

S-adenosylhomocysteine treatment of adult female fibroblasts alters X-chromosome inactivation and improves *in vitro* embryo development after somatic cell nuclear transfer

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Abstract

The poor outcome of somatic cell nuclear transfer (SCNT) is thought to be a consequence of incomplete reprogramming of the donor cell. The objective of this study was to investigate the effects of treatment with S-adenosylhomocysteine (SAH) a DNA demethylation agent, on DNA methylation levels and X-chromosome inactivation status of bovine female fibroblast donor cells and the subsequent impact on developmental potential after SCNT. Compared with non-treated controls, the cells treated with SAH revealed (i) significantly ($P < 0.05$) reduced global DNA methylation, (ii) significantly (~1.5-fold) increased telomerase activity, (iii) diminished distribution signals of methylated histones H3-3mK9 and H3-3mK27 on the presumptive inactive X-chromosome (Xi), (iv) alteration in the replication pattern of the Xi, and (v) elevation of transcript levels for X-chromosome linked genes, *ANT3*, *MECP2*, *XIAP*, *XIST*, and *HPRT*. SCNT embryos produced with SAH-treated donor cells compared with those derived from untreated donor cells revealed (i) similar cleavage frequencies, (ii) significant elevation in the frequencies of development of cleaved embryos to hatched blastocyst stage, and (iii) 1.5-fold increase in telomerase activity. We concluded that SAH induces global DNA demethylation that partially reactivates the Xi, and that a hypomethylated genome may facilitate the nuclear reprogramming process.

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Introduction

In mammalian cells, ~1–4% of cytosine nucleotides are modified by the covalent addition of a methyl group to carbon-5 of the cytosine pyrimidine ring, primarily at CpG dinucleotides. This results in the conversion to 5-methylcytosine and methylation of up to 70% of the CpG dinucleotides in the genome. These methyl groups covalently bind to the major groove of DNA and effectively inhibit transcription. Gene expression regulation by DNA methylation is proposed as the key mechanism initiating essential events in mammalian embryogenesis ranging from the establishment of imprinted signals in gametes to the unfolding of the developmental pattern in the embryo. Examples of such processes include the genome-wide global reprogramming after fertilization, the repression of transcription of genes clustered on an individual chromosome as is the case with imprinted genes or X-chromosome inactivation (XCI) and up- and down-regulation of individual genes, in a timely and tissue-specific manner (Bird 2002).

A family of DNA methyltransferases (DNMTs), whose co-substrates are DNA and S-adenosylmethionine (SAM), an important methyl donor, generally catalyzes the addition of methyl groups in most transmethylation reactions, which after transfer of the methyl group is converted to S-adenosylhomocysteine (SAH). SAH, the metabolite of SAM, has much higher affinity for the methyltransferase active site than does its precursor; therefore most cellular methyltransferase is inhibited by the accumulation of intracellular and cellular SAH. Thus, the ratio of cellular SAM and SAH has been frequently used as an indicator of DNA methylation potential (Yi *et al.* 2000, Caudill *et al.* 2001, Castro *et al.* 2005).

The importance of DNA methylation-mediated developmental regulation is particularly evident after somatic cell nuclear transfer (SCNT), a process that relies on the ability of a mature enucleated oocyte to reprogram a differentiated somatic genome to enable the re-establishment of totipotency and the post-fertilization developmental program. While this technique has been successfully applied to a number of species (Vajta 2007), the success rate in terms of the

number of live clones per manipulation remains low (1–5%) along with a high incidence of perinatal mortality and unexplained congenital malformations (reviewed by Han *et al.* 2003).

One of the proposed causes for the inefficiencies in the SCNT process is the inability to completely ‘dedifferentiate’ and reprogram the differentiated somatic donor cell. Under normal reproductive circumstances, upon fertilization, the paternal genome from sperm is demethylated by an active mechanism prior to DNA synthesis. Concomitantly, the maternal genome of the oocyte is demethylated by a passive mechanism that depends on DNA replication and then, in the embryo, DNA is *de novo* re-methylated by the blastocyst stage (Morgan *et al.* 2005). As differentiation progresses, the cells tend to establish and maintain characteristic constituent protein profiles that result from specific patterns of gene expression acquired during development and regulated, in part, by high levels of DNA methylation and histone modifications that bind to chromatin (Bird 2002, Morgan *et al.* 2005). However, it has been reported that unlike the maternal genome in the fertilized oocyte, the differentiated SCNT donor cell undergoes a reduced or incomplete passive demethylation after injection into the oocytes with a subsequent *de novo* methylation event occurring at an earlier stage compared with non-SCNT embryos (Bourc’his *et al.* 2001, Kang *et al.* 2001). The abnormal reprogramming of DNA methylation in SCNT embryos could lead to the abnormal expression of genes and result in the failed or confused reactivation of the genes that are essential for proper embryonic development. Indeed, comparison of embryos and fetuses produced by SCNT with those produced by fertilization has revealed deviations in the developmental chronology of expression of telomerase (Betts *et al.* 2001), an enzyme necessary for reestablishing telomere length in somatic cells, and in the levels of a select panel of genes associated with growth, differentiation, and metabolism (Herath *et al.* 2006, Li *et al.* 2006), including several located on the X-chromosome (Wrenzycki *et al.* 2002).

Telomerase, an enzyme whose expression is for the most part undetectable in most differentiated somatic cells is also associated with DNA methylation (Gonzalo *et al.* 2006) and modification of chromatin structure by nucleosomal histones (Blasco 2003, Gonzalo *et al.* 2006). Telomerase up-regulation has been used as an index of transcriptional activity in partially hypomethylated cells (Renaud *et al.* 2007) and the reappearance of telomerase expression in cloned embryos (Betts *et al.* 2001) has been used as a marker of reprogramming efficiency. Among clones, the status of telomere reconstruction varies widely from complete rebuilding to significantly shorter or even longer lengths than age-matched controls (Lanza *et al.* 2000, Betts *et al.* 2001). These variations presumably reflect the ability of the recipient oocyte to effectively initiate reprogramming of telomerase expression.

Similarly, some SCNT embryos and offspring have been shown to exhibit aberrations in XCI (Xue *et al.* 2002, Nolen *et al.* 2005), a process required to balance the gene dosage differences resulting from the different number of X-chromosomes in females and males. During early embryogenesis, this process is induced by X-chromosome-inactive-specific transcript (*XIST*) RNA coating one of the two X-chromosomes in every cell of a female embryo (Brockdroff 2002, Heard 2004, Chang *et al.* 2006). Immediately after *XIST* RNA coating begins, the inactivated X-chromosome (Xi) undergoes various chromatin modifications such as loss of methylation of H3 lysine 4 (H3-K4), methylation of histone H3 lysine 9 (H3-K9), and methylation of H3 lysine 27 (H3-K27), and these changes lead to transcriptional silencing (Peters *et al.* 2002, Plath *et al.* 2003, Chadwick & Willard 2004) and late replication of one of the X-chromosomes (Keohane *et al.* 1999). However, histone modification such as H3-K27 methylation is not sufficient for silencing of the X-chromosome (Plath *et al.* 2003). Hence, the inactive state is synergistically maintained through other chromatin modifications such as hypoacetylation at histone H4, macroH2A recruitment, and DNA methylation (Csankovszki *et al.* 2001). Nonetheless, the functional links between methylated DNA and histones on the X-chromosome are extremely stable and are maintained throughout all subsequent cell divisions and life (Avner & Heard 2001). On the other hand, when CpG dinucleotides of the Xi are demethylated, the fate of H3-3mK9 and H3-3mK27 distribution, replication timing, and the levels of X-linked genes expression on the hypomethylated Xi chromosome remain unclear.

