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Induction of a Senescent-Like Phenotype Does Not Confer the Ability of Bovine Immortal Cells to Support the Development of Nuclear Transfer Embryos[1]

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ABSTRACT

Previously, we reported that cloned embryos derived from an immortalized bovine mammary epithelial cell line (MECL) failed to develop beyond 12- to 16-cell stage. To analyze whether induction of a senescent-like phenotype in MECL can improve their ability to support the development after transfer into enucleated oocytes, we treated MECL with DNA methylation inhibitor 5-aza-2-deoxycytidine (Aza-C), histone deacetylase inhibitors trichostatin A (TSA), sodium butyrate (NaBu), or 5-bromodeoxyuridine and used those cells for nuclear transfer. Primary bovine fetal fibroblasts (BFF) were used as control. All agents were capable to induce features of senescence including reduced cell proliferation, enlarged cell size with a considerable proportion of cells stained positive for acidic senescence-associated β-galactosidase and G1/S cell cycle boundary arrest in MECL. Aza-C treatment induced genome demethylation. Acetylation of H3 and H4 was increased after TSA treatment in both MECL and BFF, whereas no obvious changes in global H3 or H4 acetylation were detected after NaBu treatment. Nuclear transfer experiments following diverse treatments demonstrated that the induced senescent-like phenotype of MECL did not confer their ability to support embryonic development, although 7.3% of reconstructed embryos derived from NaBu-treated cells developed to morula stage. Intriguingly, a much higher proportion of cloned embryos developed to blastocysts when using NaBu-treated BFF, compared with using untreated BFF (59% versus 26%). Our results suggest that the developmental failure of donor nuclei from bovine immortal cells could not be reversed by induction of senescent-like phenotype. The beneficial effect of NaBu on the developmental potential of cloned embryos reconstructed from BFF merits further studies.

apoptosis, early development, embryo, mammary glands, oocyte development

INTRODUCTION

In nuclear transfer experiments, freshly isolated or early-passaged primary cells are generally chosen as donor cells. To generate genetically modified animals by nuclear transfer, it is favorable to use stable cell lines that allow time for various genetic manipulations and provide an infinite source of relatively uniform donor cells. Because embryonic stem cell lines are not available in farm animal species [1], we have examined the ability of nuclei from a spontaneously immortalized bovine mammary epithelial cell line (MECL) to contribute to the development of nuclear transfer embryos. MECL nuclei failed to support the development of reconstructed embryos; no embryos developed beyond 12- to 16-cell stage. In contrast, live offspring were produced from cloned embryos derived from primary mammary gland cells [2]. The biological reason for the developmental failure of nuclear transfer embryos from bovine immortal cells remains unclear.

Most mammalian somatic cells undergo irreversible senescence after a limited number of cell divisions when cultured in vitro, a phenomenon termed cellular senescence [3] or replicative senescence. Cells in the state of senescence can be metabolically active for a long period but lose proliferating capacity, resembling terminally differentiated cells [4]. However, stem cells from some renewing tissues, embryonic germ cells, immortal cells, and most tumor-derived cells can undergo unlimited divisions when grown in vitro [5]. The ability to overcome senescence and obtain an unlimited proliferative potential is called immortalization that can be achieved spontaneously or by different stimuli. The mechanisms of immortalization still remain to be clarified. Somatic cells of near senescence or senescence have been used successfully as donors to generate cloned cattle [6, 7]. Donor cells derived from animals of different ages have similar ability to support in vitro development of nuclear transfer embryos [7]. Long-term culture of donor cells did not significantly influence the efficiency of nuclear transfer, compared with short-term cultured cells [8]. These facts indicate that senescent or aged donor cells can support the development of cloned embryos.

A variety of environmental and intracellular stimuli has been shown to induce a senescent-like state in various cell types. Cumulative experimental evidence suggests that DNA methylation and chromatin structure may play an important role in the process of senescence. In the present study, we induced senescent phenotypes by treating MECL with a DNA methylation inhibitor, histone deacetylase (HDAC) inhibitors or 5-bromodeoxyuridine (BrdU) and evaluated whether the induction of a senescent-like phenotype in MECL (in other words, the reversal of immortal phenotype) could improve the developmental ability of nuclear transfer embryos derived from those treated cells.

MATERIALS AND METHODS

Cell Culture, Treatments, and Growth Curves

A spontaneously immortalized bovine mammary cell line (MECL), which was maintained for more than 100 passages with stable morphology,

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was established and kindly provided by Dr. M. Düchler, Institute of Animal Breeding and Genetics, University of Veterinary Sciences, Vienna, Austria. Bovine primary fetal fibroblasts (BFF) were obtained from a 49-day-old female fetus of a German Simmental cow as described previously [9] and used at passage 5–6 for the following experiments. Cells were grown in Dulbecco Modified Eagle Medium (Gibco, Grand Island, NY) supplemented with 10% (v/v) fetal calf serum (Biochrom, Berlin, Germany), 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, 2 mM nonessential amino acids, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Gibco). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air.

In our pilot studies, MECL cells were exposed to various agents of different dosages and durations and used for nuclear transfer. We tested trichostatin A (TSA) at 50 ng/ml, 100 ng/ml, 300 ng/ml, or 600 ng/ml for 1 or 2 days, 5-aza-2-deoxycytidine (Aza-C) at 1 μM or 27 μM for 8 h, 33 h, 52 h, or 72 h, sodium butyrate (NaBu) at 0.5 mM, 1 mM, or 2 mM for 2, 4, or 6 days and BrdU at 20 μM or 40 μM for 5 days. With the higher concentration or longer duration of treatment, cells usually showed abnormal characteristics such as extremely large size or damaged cell membrane or became fragile to the electric pulse. Nuclear transfer embryos derived from cells with different treatments gave various cleavage rates. For example, the cleavage rates of cloned embryos from cells treated with NaBu 1 mM 48 h, 1 mM 96 h, 1 mM 144 h, 0.5 mM 96 h, or 2 mM 96 h were 62%, 77%, 68%, 70%, or 78%, respectively, but no single blastocyst was recovered. A slight decrease in cleavage rate of cloned embryos was found following Aza-C treatment of donor cells. In this study, we used a combination of Aza-C and TSA treatment. Based on the effective concentrations of these agents shown in other studies [10–14] and the results of our pilot study regarding cellular morphology after treatment as well as cleavage rates and developmental potential of nuclear transfer embryos (detail not shown), we chose the following treatments: 300 ng/ml TSA for 24 h, 1 μM Aza-C for 72 h followed by 1 μM Aza-C and 300 ng/ml TSA for 24 h, 1 mM NaBu for 96 h or 40 μM BrdU for 120 h. Additionally, nonimmortalized cells, BFF, were used as a control. All agents for the treatments were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Growth, Proliferation Assay, and Cell Marker Staining

