Rabbit somatic cell cloning: effects of donor cell type, histone acetylation status and chimeric embryo complementation

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Abstract

The epigenetic status of a donor nucleus has an important effect on the developmental potential of embryos produced by somatic cell nuclear transfer (SCNT). In this study, we transferred cultured rabbit cumulus cells (RCC) and fetal fibroblasts (RFF) from genetically marked rabbits (Alicia/Basilea) into metaphase II oocytes and analyzed the levels of histone H3-lysine 9-lysine 14 acetylation (acH3K9/14) in donor cells and cloned embryos. We also assessed the correlation between the histone acetylation status of donor cells and cloned embryos and their developmental potential. To test whether alteration of the histone acetylation status affects development of cloned embryos, we treated donor cells with sodium butyrate (NaBu), a histone deacetylase inhibitor. Further, we tried to improve cloning efficiency by chimeric complementation of cloned embryos with blastomeres from *in vivo* fertilized or parthenogenetic embryos. The levels of acH3K9/14 were higher in RCCs than in RFFs ($P < 0.05$). Although the type of donor cells did not affect development to blastocyst, after transfer into recipients, RCC cloned embryos induced a higher initial pregnancy rate as compared to RFF cloned embryos (40 vs 20%). However, almost all pregnancies with either type of cloned embryos were lost by the middle of gestation and only one fully developed, live RCC-derived rabbit was obtained. Treatment of RFFs with NaBu significantly increased the level of acH3K9/14 and the proportion of nuclear transfer embryos developing to blastocyst (49 vs 33% with non-treated RFF, $P < 0.05$). The distribution of acH3K9/14 in either group of cloned embryos did not resemble that in *in vivo* fertilized embryos suggesting that reprogramming of this epigenetic mark is aberrant in cloned rabbit embryos and cannot be corrected by treatment of donor cells with NaBu. Aggregation of embryos cloned from NaBu-treated RFFs with blastomeres from *in vivo* derived embryos improved development to blastocyst, but no cloned offspring were obtained. Two live cloned rabbits were produced from this donor cell type only after aggregation of cloned embryos with a parthenogenetic blastomere. Our study demonstrates that the levels of histone acetylation in donor cells and cloned embryos correlate with their developmental potential and may be a useful epigenetic mark to predict efficiency of SCNT in rabbits.

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Introduction

In view of the major relevance of genetically modified rabbits as animal models of human diseases and as small, but fast bioreactors for the production of therapeutic proteins, SCNT from genetically engineered donor cells would be of outstanding importance in this species. However, low efficiency of SCNT in all species studied so far greatly hindered its application in biomedicine and agriculture. As one of the key factors, the type of nuclear donor cell, which is characterized by origin and extent of differentiation, has a significant effect on the efficiency of nuclear transfer and the development of reconstructed embryos. Among the somatic cell types tested for nuclear transfer, cumulus cells appear to be the best choice with the highest

cloning efficiency and the least proportion of abnormalities in cloned animals (Tian *et al.* 2003). Whereas, diverse animal species have been cloned from different types of cultured donor cells (Wilmut *et al.* 1997, Kato *et al.* 1998, Baguisi *et al.* 1999, Polejaeva *et al.* 2000, Wakayama & Yahagimachi 2001, Galli *et al.* 2003, Shin *et al.* 2002, Woods *et al.* 2003, Zhou *et al.* 2003, Lee *et al.* 2005), in rabbits, first live cloned offspring could be produced only from freshly prepared cumulus or oviductal cells (Chesne *et al.* 2002, Inoue *et al.* 2002, Challah-Jacques *et al.* 2003), and cloning from cultured cells fibroblasts resulted only in implantation or early fetal development (Yin *et al.* 2000). However, for the purpose of generating transgenic rabbits by SCNT, donor cells must be cultured for transfection and selection to facilitate the required genetic modification. Rabbit

cumulus cells are relatively difficult to maintain in long-term culture (Dinnyes *et al.* 2001), and thus stable transfected cell clones cannot be readily established. Fetal fibroblasts, which grow rapidly and have the potential for many cell divisions in culture (Schnieke *et al.* 1997, Cibelli *et al.* 1998, McCreath *et al.* 2000), have been widely used for transgenesis via SCNT, including gene targeting (Denning & Priddle 2003, Kuroiwa *et al.* 2004).

Only recently, Li *et al.* (2006) reported production of cloned rabbits from cultured adult fibroblasts using a 'novel' SCNT protocol involving minor modifications of the previously established SCNT technique (Chesne *et al.* 2002). However, from a total 14 born cloned rabbits 9 died shortly after birth. Because of a predominantly stochastic nature of nuclear reprogramming, it is more likely that even with improved SCNT technique clones may not be free of epigenetic errors.