To further explore the mechanisms involved in dedifferentiation, we have used telomerase activity and X-inactivation as markers for reprogramming with the following objectives: (1) to investigate the effects of altered DNA methylation on telomerase activity; (2) to analyze the pattern of XCI by analyzing the distribution of inactivated X-chromosome-specific histones H3-3mK9 and H3-3mK27, the replication timing of the inactivated X-chromosome, and by measuring the transcript levels of various X-linked genes by real-time RT-PCR in hypomethylated female fibroblast cells treated with SAH; and (3) to determine the impact of SAH pretreatment of the SCNT donor cell on the *in vitro* developmental potential and telomerase activity of the resultant SCNT embryos.

Results

Global DNA methylation levels in SAH-treated cells

The amount of global DNA methylation was determined using relative fluorescence intensity (RFI) of 5-methylcytosine immunostaining emitted from the nuclei of control and SAH-treated cells. Fluorescence intensity of the non-treated control cells was considered as 100% for comparison with the treatment groups. The

nuclear fluorescence of SAH-treated cells was apparently reduced compared with control cells (Fig. 1A–F). RFI measurements demonstrated significantly ($P < 0.05$) reduced levels of methylated DNA at all concentrations tested with reduced RFI evident with increasing SAH concentrations (Fig. 1G). RFI values of cells treated with lower doses, 0.1 and 0.25 mM SAH (RFI=77.1 and 56.0% respectively), were significantly ($P < 0.05$) different from controls and each other, whereas higher doses, 0.5, 1.0, and 2.0 mM SAH (RFI=39.9, 38.9 and 39.5% respectively), were significantly ($P < 0.05$) different from controls and lower doses but not significantly ($P > 0.05$) different from each other (Fig. 1G).

Telomerase activity levels in SAH-treated cells

To analyze the impact of an altered DNA methylation state on gene expression levels, telomerase activity was assessed as an indicator of altered gene expression since previously it has been shown to be modulated by the

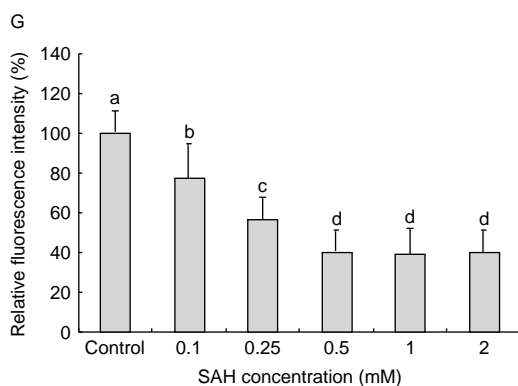
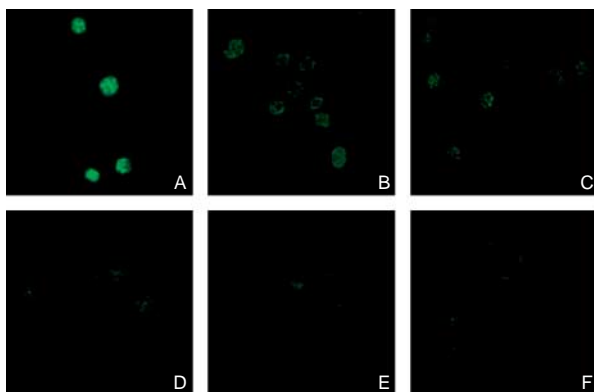


Figure 1 Immunofluorescence staining with antibody against 5-methylcytosine detected by FITC-conjugated secondary antibody (green) in the nuclei of cells treated with (A) control, (B) 0.1 mM, (C) 0.25 mM, (D) 0.5 mM, (E) 1.0 mM, (F) and 2.0 mM SAH. (G) Relative fluorescence intensity (RFI) of 5-methylcytosine immunostaining emitted from the nuclei of cells treated with each concentration of SAH. The RFI of the control cells was considered as 100% for comparison with other treatments. Bars with different letters are significantly ($P < 0.05$) different.

DNA methylation status of the catalytic subunit (telomerase reverse transcriptase, TERT) promoter (Guilleret & Benhattar 2003). The real-time quantitative telomeric repeat amplification protocol (RQ-TRAP) assay was applied to control and SAH-treated cells. Telomerase activity in the control cells was considered as 100% for comparison with other treatment groups. Relative telomerase activity (RTA) in 0.25, 0.5, 1.0, and 2.0 mM SAH-treated cells was significantly higher ($P < 0.05$) than those in control and 0.1 mM SAH groups (Fig. 2). Furthermore, RTA was significantly ($P < 0.05$) elevated with treatments of increasing SAH concentrations up to 1.0 mM, reaching levels over 150% that of controls. RTA in 2.0 mM SAH-treated cells was significantly higher ($P < 0.05$) than those in control and 0.1 mM SAH-treated cells; however, it was significantly lower ($P < 0.05$) than those in 0.5 and 1.0 mM SAH concentrations (Fig. 2).

Localization of histones H3-3mK9 and H3-3mK27 on the X-chromosomes

The localization of trimethylated histones H3 lysine 9 and 27 (H3-3mK9 and H3-3mK27) on the metaphase spreads have been used as markers of the inactivated X-chromosome (Chadwick & Willard 2004). Since treatment of fibroblasts with 1.0 mM SAH resulted in the lowest DNA methylation levels and the highest telomerase activity, only metaphase chromosomes from the control and 1.0 mM SAH-treated cells were immunostained with H3-3mK9 and H3-3mK27 antibodies and evaluated for the presence or absence of H3-3mK9 and H3-3mK27 staining on both X-chromosomes of each metaphase spread analyzed (Fig. 3). In all 80 metaphase spreads from control female cells, intense labeling of both H3-3mK9 and H3-3mK27 antibodies was detected on one X-chromosome (the presumptive inactive, Xi chromosome), but was not detected on the other (the presumptive active, Xa chromosome; Fig. 3B and C). However, in the metaphase spreads of cells treated with 1.0 mM SAH, the H3-3mK9

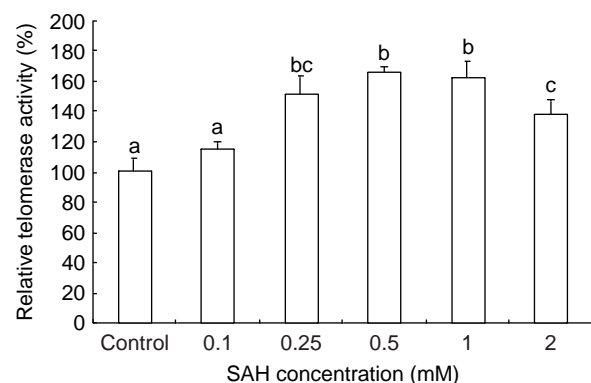


Figure 2 Relative telomerase activity (RTA) in the nuclei of cells treated with increasing concentration of SAH. RTA of the non-treated control cells was considered as 100% for comparison with SAH treatments. Bars with different letters are significantly ($P < 0.05$) different.