To measure cell growth, MECL cells were plated in triplicates at a density of 1×10^4/well in 12-well plates, incubated with different agents and harvested every 24 h up to 6 days. Cell number at the various time points was determined with a hemacytometer after trypan blue exclusion of dead cells. We used BrdU incorporation analysis to determine the proportion of proliferating cells. Untreated and treated MECL cells with 70%–80% confluence plated on gelatin-coated coverslips were incubated with 20 μM BrdU at 37°C for 45 min, washed with PBS, and fixed in ice-cold 75% ethanol for 30 min at 4°C. DNA was denatured to its single-stranded form by treatment with 2 M HCl at 37°C for 30 min. Cells were washed in PBS and incubated with 1:1000 mouse anti-BrdU monoclonal antibody (Sigma) at 37°C for 60 min. After washing, a fluorescein isothiocyanate-conjugated sheep anti-mouse immunoglobin (Sigma) was added at a concentration of 1:32, and cells were incubated at 37°C for 45 min. Finally, the slides were washed and mounted in mounting medium containing 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Proliferation capacity was not analyzed in the cells treated with BrdU because false positive cells could not be excluded. The number of BrdU-positive cells was counted in 10 fields at 200× magnification. All images were taken with a digital camera (Axiocam, Zeiss, Oberkochen, Germany) on an Axiovert 35 microscope (Zeiss).

Immunofluorescent staining with an epithelial cell maker, cytokeratin, was performed as described before [2]. Vimentin antibody was used as a negative control. MECL cells were fixed in 3:1 methanol and glacial acetic acid. The first antibodies were monoclonal anticytokeratin mouse IgG (clone 8.13, dilution 1:20, Sigma) or monoclonal antivimentin mouse IgG (clone V9, dilution 1:40, Sigma). The second antibody and mounting steps were the same as for BrdU staining (see above). All pictures were taken at 400× magnification.

Apoptosis Assay

Apoptosis was analyzed by terminal deoxynucleotidyl transferase-mediated TUNEL assay. Briefly, MECL cells that were grown on coverslips and exposed to different agents for indicated times were fixed with 4% formaldehyde in PBS and then in 80% ethanol at 4°C. The cells were washed with 50 mM Tris-HCl in saline solution, pH 7.5, permeabilized by incubation with 20 μg/ml proteinase K for 5 min, equilibrated in terminal deoxynucleotidyl transferase (TdT) buffer and incubated with TdT labeling reaction mix (Oncogene Research Product, Darmstadt, Germany) for 1 h at 37°C in a humidified atmosphere in the dark. The coverslips were mounted on glass slides with mounting medium, and slides were examined under a fluorescent microscope. Cells with fragmented DNA showed bright green signal. Hela-60 cells treated with actinomycin D served as a positive control.

Senescence-Associated β-Galactosidase Staining

Senescence-associated β-galactosidase (SA-βgal) activity was determined as described previously [15]. Subconfluent MECL cells were washed in PBS, fixed in 2% formaldehyde/0.2% glutaraldehyde/PBS for 3–5 min at room temperature, washed again in PBS, and incubated at 37°C in freshly prepared X-gal staining solution (1 mg/ml 5-bromo-4-chloro-3-indoyl β-D-galactopyranoside), 40 mM citric acid/Na phosphate buffer (pH 6.0), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 150 mM NaCl, and 2 mM $MgCl_2$ (all chemicals were purchased from Sigma) for 16 h. The proportion of blue-stained cells was counted in 10 fields at 200× magnification.

Flow Cytometric Analyses of Cell Cycle Distribution

To evaluate the effect of various treatments on cell cycle, subconfluent MECL cells were treated for indicated times. About 1×10^6 untreated or treated MECL cells were trypsinized, collected, and washed twice in cold PBS. The cells were fixed in 70% ice-cold ethanol for 1 h and then incubated with staining solution containing 50 μg/ml propidium iodide, 100 μg/ml ribonuclease A, and 1% Triton X-100 (all from Sigma) for 20 min at room temperature. Cell cycle distribution was determined by flow cytometry (FacsCalibur, Becton Dickinson, Mountain View, CA). Twenty thousand events were recorded for each sample. Cell Quest software (Becton Dickinson, Bedford, MA) was used for data analysis. All analyzed events were gated to remove debris and aggregates.

Chromosome Number Analysis

The ploidy of untreated and treated MECL was examined using a standard preparation of metaphase spreads [8]. In brief, subconfluent cells after 20 h of plating were incubated in 0.08 μg/ml demecolcine solution (Sigma) for 1 h. Cells were collected by trypsinization and treated with 0.075 M KCl at 37°C for 15 min. Following treatment, cells were fixed in 3:1 methanol and glacial acetic acid and drops of cell suspension were spread onto clean slides at a 45-degree angle. Chromosomes were stained with Giemsa Blue (Sigma) for 5 min. Then slides were gently rinsed under tap water, dried, and mounted. Chromosomes were counted with an Axiovert 35 microscope (Zeiss) at 400× magnification.

Genome Methylation Analysis and Western Blots to Detect Histone Acetylation

To isolate genomic DNA, cells treated with Aza-C 1 μM for 96 h were incubated in lysis buffer (100 mM Tris-HCl pH 8.5, 5 mM EDTA, 200 mM NaCl, 0.2% SDS, 100 μg/ml proteinase K) at 37°C overnight, and then DNA was precipitated by isopropanol, washed with 75% ethanol, and dissolved in Tris-EDTA buffer. DNA methylation study was carried out using *Msp*I or *Hpa*II (MBI Fermentas, St. Leon-Rot, Germany) restriction digest of DNA at 37°C for 4 h, and digested DNA was subjected to electrophoresis on 1% agarose gel.