There is increasing evidence that successful reprogramming of a donor nucleus after transfer into a recipient cytoplasm is largely dependent on its epigenetic state and can be influenced by *in vitro* culture conditions, including passage number, serum concentration, cell density, and physical/chemical treatment (Enright *et al.* 2003a, 2003b, Shi *et al.* 2003a). Major epigenetic characteristics of donor cells are DNA methylation and histone modifications (Shi *et al.* 2003b). Previous studies have linked developmental defects in cloned embryos to aberrant epigenetic reprogramming during early development (Dean *et al.* 2001, Kang *et al.* 2001, 2003) and proposed epigenetic modifications to the chromatin of donor cells, DNA methylation and histone H3K9 methylation, as reasonable marks of nuclear reprogramming, which correlate with developmental potential of cloned embryos (Santos *et al.* 2003). Another epigenetic modification, histone acetylation, which is closely associated with DNA and histone methylation, is involved in diverse cellular functions and processes, and may function as an epigenetic mark, a histone code, by which information about genomic function is transmitted from one generation of cells to the next (Turner 2000). Acetylated form of the histone H3-lysine 9-lysine 14 (acH3K9/14) is associated with active (euchromatin and facultative heterochromatin) chromatin configuration (Rice & Allis 2001). Staining with an antibody to acH3K9/14 can be used as a measure of euchromatic characteristics in the early embryo. Following nuclear transfer (NT), the histone acetylation status of the donor nucleus, which must be reprogrammed to an embryonic one, is affected by a number of factors and can be experimentally manipulated. For example, histone acetylation levels in bovine cumulus and fibroblast cells were shown to be affected by the stage of the cell cycle, cell origin, and cell passage numbers (Enright *et al.* 2003a). Moreover, global histone acetylation in donor cells could be increased by treatment with histone deacetylase (HDAC) inhibitor, trichostatin A

(Enright *et al.* 2003b). Treatment of donor cells with chromatin modifying agents may improve their ability to be reprogrammed by a recipient cytoplasm. Kishigami *et al.* (2006) demonstrated that trichostatin treatment leads to more than fivefold increase in success rate of mouse cloning from cumulus cells without obvious abnormalities. Previously, we demonstrated that treatment of bovine fetal fibroblasts with sodium butyrate (NaBu), another HDAC inhibitor, resulted in a more than twofold increase in the rate of cloned blastocysts compared with that of untreated cells (Shi *et al.* 2003a). Little is known as to epigenetic reprogramming in rabbits. The absence of demethylation during early development correlated with low rabbit cloning efficiency (Shi *et al.* 2004). The histone acetylation status of rabbit somatic cells, which may also contribute to a low cloning efficiency in this species, has not been studied so far.

A potential approach to improve the developmental competence of cloned embryos is aggregation with non-cloned embryonic cells. There is evidence for metabolic cooperation between cell types of different genetic origin through permeable cell junctions that enable metabolically deficient cells to function in a normal manner (Pitts & Burk 1976). Developing intercellular junctions between blastomeres of different origin might play a role in communication and subsequently enhance development of the chimeric embryos (Ducibella & Anderson 1975).

In the present study, we investigated the levels of acH3K9/14 in two types of donor cells, cumulus cells (RCC) and fetal fibroblasts (RFF), and the dynamic of acH3K9/14 immunofluorescence distribution in the corresponding cloned embryos. We assessed whether the histone acetylation status of donor cells and cloned embryos correlates with their developmental potential. We also tested the effects of modification of acH3K9/14 levels in donor cells using treatment with an inhibitor of HDACs and tried to improve *in vivo* development of cloned embryos by their aggregation with blastomeres of *in vivo* fertilized or parthenogenetic embryos.

Materials and Methods

Animal experiments were approved by the Ethical Committee for Animal Experimentation of the University of Munich and were performed in accordance with the European Union Normative for Care and Use of Experimental Animals.

Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Co.

Recipient oocyte collection

Oocytes were obtained from sexually mature out bred Zika rabbits (approximately 3.0 kg and more than 6 months old). All experiments were carried out during the

natural breeding season. Female rabbits were superovulated by injection of 100 IU pregnant mares serum gonadotrophin (Intergonan, Intervet, Unterschleissheim, Germany) intramuscularly and 100 IU human chorionic gonadotrophin (hCG; Ovogest, Intervet) intravenously 72 h later. Mature oocytes were flushed from the oviducts 15–16 h post-hCG injection in warm phosphate buffered saline (PBS) supplemented with 4 mg bovine serum albumin ml⁻¹ (BSA). Cumulus cells were removed by gentle pipetting with a small-bore pipette after treatment of oocytes with 5 mg/ml hyaluronidase in M199 (Medium 199 supplemented with 10% fetal calf serum (FCS) for 15 min at 38.5 °C.