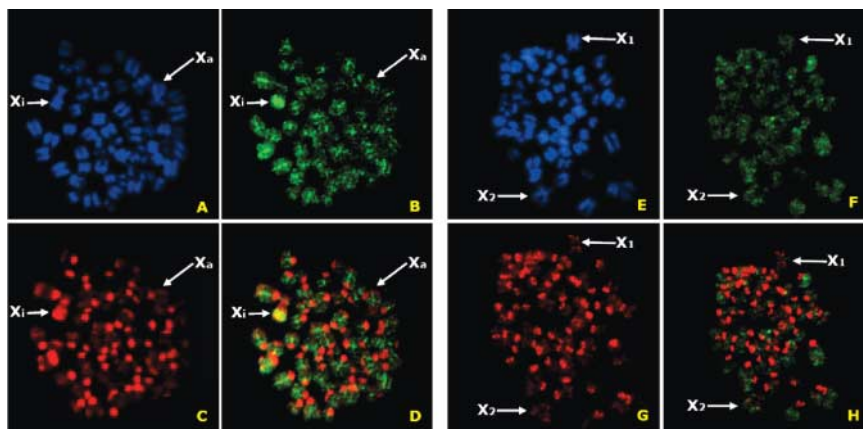


Figure 3 Distribution of H3-3mK9 and H3-3mK27 on the metaphase spread derived from (A–D) control and (E–H) 1.0 mM SAH-treated cells. Simultaneous double staining with antibodies against H3-3mK27 was detected by FITC-conjugated secondary antibody (B and F; green) and H3-3mK9 by Alexa 594-coupled secondary antibody (C and G; red). DNA was counterstained with DAPI (A and E) and the merged image (D and H) consists of H3-3mK27 (green) and H3-3mK9 (red). Distribution of both H3-3mK9 and H3-3mK27 were seen on the inactivated X-chromosome (Xi), and no distribution was detected on the activated X-chromosome (a) of control cell (B and C). But distribution of both H3-3mK9 and H3-3mK27 staining were not typically detected on both X-chromosomes (X1 and X2) of SAH-treated cells (F and G).

antibody labeling was detected on one X-chromosome in only 59.5% (52/74) of the metaphase spreads analyzed, while in 40.5% (22/74) of the cells no labeling of either X-chromosome was seen (Fig. 3G). Furthermore, the majority (82.4%; 61/74) of the cells showed no labeling with H3-3mK27 antibody, while only 17.6% (13/74) of the cells showed labeling over one X-chromosome (Fig. 3F). Combined, the pattern of X-chromosome labeling with antibodies on SAH-treated cells was significantly ($P < 0.05$) reduced compared with those of control cells with a frequency of detection of H3-3mK27 significantly ($P < 0.05$) lower than that for H3-3mK9 on SAH-treated cells (Table 1). However, for those treated cells showing incorporation of both antibodies, the pattern was similar to the untreated controls (Fig. 3B and C).

Replication timing of the X-chromosomes in SAH-treated cells

Acridine orange, a fluorescent dye, intercalates in the spirals of the DNA strand; however, bromodeoxyuridine

(BrdU), which is a structural antagonist of thymidine, interferes with the fluorescence emitted by the dye and indicates BrdU-rich (late replicating) regions of DNA. BrdU incorporation patterns on the X-chromosomes were classified as follows: zero (Z), without incorporation on either the short (p) or long (q) arm (Fig. 4A); low (L), one or few bands of BrdU incorporation on the q arm and none on the p arm; medium (M), several (> 2) reverse band acridine orange (RBA) bands on the q arm and few/none on the p arm; and high (H), numerous (5–10) bands on both the q and p arms (Fig. 4B). In the normal situation, a metaphase spread showing a pattern that was HZ was considered to indicate late replication of the inactivated or presumed inactivated X-chromosome. Reactivation of the inactivated X-chromosome would exhibit a pattern classified as ZZ indicating that both X-chromosomes replicate early in S-phase, while MZ and LZ indicate heterogeneous or partial late replication of one of the X-chromosomes. The metaphase spreads of control cells exhibited a predominantly late replication pattern for one of the two X-chromosomes characterized by an incorporation pattern

Table 1 Localization of histones H3-3mK9 and H3-3mK27 on the X-chromosomes of metaphase spreads derived from control and 1.0 mM S-adenosylhomocysteine (SAH)-treated female bovine fibroblasts.

Treatment	No. of metaphase spreads analyzed	No. (%) metaphase spreads containing one or no X-chromosome positive for trimethylated histone H3			
		H3-3mK9		H3-3mK27	
		1 Positive	0 Positive	1 Positive	0 Positive
Control	80	80 (100)*	0 (0)*	80 (100)*	0 (0)*
SAH	74	52 (59.5) [†]	22 (40.5) ^{†,‡}	13 (17.6) [†]	61 (82.4) ^{†,§}

Different superscripts within columns*[†] and row^{‡,§} indicate significant differences ($P < 0.05$).

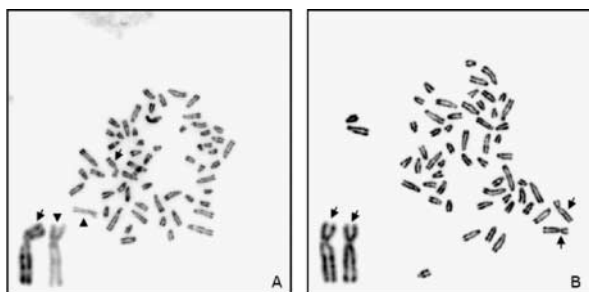


Figure 4 RBA banding replication on the X-chromosomes of cells treated with S-adenosylhomocysteine (SAH). (A) A representative example of a chromosome spread from a control cell showing no BrdU incorporation (early replicated) on the activated X-chromosome (arrows) and high incorporation (late replicated) on the inactivated X-chromosome (arrowheads). (B) Example of a spread from 1.0 mM SAH-treated cell showing no BrdU incorporation (early replicated) on both of X-chromosome (arrows). However, the pattern of incorporation was slightly different between both X-chromosomes.

that was HZ, MZ, LZ, and ZZ in 68.4% (78/114), 28.1% (32/114), 1.8% (2/114), and 1.8% (2/114) of cells respectively. However, the metaphase spreads from the SAH-treated cells primarily exhibited a heterogeneous or partial late replication pattern as follows: HZ 36.7% (87/237), MZ 20.7% (49/237), LZ 25.7% (61/237), ZZ and 16.9% (40/237) (Table 2). The frequencies of X-chromosomes with HZ, LZ, ZZ BrdU incorporation pattern in the SAH-treated cells was significantly ($P < 0.05$) different than those in the control cells. Those SAH-treated cells with X-chromosomes showing a late replication pattern (HZ) were indistinguishable from controls.

Transcript levels of X-chromosome-linked genes in SAH-treated cells

Quantitative real-time RT-PCR was used for measuring various X-linked transcripts (*ANT3*, *HPRT*, *MECP2*, *RPS4X*, *XIAP*, *XIST*, and *ZFX*) and autosomal transcripts, *ACTB* and *H2A*, in control and 1.0 mM SAH-treated cells (Fig. 5). Transcript levels of the autosomal controls, *ACTB* and *H2A*, did not differ significantly ($P > 0.05$) between the non-treated and SAH-treated fibroblasts. Although the

transcript levels of X-linked genes including *ANT3*, *HPRT*, *MECP2*, *XIAP*, and *XIST* in the SAH-treated samples tended to be slightly increased, ~1.5-fold in relation to controls, there was no significant ($P > 0.05$) difference except for *HPRT*, which was significantly higher ($P < 0.05$) in the SAH-treated samples compared with the controls. The transcript levels for *RPS4X* and *ZFX* in the SAH samples were not altered compared with controls ($P > 0.05$).

In vitro development of SCNT embryos derived from SAH-treated cells

The frequencies of fusion, cleavage to two-cell stage, and development to blastocyst stage in the SCNT embryos derived from the nuclear transfer of control and 1.0 mM SAH-treated cells are summarized in Table 3. No significant ($P < 0.05$) differences in the fusion and cleavage incidences were observed between the SCNT embryos derived from the control and SAH-treated cells (93.8% vs 93.9% and 90.5% vs 92.2% respectively). However, after culture, the percentages of embryos that reached the blastocyst and hatched blastocyst stages in the SCNT embryos derived from the SAH-treated cells were significantly ($P < 0.05$) higher than those reconstructed with the control cells (33.8 and 24.9% compared with 26.1 and 15.3% respectively). There were no significant differences in the mean number of total and ICM cells in the SCNT embryos derived from either control or SAH-treated cells (178.3 ± 35.1 and 41.4 ± 8.5 and 197.1 ± 26.5 and 43 ± 7.7 respectively).