Proteins were prepared by acid extraction. Cells were scraped from the Petri dish and collected by centrifugation. The cell pellets were washed in PBS and resuspended in lysis buffer (10 mM Hepes pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, 1.5 mM PMSF). Sulfuric acid was added to a final concentration of 0.2 M. After incubation on ice for 30 min, the acid-insoluble fraction was pelleted by centrifugation (11 000 g for 10 min at 4°C). The supernatant fraction was dialyzed overnight at 4°C in two and three changes of 0.1 M acetic acid and distilled H_2O, respectively. Ten micrograms of MECL and 30 μg BFF protein were loaded on 15% (H3) or 18% (H4) SDS-polyacrylamide gels. Proteins were blotted onto a PVDF membrane (Millipore, Eschborn, Germany), and membranes were stained using 3% Ponceau Red in 1 M acetic acid to control for equal loading of the gels or proper transfer of the proteins. Histone acetylation was investigated by Western immunoblotting techniques. Primary antibodies used were goat-antiacetylated histone H3 (Lys9/14) purchased from Santa Cruz Biotechnology, Heidelberg, Germany (sc-8655; dilution 1:200) and rabbit-antihyperacetylated histone H4 (Penta) purchased from Upstate Biotechnology (Eching, Germany) (06–

946; dilution 1:4000). Bound antibodies were detected using horseradish peroxidase conjugated IgG directed against rabbit (dilution 1:3000) or goat antiserum (dilution 1:10 000), respectively. Detection of bound secondary antibodies was performed by enhanced chemiluminescence (Amersham Biosciences, Freiburg, Germany) using the Image Station 440 CF (Kodak, Stuttgart, Germany).

Nuclear Transfer and Embryo Culture

Nuclear transfer was carried out essentially as described previously [9]. Donor cells were transferred into the perivitelline space of the oocytes that were enucleated with minimal cytoplasmic volume 16–18 h after maturation. The karyoplast-cytoplast complexes (KCCs) were exposed to a double electric pulse of 2.1 kV/cm for 10 μsec using Zimmermann cell fusion instrument (Bachofer, Reutlingen, Germany) to initiate the fusion process [16]. The fused KCCs were activated by a 5-min incubation in 7% ethanol followed by 5 h of culture in 10 μg/ml cycloheximide and 5 μg/ml cytochalasin B. KCCs were transferred into 100-μl drops of synthetic oviduct fluid supplemented with 5% (v/v) estrous cow serum, covered by paraffin oil, and cultured at 39°C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2.

Densitometry and Statistical Analysis

Quantification of the bands on Western blots was performed by the ImageQuant software package (Amersham Biosciences). The proportion of embryos at each developmental stage was compared by chi-square analysis. When *F*-test was significant ($P < 0.05$), BrdU incorporation and SA-βgal proportion were analyzed by ANOVA followed by Bonferonni post hoc test using SPSS statistical program (SPSS Inc., Chicago, IL). A value of $P < 0.05$ was considered significant. The data were presented as mean ± SD.

RESULTS

Growth Inhibition of MECL after Treatment

We analyzed the effect of various treatments on MECL growth. As shown in Figure 1, Aza-C or TSA considerably inhibited cell growth. In the NaBu or BrdU groups, cell growth was slower as compared with the untreated MECL and later nearly arrested. The effect of these agents on cell proliferation was examined by measuring BrdU incorporation that correlates with DNA synthesis and proliferation. The proportions of BrdU-positive cells in TSA-, Aza-C+TSA-, and NaBu-treated cells were significantly decreased as compared with nontreated cells, indicating a low proliferative index (Fig. 2).

Induction of Senescent-Like Phenotype in MECL

Senescence is associated with altered cellular morphology and size [17]. Morphological changes of MECL were observed. After all the treatments, cells had an increased cell size and a more flattened appearance (Fig. 3A). We next investigated whether the treated cells underwent biochemical senescence by staining for SA-βgal. Senescent cells have increased endogenous SA-βgal activity, which is not detected in terminally differentiated or quiescent cells [15]. We observed that SA-βgal activity was significantly increased in MECL treated with various agents. BrdU treatment resulted in the highest proportion of SA-βgal-positive cells (Fig. 3B).

Cell Cycle Arrest at the G1/S Boundary

Because senescence is associated with cell cycle arrest, we evaluated the effect of various treatments on the cell cycle by using propidium iodide staining of DNA. Flow cytometric analysis for the DNA content indicated that the cell population in the S and G_2/M phases decreased after the cells were subjected to different treatments, and this

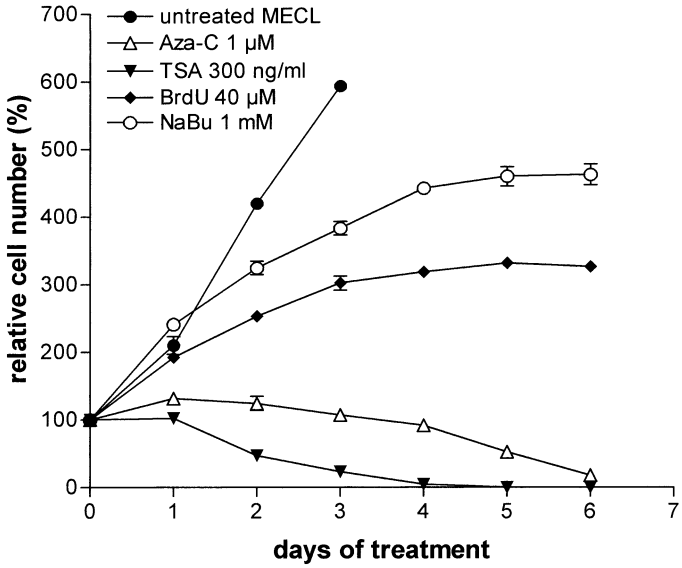

FIG. 1. Growth curve of treated and untreated MECL. TSA and Aza-C significantly inhibited the proliferation of MECL. NaBu and BrdU block the proliferation of MECL. The relative cell number at each time point shown on the growth curves represents the mean value ± SD of triplicates normalized to the cell number at Day 1.

reduction was accompanied by an increase in the proportion of cells in the G_0/G_1 phase. Whereas in untreated MECL, 40% of the cells were in G_2/M, 12% in S, and 48% in G_0/G_1, treatment with Aza-C+TSA resulted in a decrease of G_2/M and S populations to 24% and 3%, respectively, and an increase of the G_0/G_1 fraction to 73%. Similar results were obtained after treatment with TSA, NaBu, or BrdU (Fig. 4). These data indicate that the various treatments in this study inhibited the DNA synthesis via inhibition of the cell cycle at G_0/G_1 to S boundary.