Induction of metaphase II protrusion and enucleation

Denuded oocytes were treated with 0.6 µg demecolcine ml⁻¹ between 40 min and 2 h (Yin *et al.* 2002). The resulting metaphase II protrusion with little underlying cytoplasm was removed in M199 supplemented with 7.5 µg cytochalasin B ml⁻¹ (CB) and 0.6 µg demecolcine ml⁻¹ using an enucleation pipette. Enucleated oocytes were kept in M199 and later used as recipient cytoplasts.

Preparation of somatic donor cells

Heterozygous Alicia (Ali)/Basilea (Bas) rabbits, donated by Therapeutic Human Polyclonals, Inc., (Sunnyvale, CA, USA) were used as donors of somatic cells. Ali is a rabbit strain, which has a variant of the allotype allele a2 associated with the H chain of immunoglobulin molecules. Bas is another rabbit strain with a mutant gene at the Ab allotypic locus controlling the synthesis of allotypic specificities (Ab4, Ab5, Ab6, Ab9, Ab4v, and Ab95) of κ-immunoglobulin light chains (Garcia *et al.* 1982, Kelus & Weiss 1986).

Rabbit cumulus cells (RCC)

In vivo matured oocytes (Ali/Bas) were collected from superovulated donors. Cumulus cells were recovered from cumulus-oocyte complexes after hyaluronidase treatment, pooled, and cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% (v/v) FCS, 2 mmol/l pyruvic acid, 2 mmol/l L-glutamine, 0.1 mmol/l 2-mercaptoethanol, 2 mmol/l non-essential amino acids, 100 IU penicillin ml⁻¹, and 100 µg streptomycin ml⁻¹ in a humidified atmosphere of 5% CO₂ in air at 38.5 °C. Confluent cells at passages 1–5 were used as nuclear donors.

Rabbit fetal fibroblasts (RFF)

Fetal fibroblasts were established from an individual Ali/Bas female fetus 15 to 16 days post-coitum and cultured in DMEM. Confluent fetal fibroblasts from passages 1 to 8 were either directly used as donors for NT (RFF) or split as 1:4 of their confluent density and

then treated with 1 mmol/l NaBu prepared in cell culture medium for 72 h (RFF-NaBu cells). After 3 days of culture, RFF-NaBu cells reached 80–90% confluence and were larger than non-treated RFFs.

Prior to nuclear transfer, attached cells were trypsinized, and suspended in M199 after three times wash.

Detection of histone acetylation levels in donor cells

Confluent RCCs at passage 3, and RFFs and RFF-NaBu cells at passage 5 were used to evaluate their histone acetylation levels. Proteins were prepared by acid extraction. Adherent cells were washed three times with ice-cold PBS, scraped from the culture dish and collected by centrifugation at 1200 g for 10 min. The washed cells were suspended in 1 ml of ice-cold lysis buffer (10 mmol/l Tris-HCl, 50 mmol/l sodium bisulfite, 1% Triton X-100, 10 mmol/l MgCl₂, 8.6% sucrose, pH 6.5) and homogenized by passing them 20 times through a 20-gauge needle. The nuclei were collected by centrifugation at 1200 g for 10 min, washed three times with lysis buffer, and once with TE (Tris-HCl/EDTA) buffer (10 mmol/l Tris-HCl, 13 mmol/l EDTA, pH 7.4). The pellet was suspended in 0.1 ml of ice-cold autoclaved H₂O using a Vortex mixer, and concentrated H₂SO₄ was added to the suspension to a final concentration of 0.2 N, followed by incubation at 4 °C overnight. After centrifugation for 5 min at 1200 g, the supernatant was mixed well with 1 ml of acetone in a new Eppendorf tube, and then incubated at –20 °C overnight. Coagulated material was collected by centrifugation for 5 min at highest speed and the pellet was allowed to air dry. The acid soluble histone fraction was dissolved in 50 µl of autoclaved H₂O. Protein was quantified using Bradford Reagent, and Varian u.v.–visible spectrometer (Cary WinUV 50Bio, Varian, Darmstadt, Germany) was used to measure the absorbance at 595 nm.

Ten micrograms of protein were loaded on 15% SDS-polyacrylamide gels. Proteins were blotted onto PVDF membrane (Millipore, Eschborn, Germany). Histone acetylation was investigated by Western immunoblot analysis. Primary antibodies were goat-anti-acetylated histone H3 (Lys 9/14) (sc-8655; Santa Cruz Biotechnology, Heidelberg, Germany; dilution 1:350). Bound antibodies were detected by using donkey anti-goat IgG-AP (sc-2310; Santa Cruz Biotechnology; dilution 1:500). Detection of bound secondary antibodies was performed by developing the membrane with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) blue liquid substrate membrane detection solution in a dark room. As control, cells were incubated with primary or secondary antibodies alone.