Telomerase activity levels in SCNT embryos derived from SAH-treated cells

To evaluate the extent of nuclear reprogramming in the SCNT embryos, telomerase activity was investigated at the blastocyst stage of SCNT embryos derived from the SAH-treated and non-treated cells on day 9 by the RQ-TRAP method. Telomerase activity in the SCNT embryos derived from the control cells was considered as 100% for comparison with SCNT embryos derived from the SAH-treated cells. RTA in the SCNT embryos derived from the SAH-treated cells was significantly ($P < 0.05$) increased, ~1.5-fold higher in relation to controls (Fig. 6).

Table 2 Degree of bromodeoxyuridine (BrdU) incorporation on the X-chromosomes of metaphase spreads derived from control and 1.0 mM S-adenosylhomocysteine (SAH)-treated cells.

Treatment	No. of metaphase spreads analyzed	No. (%) of metaphase spreads with varying levels of BrdU incorporation on the X-chromosomes			
		High, Zero (HZ)	Medium, Zero (MZ)	Low, Zero (LZ)	Zero, Zero (ZZ)
Control	114	78 (68.4)*	32 (28.1)*	2 (1.8)*	2 (1.8)*
SAH	237	87 (36.7) [†]	49 (20.7)*	51 (25.7) [†]	40 (16.9) [†]

Different superscripts*[†] within columns indicate significant differences ($P < 0.05$). Zero (Z), no BrdU incorporation on either the short (p) or long (q) arm. Low (L), one or few bands of BrdU incorporation on the q arm and none on the p arm. Medium (M), several bands of BrdU incorporation on the q arm and a few on the p arm. High (H), numerous bands of BrdU incorporation on both the q and p arms.

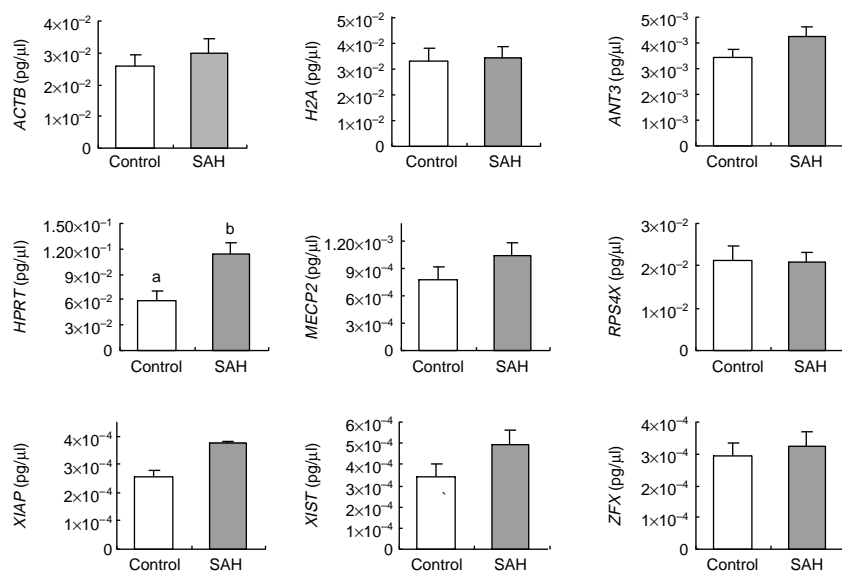


Figure 5 Transcript levels of X-linked genes by real-time RT-PCR in the control and 1.0 mM SAH-treated cells. Bars with different letters are significantly ($P < 0.05$) different.

Discussion

Mounting evidence strongly suggests that the developmental failures and abnormalities of SCNT embryos may be due to incomplete epigenetic reprogramming of the differentiated donor somatic genome (reviewed by Morgan *et al.* 2005). Cloned embryos closely resemble donor cells in their overall genomic methylation status generally being hypermethylated compared with their *in vitro* or *in vivo* fertilized counterparts (Bourc'his *et al.* 2001, Dean *et al.* 2001, Kang *et al.* 2001). Our data have demonstrated that SAH treatment of fibroblasts induces global DNA demethylation, elevates telomerase activity, and partially reactivates the inactivated X-chromosome. Moreover, the use of SAH-induced hypomethylated donor somatic cells increases the developmental potential for SCNT embryos that also exhibit greater telomerase activity levels, indicative of an enhanced dedifferentiation (nuclear reprogramming) of the transplanted somatic cell genome.

Global demethylation

DNA demethylating drugs can be grouped into two classes according to their mode of action. Nucleoside

analogs, such as 5-aza-2'-deoxycytidine (5-aza-dC), are incorporated into the DNA of replicating cells, where they result in the formation of covalent complexes with DNMTs (Christman 2002). On the other hand, non-nucleoside compounds, such as SAH (an analog of the universal methyl donor metabolite SAM), inhibit DNMT enzymatic activity without being incorporated into the DNA (De Cabo *et al.* 1994). Although 5-aza-dC is cytotoxic causing either cell cycle arrest (senescence) or apoptosis, especially at high doses, SAH does not appear to elicit any cytotoxic effects (De Cabo *et al.* 1994, Nieto *et al.* 2004). This attribute was the primary reason for choosing SAH treatment to reduce DNA methylation levels in fibroblast donor cells in this study.

Estimation of the RFI of 5-methylcytosine immunostaining emitted from the nuclei is one of several, albeit somewhat insensitive, ways that has been used, in a variety of contexts, to estimate global DNA methylation status of cells and embryos (Enright *et al.* 2005). We used this approach to qualitatively determine whether SAH treatment administered over two cell culture passages has the capacity to alter genome-wide methylation levels. Cellular or intracellular high SAH concentration or decreased SAM/SAH ratio induces global hypomethylation of DNA, RNA, proteins, and phospholipids of cells

Table 3 *In vitro* development of somatic cell nuclear transfer (SCNT) embryos derived from control and 1.0 mM S-adenosylhomocysteine (SAH)-treated donor cells.

Treatment	No. of oocyte fused	No. (%) of embryos developed to			Total cell no. (ICM cell no.)
		2-cell	Blast	H-blast ^a	
Control	883/941 (93.8)	698/771 (90.5)	201/771 (26.1)*	118/771 (15.3)*	178.3 ± 35.1 (41.4 ± 8.5)
1.0 mM SAH	828/884 (93.7)	725/786 (92.2)	266/786 (33.8) [†]	196/786 (24.9) [†]	197.1 ± 26.5 (43.4 ± 7.7)

Percentages with different superscripts within columns indicate significant ($P < 0.05$) differences.

^aHatching blastocysts.