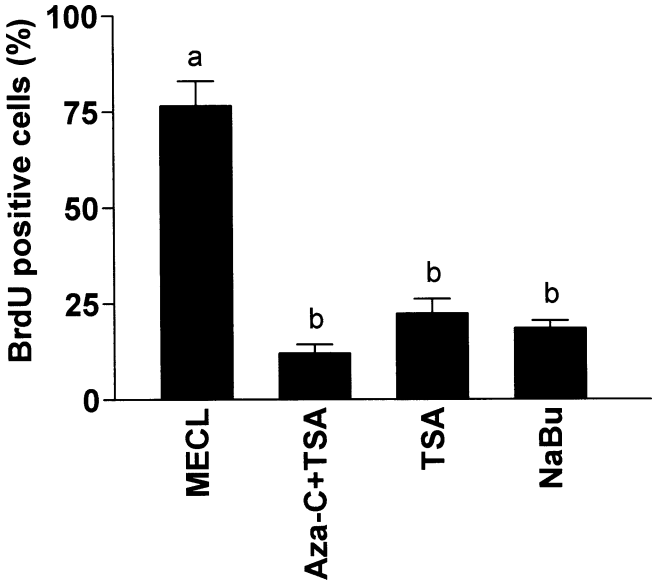

FIG. 2. Determination of DNA synthesis by measuring BrdU incorporation in untreated MECL and MECL treated with 1 μM Aza-C for 72 h followed by 1 μM Aza-C and 300 ng/ml TSA for 24 h, 300 ng/ml TSA for 24 h, or 1 mM NaBu for 96 h. Quantification of BrdU immunofluorescent staining in untreated and treated MECL, showing the proportion of BrdU-positive nuclei. The data represent the mean ± SD of three independent experiments. Values in the columns labeled with b are significantly decreased, compared with that in MECL column labeled with a ($P < 0.01$).

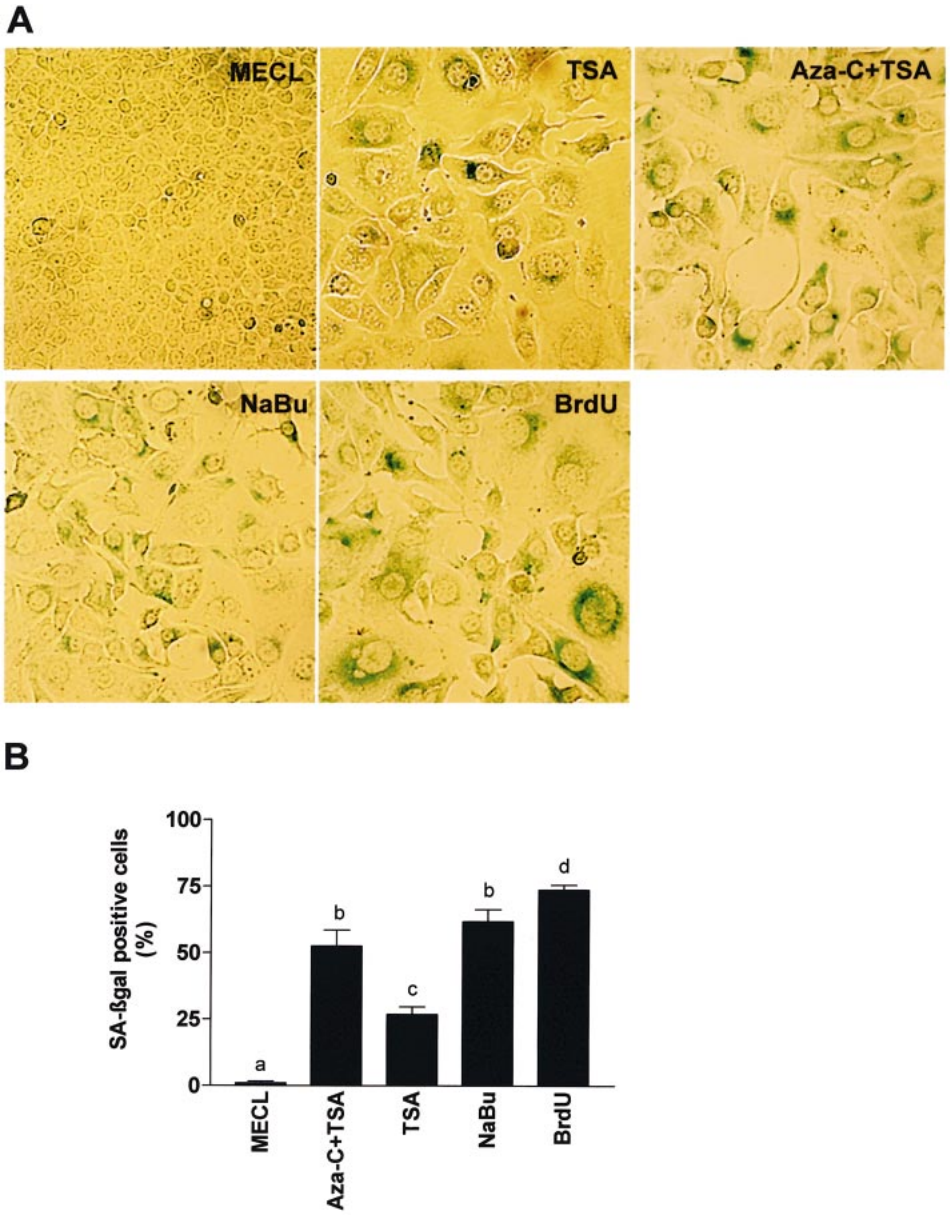

FIG. 3. Induction of morphological and histochemical features of senescence in untreated MECL and MECL treated with 1 μM Aza-C for 72 h followed by 1 μM Aza-C and 300 ng/ml TSA for 24 h, 300 ng/ml TSA for 24 h, 1 mM NaBu for 96 h, or 40 μM BrdU for 120 h. **A**) Cell morphology and SA-βgal activity in untreated and treated MECL. Treated MECL are enlarged, more spread, and stained for SA-βgal activity (cytoplasmic blue staining). **B**) Quantification of the results presented in **A**, showing the percentage of SA-βgal-positive cells. The data represent the mean ± SD of three independent experiments. Cells are photographed at the same magnification (200×). Values labeled with different letters differ significantly ($P < 0.05$).

