, 90% of cloned embryos show biallelic expression of Xist RNA, indicating that imprinted XCI does not occur in SCNT blastocysts.

**Genetic factors controlling XCI**

Two factors important for XCI are Ezh2 and Eed (Erhardt et al., 2003; Wang et al., 2002). Eed localization is markedly different in somatic nuclei and preimplantation embryos. While not associated with the inactive X chromosome in somatic cells, Eed coats the X chromosome during the initial stages of XCI in the developing embryo (Mak et al., 2002, 2004; Silva et al., 2003). In order to recapitulate normal development, Eed must be re-directed to the inactivating X chromosome. Our immunohistochemical results show that Eed accumulates in discrete foci in many cells of cloned blastocysts. The presence of single foci of Xist or Eed localization suggests that XCI is successful in many cells in the SCNT embryo. However, there are still a large number of cells that mislocalize or have no Eed localization (~40%), suggesting that the epigenetic machinery responsible for XCI is aberrant in these cells. Additionally, in some cells, two Xist or two Eed foci were observed, suggesting that inactivation of two X chromosomes occurred. DNA FISH data suggest that approximately 10% of cells in the cloned embryos have supernumerary X chromosomes, a number that is similar to the percent of cells with two Xist or two Eed signals. The presence of supernumerary X chromosomes in cloned embryos has not been reported in mice. However, previous studies in bovine, rabbit, and primates have identified a high degree of aneuploidy and tetraploidy in cloned animals (Booth et al., 2003; Shi et al., 2004; Simerly and Navara, 2003).

We performed a preliminary study of murine SCNT blastocysts to determine ploidy, as the presence of blastomeres with two inactive Xs (but not three) and cells with four X chromosomes suggested that there were tetraploid cells. We observed that cloned blastocysts contained a significant number (~5%) of tetraploid cells, a frequency that is comparable to the proportion of cells with two inactive X chromosomes (11.0% two Xist and 8.5% two Eed signals) (data not shown). There were no tetraploid cells in control blastocysts (0% tetraploidy, 0.9% two Xist, and 0.2% two Eed signals). No haploid cells were observed in either control or SCNT blastocysts. Thus, the presence of tetraploid cells and two inactivated X chromosomes indicates normal XCI, while the large number of SCNT blastocysts with no Xist signal in the absence of haploid further points to the inability of SCNT blastocysts to regulate XCI correctly. Many groups have reported significant developmental delays and reduced cell numbers in cloned animals (Boiani et al., 2002, 2003; Bortvin et al., 2003; De Sousa et al., 2002). Our data indicate that this may be due in part to aneuploidy/tetraploidy and should be investigated further.

**XCI in mid- and late-gestation embryos**

In mid- and late-stage mouse embryos, there are two patterns of XCI. The placenta, which is derived from trophectodermal cells, exhibits imprinted XCI, while the embryo, originating from the ICM, exhibits random XCI. Analysis of the late-stage SCNT fetuses revealed that the Xce effect is preserved in the embryo proper. This indicates that the cells comprising the embryo reactivate the somatic X chromosome as development progresses and successfully undergo random XCI in a manner dictated by the Xce genotype. Although it appears that SCNT embryos exhibit developmentally appropriate random XCI, it is also possible that SCNT fetuses with erroneous XCI are eliminated leaving a population of late-stage embryos that exhibit successful XCI. Alternatively, cells within the developing embryo with incorrect XCI may be eliminated from the fetus as development progresses. In either case, only fetuses with correct XCI would be observed at later stages of development.

In contrast to the SCNT fetuses that have apparently normal XCI, the corresponding placentae from these clones display a variable pattern of XCI. We observed that placentae derived from mid- and late-gestation cloned concepti displayed the same variable XCI patterns as were present in SCNT blastocysts, possibly indicating that there is little change in these early patterns. One day 19.5 fetus had a placenta with biallelic X-linked gene expression, suggesting that imprinted XCI is not necessary for a functional placenta. However, the placenta of this animal was extremely large (0.41 g versus 0.12 g in wild type), a common finding in cloned animals (Chavatte-Palmer et al., 2002; Heyman et al., 2002a,b; Renard et al., 2002; Wakayama et al., 1998). The inability to inactivate an X chromosome may contribute to this phenotype, or may be simply one of a range of epigenetic abnormalities in SCNT placentae.

While abnormal expression of X-linked genes was observed in all stages of clone development, it is clear that normal XCI is occurring to some degree in cloned embryos. In fact, at each stage of development, we observed at least one embryo that appeared to have an XCI pattern indistinguishable from that seen in vivo-derived embryos, suggesting that XCI can occur in a SCNT embryo. Overall, however, our data indicate that SCNT embryos are unable to regulate XCI consistently, resulting in embryos that contain a mixture of cells with varying degrees of XCI. Viable clones may be those that most closely approximate a normal XCI pattern, whether through an accidental stochastic event or bona fide reprogramming.

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