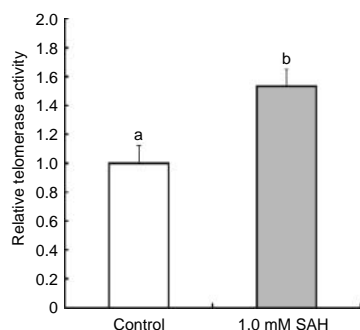


Figure 6 Relative telomerase activity (RTA) of SCNT embryos derived from control and 1.0 mM SAH-treated donor cells. The RTA of SCNT embryos derived from the control donor cells was considered as 100% for comparison with SAH-treated donor cells. Bars with different letters are significantly ($P < 0.05$) different.

and tissues, since high-affinity binding of SAH to the active site of cellular methyltransferases results in product inhibition of the enzyme (Yi *et al.* 2000, Caudill *et al.* 2001, Castro *et al.* 2005). Here, we have detected high levels of DNA demethylation in fibroblasts subsequent to daily treatment with SAH (up to 1.0 mM SAH) over two cell passages (~10 days). The level of DNA demethylation appeared to plateau at an RFI of about 40% of controls with treatment of 0.5 mM SAH and higher, suggesting a threshold concentration for SAH treatment. Similar observations have been made with other demethylating agents such as 5-aza-dC in which higher concentrations limit demethylation possibly due to cytotoxicity (Enright *et al.* 2005). 5-Aza-dC induces a dose-dependent reduction in global DNA methylation levels, but also inhibits cell proliferation with elevated chromosomal abnormalities and apoptosis levels at high (>0.5 μ M) concentrations (Mohana Kumar *et al.* 2006). Alternatively, it has been reported in HepG2 cells that a reduction in global DNA methylation of ~30% was induced by 1 mM SAH for 24 h (Hermes *et al.* 2004) and 1 mM SAH for 3 days in mouse fibroblasts without cytotoxicity (Nieto *et al.* 2004). This may be due to the short exposure to SAH in those studies, as SAH is rapidly oxidized to the sulfoxide in alkaline solutions (De Cabo *et al.* 1994). We have also observed that 5-azacytidine-treated fibroblasts arrested at G1/S-phase of cell cycle, while SAH treatment did not appear to have any effect on cell proliferation (data not shown).

Effect of SAH treatment on telomerase activity

Telomerase activity levels were assessed in the SAH-treated fibroblast donor cells as a marker for gene activation by global DNA demethylation. Telomerase activity is tightly regulated by the expression of TERT and telomerase RNA component (TERC), two main subunits of the telomerase holoenzyme. TERT seems to be the rate-limiting factor for telomerase activity with hTERT expression apparently regulated by the methylation

status of its promoter (Cong *et al.* 1999, Shin *et al.* 2003). Although there have been some discrepancies (Guilleret & Benhattar 2003), generally CpG island methylation of the hTERT promoter is associated with low/no telomerase activity (Bechter *et al.* 2002, Shin *et al.* 2003). Treatment of human cell lines with 5-aza-dC induces expression of hTERT, suggesting a potential role for DNA methylation contributing to hTERT repression in some cells (Dessain *et al.* 2000). Recently, it has been reported that lack of DNMTs results in increased telomerase activity and subtelomeric DNA hypomethylation associated with elongated telomeres in DNMT-deficient mouse embryonic stem cells (Gonzalo *et al.* 2006). In addition, alterations in histone tails of chromosome have been demonstrated to negatively regulate telomere length, and loss of these heterochromatic marks such as di- and trimethylation of H3-K9 leads to telomere elongation in mouse cells (Garcia-Cao *et al.* 2004), suggesting that DNA modification of compacted chromatin status and DNA methylation itself are fundamental factors for negative control of telomerase activity and/or its access to telomeres in mammalian cells. In our studies, telomerase was increased in adult bovine fibroblasts traditionally known to be telomerase negative (Betts *et al.* 2001). Our detection of telomerase activity in non-treated control fibroblasts may be due to the greater sensitivity of using the real-time RQ-TRAP method of telomerase detection (Perrault *et al.* 2005). Recently, telomerase has been found, in normal human somatic cells, to maintain telomere structure as a postulated means for chromosomal stability (Masutomi *et al.* 2003). Increased telomerase activity induced by SAH treatment may be due to transcriptional activation or up-regulation of the TERT and/or TERC genes, as a result of DNA hypomethylation and/or histone modifications. Up-regulation of telomerase activity plateaus at a similar dose of SAH (0.5 mM) to that at which DNA demethylation levels off indicating some relationship between the amount of global DNA methylation and the telomerase activity levels. We suggest that SAH treatment induces high DNA hypomethylation and up-regulation of telomere activity without apparent arrest and apoptosis of the cell.

Effect of SAH treatment on XCI

The XCI status was assessed in the SAH-treated female fibroblast donor cells as a marker for associated epigenetic alterations induced by global DNA demethylation. XCI is a multistep epigenetically regulated process that begins soon after fertilization and is maintained in all female somatic cells throughout pre- and postnatal life (Heard 2004, Chang *et al.* 2006). Key to this gene silencing process is the establishment and maintenance of DNA and histone methylation, which alters the replication profile and transcriptional activity in the hypermethylated X-chromosome (Csankovszki

et al. 2001). This process is initiated by the transcription of a non-coding sequence of RNA from the XIST (X-inactive-specific transcript) from the locus on the X-chromosome to be inactivated. Subsequently, transcription of the majority of genes on that chromosome is reduced, histones H3 lysine 9 and lysine 27 become methylated, and the inactivated X-chromosome becomes late replicating. Hypermethylation of CpG-rich areas of the inactive X-chromosome occurs as the last of the series of events in the inactivation process and is thought to play a role in the maintenance of the inactivation process rather than initiating it (reviewed by *Chang et al.* 2006). Examination of the X-chromosomes of cells treated with the demethylating agent 5-aza-dC has previously shown that the replication pattern is altered with a trend toward synchronous replication of the two X-chromosomes (*Jablonka et al.* 1987, *Haaf et al.* 1988) and an increase in the transcriptional activity of the X-linked gene, *HPRT* (*Sasaki et al.* 1992). The effect of SAH-induced demethylation in this context has not been previously reported. In the present study, SAH treatment of female fibroblasts induced the removal of histones H3-3mK9 and H3-3mK27 trimethylation on one of the X-chromosomes, the presumptive Xi, in over 40% and 82% of cells respectively. It has been demonstrated previously that DNA methylation is controlled by histone H3-K9 methylation (*Tamaru & Selker* 2001, *Lehnertz et al.* 2003). Conversely, the distribution of H3-3mK27 is directly influenced by DNA methylation, and the allocation of H3-2mK9, H3-1mK27, and H3-2mK27 histone modifications were not affected by changes in the methylation status of the DNA (*Mathieu et al.* 2005). Since methylation of histones H3-3mK9 and H3-3mK27 is achieved by histone methyltransferase as well (*Peters et al.* 2003, *Plath et al.* 2003), removal of H3-3mK9 and H3-3mK27 from the presumptive inactivated X-chromosome may be also be due to the inhibition of histone methyltransferase, independent of the methylation status of the X-chromosome DNA. In earlier studies, the level of methylated H3-K9 histones was reduced in cells treated by increasing concentrations of SAH indicating that SAH or methyl donor deficiency inhibits H3 lysine 9 methylation activity (*Kim et al.* 2003). However, *Fahrner et al.* (2002) have suggested that methylation of H3-K9 is inhibited by 5-aza-dC, which is a DNMT inhibitor, and not histone methyltransferase inhibitor. This implies that removal of both H3-3mK9 and H3-3mK27 methylation could also be related to SAH-induced DNA hypomethylation. Interestingly, we have shown that the distribution pattern of these trimethylated histones, H3-3mK9 and H3-3mK27, on the presumptive Xi of SAH-treated cells is different (not entirely overlapping), suggesting that their establishment/maintenance may be modulated by other unknown factors.