Evaluation of Apoptosis

Apoptosis can cause DNA damage, resulting in a lower efficiency of nuclear transfer [18]. DNA fragmentation is an early feature of apoptosis [19]. To exclude a proapoptotic effect of the various treatments, we examined DNA fragmentation by TUNEL assay. Three random fields of 200 cells were scored to determine the proportion of cells undergoing apoptosis. No detectable apoptosis was found in untreated MECL and after BrdU treatment. The proportions of apoptotic cells in TSA, Aza-C+TSA, and NaBu groups were less than 2% (data not shown). This was further confirmed by cell cycle analysis. The cells with less than 2N DNA content, characteristic of apoptotic cells, were virtually absent in untreated and treated groups (Fig. 4).

Cellular Ploidy and Differentiation Status of MECL

In our earlier studies, MECL appeared to be karyotypically normal and stable during the course of continuous passage [2]. Because aneuploidy, which can affect embryonic development, might be the underlying cause of immortalization and could be induced by some chemical reagents, chromosome content of MECL before and after treatment was determined. The results are summarized in Table 1; 84% of MECL cells were diploid. No significant changes of cellular ploidy were found after treatment as compared with untreated cells. All cells from untreated MECL stained positive for the epithelial cell marker, cytokeratin. After various treatments, no obvious changes in staining intensity and proportion of positive cells were found (Fig. 5);

Genome Methylation and Histone Acetylation

Genomic DNA was demethylated after Aza-C treatment in both MECL and BFF, as shown in Figure 6. Among the core histones, H3 and H4 are the principal targets of regulatory posttranscriptional modifications. By Western blot, we compared the effects of TSA and NaBu on global histone H3 and H4 acetylation (Fig. 7). After TSA treatment, a marked increase of histone H3 and H4 acetylation in both

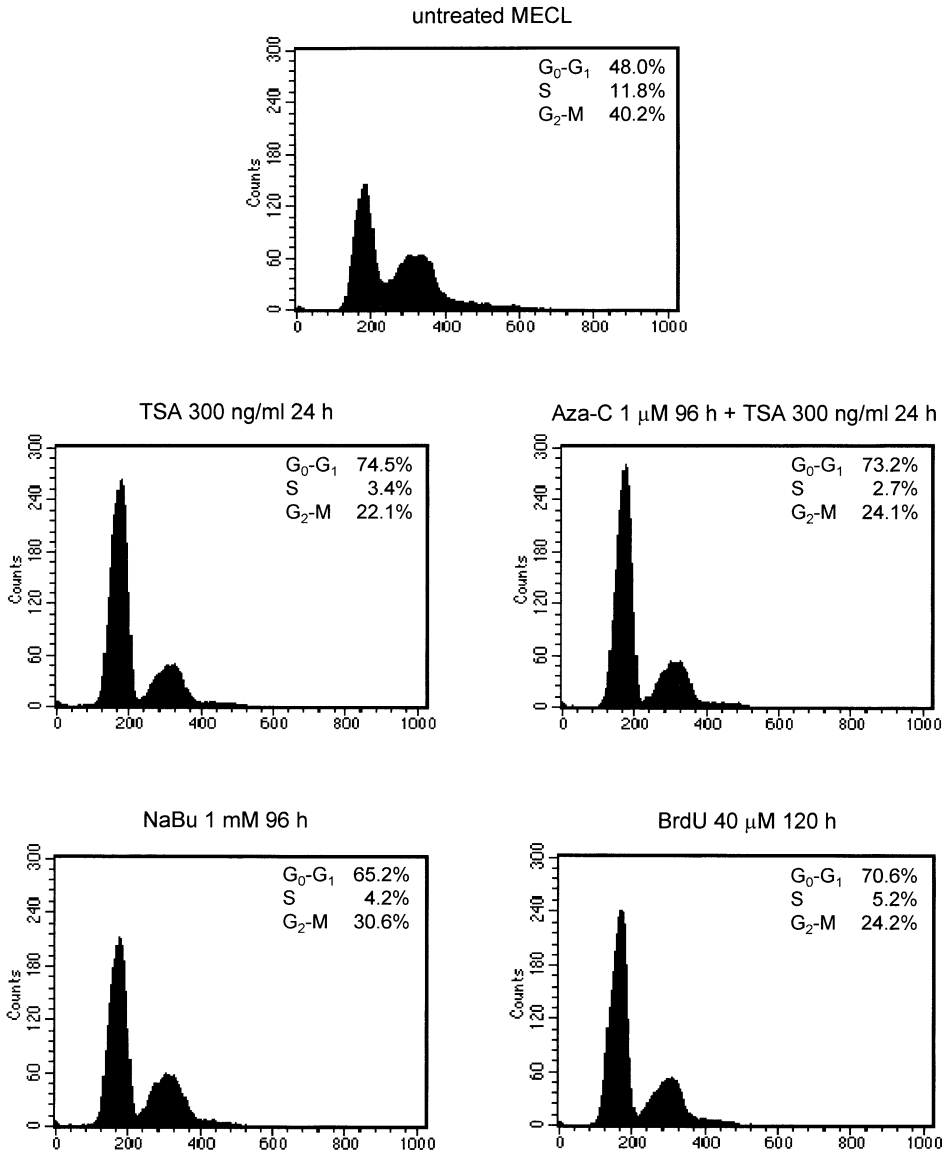

FIG. 4. Effect of various treatments on cell cycle. Compared with untreated MECL, cells treated with different agents for the indicated time display similar changes in cell-cycle profiles, with a prominent G_1/G_0 arrest and reduction of cells in S and G_2/M phases. The proportion of cells in each phase is indicated.

MECL and BFF was observed, whereas NaBu treatment of two durations had no obvious effect on histone H3 or H4 acetylation.

In Vitro Development of Early Nuclear Transfer Embryos

After various treatments, we performed nuclear transfer experiments using untreated and treated immortal and primary cells as nuclear donors. Results are summarized in Table 2. In the groups of MECL cells treated with Aza-C+TSA, TSA, and BrdU, reconstructed embryos started to degenerate as early as at two-cell stage. In contrast, the early stage of nuclear transfer embryos derived from NaBu-treated cells was morphologically healthy, showing signs of degeneration not earlier than at 16-cell stage. Some embryos developed to the morula stage but not further. As shown earlier in this study, no further improvement of development was achieved with other concentrations or durations of NaBu treatment. As a control, we also treated BFF with Aza-C, TSA, or NaBu and used those cells for nuclear transfer. TSA had no effect on the embryonic development. The developmental ability was significantly decreased when using the combination of Aza-C and TSA. Interestingly, a much higher proportion of nuclear transfer embryos derived from BFF treated with NaBu developed to blastocyst stage as compared with those derived from untreated BFF (59% versus 26%; $P < 0.05$).