In general, transcriptionally active euchromatin replicates during the first half of S-phase, whereas silent

heterochromatin replicates during the second half. The mostly heterochromatic Xi is also characterized by late replication, compared with its activate homolog with early replication timing (*Hansen et al.* 1996, *Mostoslavsky et al.* 2001). The shift from synchronous replication to asynchronous (late) replication of the X-chromosomes in female embryos of cattle and other species during development has been shown to occur after the initial expression of XIST (*De La Fuente et al.* 1999, *Heard* 2004) and associated with H3 trimethylation at lysine 9 (*Chadwick & Willard* 2004) and 27 (*Plath et al.* 2003, *Heard* 2004) localized over the late replicating regions. Treatment of cells with SAH shifted the replication pattern of the presumptive Xi from late to intermediate or early replication in 63.9% of cells examined (vs 31.7% in untreated cells). However, it should be noted that determining the replication pattern of the X-chromosomes with BrdU is a sensitive method that requires a high degree of synchronization of the cells at the time of labeling (*Di Bernardino et al.* 2002) and fixation such that even in untreated samples we detected asynchronous replication considered to result from the incomplete synchronization of the cell cultures at the time of BrdU administration (*Ponce de Leon et al.* 1992) in approximately one-third of the cells examined. Nonetheless, using this as a baseline value, SAH treatment clearly altered the X-chromosome replication pattern. Change in replication timing coincided with the immunochemical identification of changes in cytochemically detectable methylated domains of H3-3mK9 and H3-3mK27. Alteration of DNA methylation by 5-aza-dC and histone methylation patterns with inhibitors of histone deacetylases have also been associated with alteration in replication timing of the late replicating X-chromosome (*Bickmore & Carothers* 1995) suggesting that, as observed in the present study, disruption of both global DNA methylation and histones H3 lysine 9 and 27 methylation patterns is associated with heterochromatic DNA replication patterns. Although the methylation status of the inactivated X-chromosome was not specifically assessed in this study, the shift to early replication of the presumptive Xi in SAH-treated cells corroborates the observed reduction in lysine trimethylation of H3 histones and predicts that the inactive X-chromosome DNA was demethylated based on the known series of events for the inactivation process (*Chang et al.* 2006).

The main physiological outcome of X-inactivation is the dosage compensation for X-linked genes that manifests as transcriptional silencing of the majority of the loci on the Xi (*Chang et al.* 2006). Alteration in mechanisms that initiate or maintain the X-inactivation would be expected to alter the expression pattern of X-linked genes in female cells (*Sasaki et al.* 1992, *Tinker & Brown* 1998). Generally, gene expression in demethylated cells has been shown to increase by the reactivation of silenced genes, as a result of CpG hypomethylation. DNA demethylation by SAH treatment showed up-regulation of genes related to cell

growth/maintenance, metabolism and oxidoreductase activity induced in the SNU-16 gastric cancer cells (Lee *et al.* 2004), and gene expression has been also increased in human fibroblasts treated with 5-aza-dC (Liang *et al.* 2002). X-linked genes, such as *G6PD*, *HPRT*, *PGK*, and *TIM*, have been shown to be fully re-expressed on the Xi reactivated with synchronous replication timing by hybridization of chorionic villi cells from term placentas with mouse A9 cells (Luo *et al.* 1995) and *HPRT* mRNA has been highly increased on an inactive human X-chromosome in a somatic cell hybrid with demethylation of the *HPRT* CpG promoter, following exposure to 5-aza-dC (Sasaki *et al.* 1992). The locus of the *XIST* gene is methylated in the active X-chromosome; however, after 5-aza-dC treatments, the *XIST* gene is expressed in the mouse/human somatic cell hybrid (Tinker & Brown 1998). In the present study, only *HPRT* showed a significant increase in mRNA levels subsequent to SAH treatment. Interestingly, several of the genes, including *XIST*, showed a notable, albeit non-significant, increase in the level of expression in treated samples. *HPRT* is transcribed only from the active X-chromosome, but *RPS4X* and *ZFX*, both of which had expression levels that were unchanged by treatment, have functional Y-homologs, and are transcribed from both the active and inactive X-chromosome (Boggs & Chinault 1994, Carrel *et al.* 1996, Brown & Chow 2003). It is likely that gene loci escaping X-inactivation such as *RPS4X* and *ZFX* are not methylated on either of the X-chromosomes and hence were not affected by DNA demethylation with SAH, whereas the transcript levels of other X-linked genes were slightly increased by SAH treatment. The non-significant elevation in the expression of X-linked genes might be a reflection of the heterogeneous population of cells in culture following treatment since only slightly more than half of the cells examined show alterations in replication timing and histone methylation patterns on the presumptive inactive X-chromosome. Moreover, since both of these events are downstream of transcriptional silencing, it is possible that SAH treatment, at the dose and times investigated here, was only able to partially reverse X-inactivation. This possibility was further supported by the fact that *XIST* expression, the initiating step in X-inactivation, was not reduced as might be expected if X-inactivation was fully reversed (Tinker & Brown 1998).

Effect of SAH treatment on SCNT embryo development

Epigenetic reprogramming is a very important process in fertilization-derived and cloned embryo development (reviewed by Morgan *et al.* 2005). After fertilization, nuclear reprogramming, which erases some of the epigenetic modifications inherited from the gametes and establishes new zygotic patterns, is an essential process of normal embryogenesis and involves DNA demethylation and methylation events as well as other chromatin alterations (Morgan *et al.* 2005). In

fertilization-derived bovine embryos, the paternal genome is actively demethylated at the zygote stage while methylation of the maternal genome is reduced passively upon cleavage up to the eight-cell stage, followed by a *de novo* methylation event at the 16-cell stage (Dean *et al.* 2001, Reik *et al.* 2001). However, in the SCNT bovine embryos, active demethylation occurs at the one-cell stage with no further demethylation occurring subsequently (Bourc'his *et al.* 2001, Kang *et al.* 2001). At the morula stage, all nuclei of blastomeres appeared highly methylated, resembling those of the differentiated somatic donor cells. It has been suggested that epigenetic reprogramming occurs aberrantly and incompletely in a significant portion of cloned embryos (Dean *et al.* 2001) resulting in aberrant gene expression and abnormal development (Morgan *et al.* 2005). Moreover, it has been shown that H3-K9 methylation is reprogrammed in parallel with DNA methylation and reveals an association between epigenetic marks and developmental potential (Santos *et al.* 2003). The majority of cloned embryos in that study exhibited H3-K9 hypermethylation associated with DNA hypermethylation suggesting genome-wide failure in reprogramming dependent on the donor cell type (Santos *et al.* 2003).

Donor cell treatment with DNMT inhibitor, 5-aza-dC and/or the histone deacetylase inhibitor, Trichostatin A have been used to induce widespread reductions in DNA methylation and increase in histone acetylation levels respectively in donor cells before nuclear transfer (Enright *et al.* 2003, 2005, Shi *et al.* 2003). Although 5-aza-dC-treated cells reduced DNA methylation levels, the donor cells are induced to undergo senescence by the deleterious side effects of 5-aza-dC treatment and subsequent *in vitro* development does not improve for the SCNT embryos derived from them (Enright *et al.* 2003, Shi *et al.* 2003). 5-Aza-dC treatment at much lower concentrations (0.01 μ M) did not impose deleterious effects on the development of embryos cloned from treated cells, although these embryos were not evaluated for pregnancy outcomes (Enright *et al.* 2005). Although embryo transfers and pregnancy monitoring still have to be carried out, we have demonstrated a significant improvement in the frequency of blastocyst development for embryos cloned with SAH-treated donor cells compared with non-treated controls.

To support the supposition that nuclear reprogramming was improved in embryos cloned from SAH-treated donor cells, we measured telomerase activity in the resultant blastocysts. Generally, the presence of telomerase activity is associated with the undifferentiated state and cellular immortality, while cellular aging and senescence result in cells that lack telomerase (reviewed by Greider 1998). Telomerase levels are quite high in mammalian embryos (Betts & King 1999) but diminish during cellular differentiation (Yamada *et al.* 1998), thus making it a good marker for assessing nuclear reprogramming efficiency during cloned embryo development

(Betts *et al.* 2001). We have demonstrated that using hypomethylated donor somatic cells increases the developmental potential of the resultant SCNT embryos possibly by enhancing the nuclear reprogramming efficiency as evident by increased telomerase activity levels. Changes to the DNA methylation and histone methylation status of donor somatic cells by SAH treatment may contribute to enhanced nuclear reprogramming efficiency of the SCNT embryos by producing epigenetic characteristics more like those of normal fertilized embryos. Alternatively, the observed improvement in development for embryos cloned from the SAH-treated donor cells may be due solely by the up-regulation of telomerase itself. Telomerase provides chromosomal/genomic stability and increased levels during early development may provide the SCNT embryos some protection against apoptosis or to better enable DNA repair mechanisms aiding development in suboptimal culture environments (Izbicka *et al.* 1999). In addition, up-regulation of telomerase has been shown to increase proliferation, cell survival, and possibly alter cell state to a more undifferentiated, progenitor-like condition (Perrault *et al.* 2005).