DISCUSSION

Successful cloning depends mainly on the intact genetic information of the donor nuclei; even cell viability is not an absolute requirement [20, 21]. Several lines of evidence indicate that somatic cells with senescent phenotype could serve as donor cells in nuclear transfer. Cells at a quiescent

TABLE 1. Chromosome analysis of nontreated and treated bovine immortal mammary cells.

	Chromosome number		
Treatment*	60 (%)	Abnormal (%)	Spreads counted
Nontreated	71 (84)	14 (16)	85
TSA	64 (85)	11 (15)	75
Aza-C+TSA	47 (78)	13 (22)	60
NaBu	65 (81)	15 (19)	80
BrdU	56 (80)	14 (20)	70

* Aza-C+TSA, Aza-C, 1 μM for 96 h and TSA 300 ng/ml for 24 h; TSA, 300 ng/ml for 24 h; NaBu, 1 mM for 96 h; BrdU, 40 μM for 120 h.

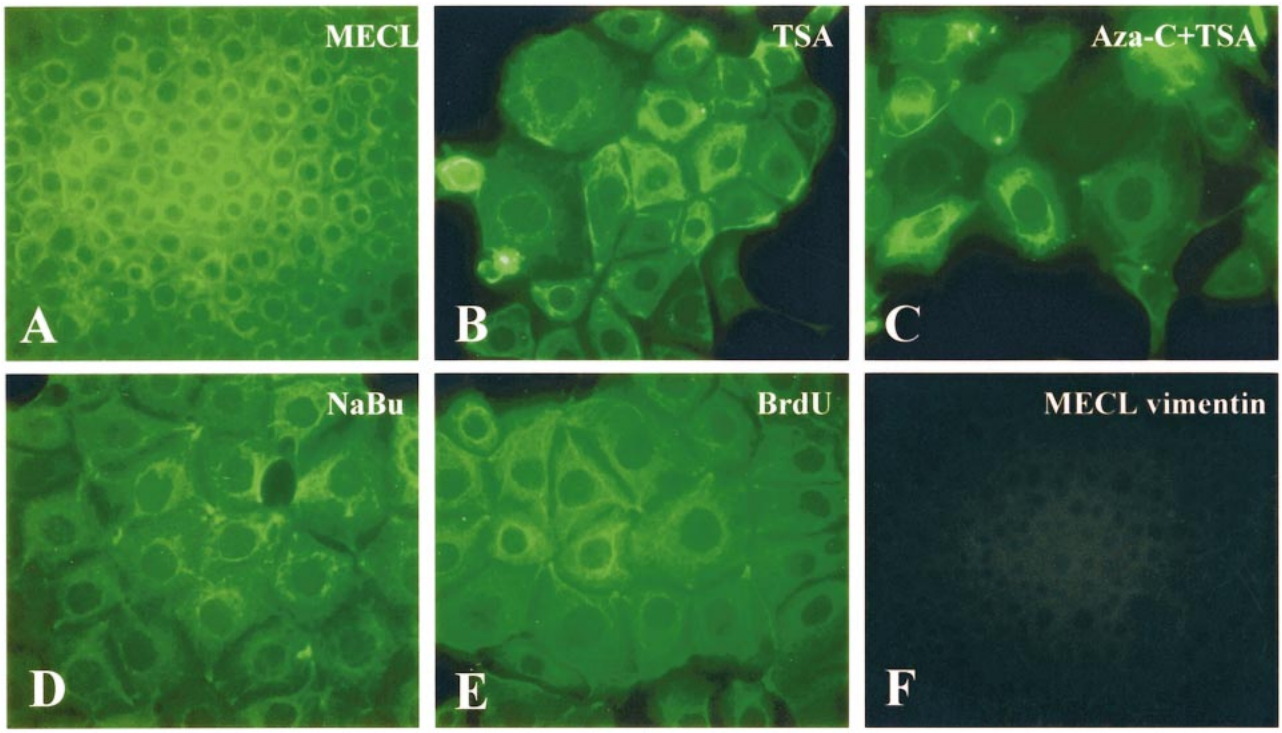

FIG. 5. Immunofluorescent staining of cytokeratin in untreated and treated MECL cells. **A**–**E**) Expression of cytokeratin in MECL cells following different treatments. **F**) MECL cells are not stained by antibody specific for vimentin. Photographs are taken at the same magnification (400×).

stage (G_0) induced by serum starvation for 5 days up to 2 wk are commonly used as nuclear donors, and nuclear transfer embryos derived from such cells even exhibit improved development to the blastocyst stage [9, 22, 23]. Cells that entered replicative senescence featured by expressing markers of senescence, such as early population doubling level complementary DNA-1 and senescent morphology, have been used successfully to produce offspring following cloning [6]. In the present study, we investigated whether induction of a senescent phenotype in immortalized bovine MECL cells improves their developmental potential after nuclear transfer.

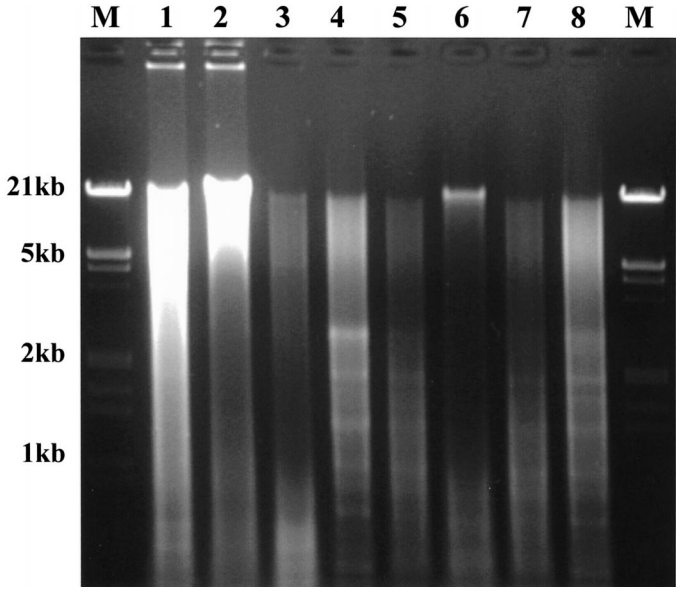

FIG. 6. DNA methylation analysis in MECL and BFF by *Msp*I and *Hpa*II digestion. Lane 1, untreated MECL digested with *Msp*I; 2, untreated MECL digested with *Hpa*II; 3, Aza-C treated MECL digested with *Msp*I; 4, Aza-C treated MECL digested with *Hpa*II; 5, untreated BFF digested with *Msp*I; 6, untreated BFF digested with *Hpa*II; 7, Aza-C treated BFF digested with *Msp*I; 8, Aza-C treated BFF digested with *Hpa*II. M, Molecular weight marker.

Senescence is a complex and heterogeneous process that can be induced by telomere dysfunction, introduction of oncogenes, DNA damage agents, oxidative stress, or epigenetic alterations [24]. Replicative senescence in cultured somatic cells is thought to depend on a cell division-counting mechanism or cell cycle checkpoint response to inappropriate culture conditions [25, 26]. Although senescence mainly represents a genetically governed process, epigenetic regulation as an alternative mechanism for mediating gene expression in cells has attracted increasing attention. DNA methylation generally declines with cellular senescence [27] and immortal cell lines usually maintain a constant level of DNA methylation [28]. The loss of cytosine methylation or aberrant de novo methylation has been proposed to be an alternative counting mechanism for cellular aging [29]. Increased methylation is required for initiation of cell transformation such as immortalization [30]. Treatment with demethylating agents led to G_1 cell cycle arrest, increased expression of a senescence-associated marker, and cyclin-dependent kinases in hepatocellular or oral carcinoma cell lines [31, 32].

Histone acetylation is an important regulator of transcription in eukaryotic cells. In general, histone acetylation is associated with transcriptionally active chromatin, whereas histone deacetylation is related to transcription repression. There is a considerable decrease in the levels of HDAC1 or HDAC2 during replicative senescence in human fibroblasts [33]. Normal human fibroblasts treated with HDAC inhibitors displayed a phenotype characteristic of senescent cells and a reduction in proliferative life span [10]. NaBu and TSA are two common HDAC inhibitors that can lead to the accumulation of highly acetylated his-

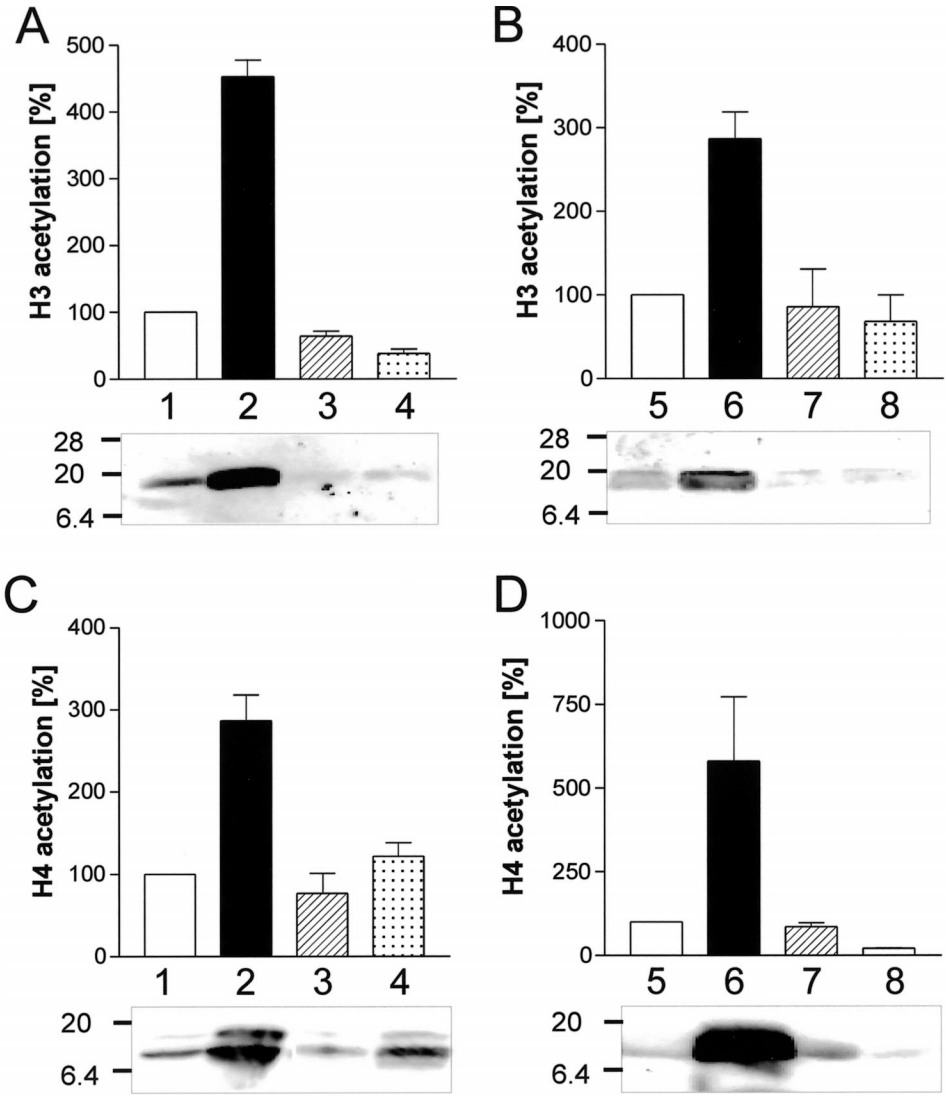

FIG. 7. Global histone H3 and H4 acetylation status in untreated and treated MECL (**A** and **C**) and BFF (**B** and **D**). Treatment with 300 ng/ml TSA for 24 h (lanes 2 and 6), but not with 1 mM NaBu for 96 h (lanes 3 and 7) or 48 h (lanes 4 and 8), increases H3 and H4 acetylation up to 6-fold if compared with untreated cells (lanes 1 and 5). Data are expressed in relation to untreated controls and show means from two (H3) or three (H4) experiments (the error bars are SEM). The inserts display representative sections from respective Western immunoblots. The molecular weight markers are indicated in kilodaltons.

tones. Both agents are inducers of growth arrest, cell death [11, 34], and differentiation [35, 36] in a variety of immortal and tumor cell lines. Demethylation agents and HDAC inhibitors act synergistically to reactivate genes silenced in cancer [12]. Recent studies also show that the regulation of higher-order chromatin structures by DNA methylation and histone modification is crucial for reprogramming genome during early embryogenesis [37]. BrdU incorporates into DNA to substitute thymidine and changes the interaction between DNA and DNA-binding proteins, which might change the chromatin configuration [38]. It has been shown that BrdU affects immortalization pathways and induces a senescent-like phenotype in any type of immortal cells [13].