Even though the use of hypomethylated donor cells increases the developmental potential of SCNT embryos during pre-implantation development and holds promise for the improvement of SCNT embryo developmental potential, the possibility for developmental abnormalities related to aberrant expression of X-linked and imprinted genes exists. Further exploration of development beyond the blastocyst stage in terms of gene expression in addition to fetal anatomy and physiology is required.

Materials and Methods

All chemicals and reagents used in this study were purchased from the Sigma Chemical Company and Gibco BRL, unless otherwise stated.

Preparation and demethylation treatment of cells

Primary cultures of bovine female adult crossbred beef ear skin fibroblasts (~4 years old) were established from a piece of ear tissue and stored in LN₂ until required. For each experiment, frozen cells were thawed and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin–streptomycin (10 000 IU and 10 000 µg/ml respectively; Invitrogen). The cells at the third passage were cultured in DMEM plus 10% FBS and 1% Pen-Strep containing 0 (control), 0.1, 0.25, 0.5, 1.0, and 2.0 mM SAH in 4-well dishes (Nunc, Roskild, Denmark) for an additional two passages until confluence (5 days) with the culture media being changed daily with fresh DMEM containing respective concentrations of SAH. The cells were then dissociated in 0.25% trypsin with 0.04% EDTA and immediately analyzed for global demethylation or frozen at –80 °C for future analysis of RTA.

Quantification of global DNA methylation

The immunostaining method for the detection of 5-methylcytosine was used as described previously by Castilho *et al.* (1999). Briefly, fully confluent SAH-treated cells at various concentrations were exposed to warm 0.075 M KCl for 20 min, followed by fixation in methanol and acetic acid (3:1) overnight at 4 °C. The fixed cells were spread onto pre-cleaned wet slides and air-dried. The slides were treated with RNase (10 µg/ml) and pepsin (0.1 mg/ml) for 1 h at 37 °C, dehydrated in 70% and 100% ethanol and air-dried. The slides were treated with 4 M HCl for 15 min at room temperature (RT), washed with PBS, and blocked for 30 min at RT in PBST (PBS containing 0.05% (v/v) Tween 20) supplemented with 1% (v/v) BSA (essentially fatty acid free). The slides were incubated with mouse MAB against 5-methylcytosine (1:200 dilution; VWR Laboratories, Mississauga, ON, Canada) in PBS at 37 °C for 1 h, followed by three washes with PBST and incubation in PBS containing an FITC-conjugated anti-mouse secondary antibody (1:200 dilution; Abcam, Cambridge, MA, USA) at 37 °C for 1 h. Finally, the slides were washed with PBST and mounted with Vectashield mounting medium (Vector Laboratory Canada Inc., Burlington, USA) containing 1 µg/ml 4',6'-diamidino-2-phenylindole (DAPI) for staining DNA. The slides were observed under a Leitz fluorescence microscope (Leica, Wetzlar, Germany). Openlab Leica image processing software (Improvision, Lexington, MA, USA) equipped with the microscope was used for image acquisition and quantitative measurements of the RFI emitted by each individual nucleus. Every slide was analyzed using the same optical conditions. At least 100 nuclei were analyzed for each SAH treatment.

Relative-quantitative telomerase repeat amplification protocol (RQ-TRAP)

The RQ-TRAP assay was modified from a conventional TRAP assay for use on the LightCycler 3.0 (Roche) as described previously by Betts *et al.* (2006). Briefly, after harvesting 1×10^5 cells treated with various SAH concentrations and SCNT embryos developed to blastocyst on day 9, samples were either immediately frozen at –80 °C for future analysis or lysed. The samples were lysed in 0.5% (v/v) 3-[(3-cholamidopropyl) dimethylammonio] propane-sulfonic acid lysate buffer (pH 7.5) supplemented with 10 mM Tris–HCl, 1 mM MgCl₂, 1 mM EGTA, 0.1 mM benzamide, 5 mM of 2-mercaptoethanol, and 10% glycerol at a concentration of 250 cells/µl for 30 min on ice. Following lysis, the samples were centrifuged for 20 min at 12 000 g at 4 °C to remove cell debris. Eighty percent (by volume) of the lysis was then transferred to a fresh microcentrifuge tube and analyzed using RQ-TRAP. Each run included measurements of 1000, 100, 10, and 1 293T (Chemicon/Millipore, Lake Placid, NY, USA) telomerase-positive control cell(s) for the generation of a standard curve. As a negative control, a portion of the 293T sample was heat inactivated by incubation for 10 min at 85 °C. The RQ-TRAP was optimized using the PCR reagent LightCycler FastStart DNA Master SYBR Green I (Roche), according to the manufacturer's protocol, containing 2.5 mM MgCl₂, 0.02 µg primer TS (5'-AAT CCG TCG GAG CAG AGT T-3'), 0.04 µg primer ACX (5'-GCG CGG CTT ACC CTT ACC CTT ACC CTA ACC-3'), and 2 µl of each 250 cells/µl sample to be analyzed.

The assay run included 20-min incubation at 25 °C, followed by 10-min incubation at 94 °C, and 40 cycles of PCR at 94 °C for 30 s and 60 °C for 90 s. All the samples were quantified using the LightCycler Quantification Software's (Roche) second derivative method of crossing point (Cp) determination, which was then compared with the 293T cell standard curve generated, and activity expressed as RTA to 293T cells on a per cell basis.

Distribution analysis of H3-3mK9 and H3-3mK27

Metaphase chromosomes and immunostaining of H3-3mK9 and H3-3mK27 were prepared as described previously with minor modifications by Belyaev *et al.* (1996). Briefly, the cells at the third passage were treated with 0 (control) and 1.0 mM SAH during the two passages until confluence. After reseeding, the growing cells treated with 0 (control) and 1.0 mM SAH were arrested at M-phase with 0.05 µg/ml demecolcine (colcemid) treatment for 2 h. Mitotic cells were collected by vigorously shaking the flasks and treated with warm KCl (0.075 mol/l) for 10 min, and then spread onto the slides using a Shandon Cytospin Centrifuge (Thermo-Fisher, Whitby, ON, Canada) at 500 *g* at 10 min. The slides were immersed in KCM buffer (120 mM KCl, 20 mM NaCl, 10 mM Tris-HCl (pH 8), 0.5 mM EDTA, and 0.1% Triton X-100) for 10 min, followed by fixation in 4% formaldehyde (in PBS) for 5 min, washed with 0.04% Photoflo (in H₂O; Kodak) for 5 min, and air-dried. The slides were blocked for 30 min at RT in PBST supplemented with 1% BSA and incubated in PBST containing mouse rabbit anti-H3-3mK9 (1:100 dilution; Abcam) and mouse anti-H3-3mK27 (dilution 1:100; Abcam) overnight at 4 °C and incubated in PBS containing a anti-rabbit Alexa 594-coupled antibody (1:100 dilution; Abcam) for the detection of H3-3mK9 and FITC-conjugated anti-mouse secondary antibody (1:100 dilution, Abcam) for the detection of H3-3mK27 at 37 °C for 1 h. Finally, the slides were washed with PBST and mounted with Vectashield mounting medium containing 1 µg/ml DAPI for staining DNA. Image acquisition and analysis were carried out as described above.