TABLE 2. In vitro development of nuclear transfer embryos derived from nontreated and treated bovine immortal mammary cells and primary fetal fibroblasts.*

Treatment	Transferred	Fused† (%)	Cleaved (%)	12–20 cells (%)	Blastocyst (%)
MECL cells					
Nontreated	120	101 (84)[a]	61 (60)[a]	1 (2)[a]	0[a]
TSA	94	84 (89)[ab]	66 (77)[b]	2 (3)[a]	0[a]
Aza-C+TSA	211	181 (86)[a]	115 (64)[a]	7 (4)[a]	0[a]
NaBu	132	124 (94)[b]	96 (77)[b]	23 (19)[b]	9 (7.3)[b‡]
BrdU	135	123 (91)[ab]	65 (53)[a]	2 (1.6)[a]	0[a]
BFF cells					
Nontreated	108	105 (97)[a]	67 (64)[a]	n.d.	27 (26)[a]
TSA	105	99 (94)[a]	76 (77)[a]	n.d.	28 (28)[a]
Aza-C+TSA	95	93 (98)[a]	43 (46)[b]	n.d.	7 (7.5)[b]
NaBu	84	82 (98)[a]	79 (96)[c]	n.d.	48 (59)[c]

* Within each cell type, values with different superscripts (a,b,c) differ significantly (chi-square, $P < 0.05$). n.d., Not determined.
† Fusion rates are calculated based on the numbers of reconstructed embryos. Other rates are calculated based on the numbers of fused embryos.
‡ The proportion of morulae.

In our study, after various treatments, MECL cells exhibited a senescent-like phenotype, such as reduced growth ability, a significantly increased proportion of cells with enlarged cytoplasm and SA-βgal activity, and the arrest of cells at the G_1/S cell cycle boundary. These phenotypes are the features of cellular senescence [39, 40]. Our treatments did not induce detectable apoptosis, obvious changes of cellular ploidy, and the expression of the cell type-specific marker cytokeratin. However, MECL cells treated with Aza-C+TSA, TSA alone, or BrdU, like untreated cells, failed to support the development of cloned embryos. Of particular interest is that the nuclear transfer embryos derived from NaBu-treated MECL cells could develop further to the morula stage, but all the morulae underwent degeneration and no blastocyst was obtained. Although all the treatments in this study could convert MECL to a state resembling senescence, the molecular pathways involved in these responses are largely unknown. We do not know whether the senescent phenotype induced by any of these treatments was the same as replicative senescence except decreased cell proliferation, cell cycle arrest, morphological changes, and expression of SA-βgal. One intriguing finding in our study is that treatment of primary fetal fibroblasts BFF with NaBu resulted in a more than two-fold increase in the rate of developing to blastocyst, compared with that of untreated BFF. However, TSA had no positive effect on the development of cloned embryos.

Surprisingly, although both TSA and NaBu are HDAC inhibitors, our results showed that TSA induced significant hyperacetylation of histone H3 and H4 in both immortal cells and primary fibroblasts, whereas NaBu had no obvious effect on global histone H3 and H4 acetylation. The differential effect of TSA and NaBu has been found also in other cell lines, and different cell types can differ markedly in the extent of histone hyperacetylation induced by HDAC inhibitors [41]. TSA, a hydroxamic acid, is a more potent HDAC inhibitor [42]. TSA binds directly to the catalytic site of HDAC and is more specific than NaBu in inhibiting HDAC activity. Butyrate belongs to short-chain fatty acids derived from bacterial metabolism of dietary fiber in the colon. Butyrate is important for proper epithelial cell regulation but was also found to have antiproliferation and differentiation-inducing activity on various human neoplastic cells and some primary cells such as epithelial cells, hepatocytes, and liver cells [43–45]. In distinct cellular systems, NaBu has inhibitory effects not only on histone deacetylation but also on histone phosphorylation and DNA hypermethylation [46, 47]. These observations suggest that rather than histone acetylation, other functions of NaBu might explain its beneficial effect on embryonic development. However, the lack of a clear effect of NaBu on global histone acetylation does not exclude more subtle changes in the acetylation status of histones in specific chromatin regions, which deserves further investigation.

When MECL cells were treated with Aza-C or TSA, the cell number significantly decreased from Day 4 or Day 2, respectively, indicating a toxic effect of these two agents. Although we chose the time of treatment for nuclear transfer experiment just before the cells died and cells still showed a relatively normal morphology, toxic effect cannot be excluded. With the combined treatment Aza-C+TSA in BFF, the developmental potential was actually decreased. In another study, the reduction in blastocyst rate obtained after nuclear transfer with Aza-C-treated fetal fibroblasts was also observed, although a considerable demethylation was induced after Aza-C treatment [14]. Our results indicate that a marked DNA demethylation induced by Aza-C and histone hyperacetylation induced by TSA treatment of donor cells are not beneficial for development after nuclear transfer. The improved development of nuclear transfer embryos derived from NaBu-treated primary fetal fibroblast is quite interesting and merits further investigation.

The analysis of various aspects and types of immortalization and senescence has turned out to be very informative regarding numerous in vivo processes, and particularly to carcinogenesis, given that the acquisition of unlimited proliferative potential is probably a critical step for the development of cancerous lesions [48]. The developmental failure of immortal cells after nuclear transfer may be due to the mutations or deregulation of genes that are essential for the early embryonic development that occurred during the course of immortalization. Because different types of immortal or tumor cells have acquired their abnormal status by distinct and multiple molecular mechanisms, it would be worthwhile to extend this study to other cell types or trigger senescence by other stimuli. Nuclear transfer provides a unique experimental system to study reprogramming mechanisms that may finally revert immortal or tumor cells to normal development.

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