Replication timing assay

The replication timing of the X-chromosomes was analyzed using RBA orange of metaphase spreads as described previously by Di Bernardino *et al.* (2002). Briefly, after trypsinization of the cells previously treated with 0 (control) and 1.0 mM SAH for the two passages until confluence, the cells were cultured for 3 h and then synchronized at the middle of S-phase with 300 µg/ml thymidine for 16 h. This cell cycle block was released with two washes with fresh DMEM, and then the cells were allowed to resume their cell cycle in DMEM, supplemented with 20 µg/ml BrdU, a thymidine analog. After 7 h of culture, cells at metaphase were harvested and treated with warm 0.075 M KCl for 20 min, followed by fixation in cold methanol:acetic acid (3:1, v/v) overnight at 4 °C. The fixed cells were spread onto pre-cleaned wet slides, air-dried, and stained with 0.1% acridine orange solution in PBS (pH 7.0) for 10 min, washed thoroughly in tap water, mounted in phosphate buffer, and sealed with nail cement. Image acquisition and analysis were carried out as described earlier.

Quantitative analysis of transcripts by real time RT-PCR

Total RNA of the fully confluent control and the SAH-treated cells was extracted using the QIA shredder column and Qiagen RNeasy Micro Kit (Qiagen). Homogenization, isolation, precipitation, and purification of RNA were performed, according to the manufacturer's procedure with an extra step of DNase I treatment for the removal of DNA contamination. The concentration of extracted total RNA was determined by measuring the absorbance at 260 nm in a spectrophotometer. Two micrograms of total RNA was synthesized for the first-strand cDNA with Omniscript RT Kit (Qiagen). Each of the cDNA samples contained 2 µl of 10 µM oligo(dT)₁₂₋₁₈ primer (Invitrogen), 1 µl of 10 U/µl RNase inhibitor (Fisher Scientific, Whitby, ON, Canada), 2 µl RT buffer, 2 µl dNTP, and 1 µl Omniscript (Qiagen), adjusted to a total volume of 20 µl using H₂O. The cDNA samples were then incubated in a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, MA, USA) at 42 °C for 1 h, followed by 5 min at 95 °C to inactivate the enzyme. A total of three RT reactions were used for each RNA sample. The real-time RT-PCR was carried out using the LightCycler 3.0 and the LightCycler FastStart DNA Master SYBR Green I (Roche), according to the manufacturer's protocols. Each reaction mix contained 2 µl of the cDNA reaction, 2 µl of the FastStart DNA Master SYBR Green I reaction mix, 3 mM MgCl₂, and 2 µl of each the forward and reverse primers (0.1 µg/µl), adjusted to a total volume of 20 µl using H₂O. X-linked genes (*ANT3*, *HPRT*, *MECP2*, *RPS4X*, *XIAP*, *XIST*, *ZFX*) and autosomal genes (*ACTB*, *H2A*) were analyzed for the pattern of the transcripts by quantitative real-time RT-PCR. Primer sequences, the size of amplified products, and the GenBank accession numbers are shown in Table 4. The annealing and acquisition temperatures of primer were used as described previously by Nino-Soto *et al.* (2005). In each of the cDNA samples, at least five replicates of PCRs were carried out.

SCNT and embryo culture

Cells at the third passage were treated with control and 1.0 mM SAH for an additional two passages until confluence and dissociated in 0.25% trypsin in EDTA immediately before nuclear transfer. Immature bovine oocytes obtained from slaughterhouse ovaries were *in vitro* matured in TCM-199 containing 2.5 mM Na pyruvate, 1 mM L-glutamine, 1% (v/v) Pen-Strep, 2% steer serum (PAA Laboratories, Etobicoke, ON, Canada), 1 µg/ml estradiol-17β, 0.5 µg/ml follicle-stimulating hormone, and 1 µg/ml luteinizing hormone (USDA National Hormone and Peptide Program, UCLA Medical Center, Torrance, CA, USA) at 38.5 °C in a humidified atmosphere of 5% CO₂ in air. After 18 h of culture, the oocytes denuded by vortexing for 2 min in 3% (v/v) sodium citrate solution were enucleated in HEPES-buffered Tyrode's salt solution with albumin, lactate, and pyruvate (HEPES-TALP), and successfully enucleated oocytes were selected by a short 1–2 s exposure of the pipette containing aspirated cytoplasm stained with 5 µg/ml bisbenzimidazole (Hoechst 33342) to u.v. light. Donor cells with intact membranes were selected and transferred to the perivitelline space of each enucleated oocyte. Oocyte-donor cell complexes were fused with two electric pulses at

Table 4 Sequence-specific primers used for RT-PCR.

Gene	Primer sequences (5'–3')	PCR product band size (bp)	Annealing temperature (°C)	Acquisition temperature (°C)	GenBank accession number
ACT-B	F-CGTGACATTAAGGAGAAGCTGTGC R-CTCAGGAGGAGCAATGATCTTGAT	374	63	86	BC008633
H2A	F-GTCGTGGCAAGCAAGGAG R-GATCTCGGCCGTTAGGTACTC	182	59	87	U62674
ANT3	F-TTCCCTGTGCTTCGTCTACC R-TGCCCTTGATCATGATGTCG	383	60	89	NM_001636
HPRT	F-CGAGATGTGATGAAGGAGATGG R-TTCAAATCCAACAAGTCTGG	397	61	81	NM_001034035
MECP	F-GGGACCCATGTATGATGACC R-ATGTGTCGCCTACCTTTTCG	173	60	81	NM_004992
RPS4X	F-ATTAAGATCGATGGCAAAGTCC R-AAAAGAACCTGGATGTCTCTCC	408	59	80	XM_591678
XIAP	F-GGCGACACTTTCCTAATTGC R-AAGCATAAAATCCAGCTCTTGC	213	58	78	NM_001167
XIST	F-CCTTGTCATGTGGATATCATGG R-AATGTCCTTGGAAAGACTTTGG	224	59	84	NR_001464
ZFX	F-TCTATCCTTGCATGATTTGTGG R-AGAGTCTGCGGACCTATATTCC	494	57	83	NM_003410

1.0 kV/cm for 15 μ s in 0.26 M mannitol solution supplemented with 50 μ M MgCl₂ using an ECM 2001 BTX cell manipulator (VWR Laboratories) equipped with electrode needles. After 1 h, the eggs were assessed for successful fusion. Fused oocytes were subsequently activated with 5 μ M ionomycin in HEPES-TALP for 5 min, followed by exposure to *in vitro* culture (IVC) medium containing 10 μ g/ml cycloheximide (CHX). After incubation with CHX for 5 h at 38.5 °C, reconstructed embryos were transferred to the IVC medium, which was modified synthetic oviduct fluid (mSOF) supplemented with 2.9 μ g/ml EDTA and 8 mg/ml BSA up to day 3. On day 3 of the culture, the embryos were transferred to mSOF supplemented with 8 mg/ml BSA and MEM amino acids until day 9 (Mastromonaco *et al.* 2004). All IVC culture was at 38.5 °C in a humidified atmosphere of 5% CO₂, 5% O₂, and 90% N₂. Cleavage and blastocyst frequencies were recorded on days 2 and 9 respectively. Total and inner cell mass cell numbers and RTA levels of the SCNT embryos developed to hatched blastocyst on day 9 were determined by the differential staining method (Thouas *et al.* 2001) and RQ-TRAP assay described earlier.

Statistical analysis

Differences among treatments were analyzed using one-way ANOVA, those in the RFI, RTA, and transcript level of X-chromosome genes using Student's *t*-test, and those in the incorporation of H3-3mK27 and H3-3mK9, late replication, and development *in vitro* into blastocyst stage using χ^2 test. *P* < 0.05 was used as the level of significance.

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