Nuclear transfer, fusion and activation

Transfer of donor karyoplast was carried out essentially as described previously (Zakhartchenko *et al.* 1999).

Briefly, an individual nuclear donor cell was introduced under the zona pellucida of enucleated oocyte in M199. Karyoplast–cytoplasm complexes (KCC) were manually aligned in a fusion chamber consisting of two wire electrodes 200 μm apart, overlaid with Eppendorf fusion medium and then fused with an Eppendorf Multiporator (Hamburg, Germany) using double direct current of 1.95 kV/cm, 25 μs . After 20–40 min incubation in M199, fused KCC were activated by the same electric pulses as for fusion, then immediately incubated for 1 h in 1.9 mmol/l 6-dimethylaminopurine and 5 μg CB ml^{-1} prepared in Menezo B2 medium (INRA, Paris, France) containing 10% FCS in a humidified atmosphere of 5% CO_2 in air at 38.5 °C.

Detection of acH3K9/14 immunofluorescence in *in vivo* fertilized and cloned embryos

In vivo fertilized and cloned embryos, which were freed of zona pellucida at desired stages, were washed three times in PBS, fixed for 15 min in 4% paraformaldehyde in PBS, and permeabilized with 0.2% Triton X-100 in PBS for 30 min at room temperature. After washing with 0.1% Triton X-100 and 0.1% Tween-20 in PBS, embryos were blocked overnight at 4 °C in 1% BSA and 0.05% Tween-20 in PBS (blocking solution), then incubated with goat-anti-acetylated histone H3 (Lys 9/14) antibody in fresh blocking buffer overnight at 4 °C. After thorough wash in fresh blocking buffer, embryos were incubated with donkey anti-goat IgG-AP antibody in fresh blocking buffer for 1 h at room temperature. Embryos were washed again and transferred in minimum volume of blocking solution into 6 μl Vectashield mounting medium with 4', 6-diamino-2-phenylindole (DAPI) (Vector laboratories, Grünberg, Germany). Observations were performed with a fluorescent microscope (Axiovert 200M; Zeiss, Hallbergmoos, Germany). Images were recorded digitally with a high-resolution CCD camera under identical exposure time. Six to fifteen embryos were analyzed per each stage of development. As control, embryos were incubated with primary or secondary antibodies alone. Both normal and cloned embryos as well as all embryos at different stages were stained simultaneously to reduce experimental variability.

Embryo culture and transfer

After activation, reconstructed embryos were cultured overnight in B2 medium at 38.5 °C and 5% CO_2 in air. Using the laparoscopic technique described by Besenfelder & Brem (1993), 4- to 10-cell stage cloned embryos were transferred through the infundibulum into each oviduct of recipient females which were induced into pseudopregnancy status either by mating to vasectomized males or injection of 80 IU hCG at 20–22 h after injecting the females used as oocyte donors. Pregnancy was

determined by palpation around 2 weeks after embryo transfer. To assess the developmental potential to blastocyst, cloned embryos were cultured in B2 medium for up to 6 days. As control for embryo transfer technique, 4- to 10-cell stage *in vivo* fertilized embryos were transferred into recipients and either flushed on day 5 after transfer or left to determine pregnancy and offspring rates.

Chimeric embryo complementation

To improve *in vivo* development of embryos cloned from NaBu-treated cells, we aggregated them with blastomeres of *in vivo* fertilized or parthenogenetic embryos.

Superovulated Zika females were mated with fertile Zika males followed by i.v. injection with 100 IU hCG. *In vivo* fertilized embryos were collected by flushing oviducts with mPBS 16 h after hCG injection, and then cultured in B2 medium. To prepare parthenogenetic embryos, cumulus-free oocytes were activated and cultured under the same conditions as used for nuclear transfer embryos.

Six- to 12-cell *in vivo* fertilized or parthenogenetic embryos were treated with 0.5% pronase in mPBS for seconds to remove the zona pellucida, and then incubated in 0.25% trypsin–0.01% EDTA in mPBS for 2 min to separate individual blastomeres. A single or two blastomeres were introduced under the zona pellucida of 4- to 10-cell stage cloned embryos in M199 containing 7.5 μg CB ml^{-1} . After thorough washing, first in M199 and then in B2 medium, aggregation embryos were cultured in B2 medium either till embryo transfer within 1 h or up to 6 days for the assessment of development to blastocyst.

Ali/Bas genotyping of fetuses and offspring

Genomic DNA from nuclear donor cells as well as tissues from recipient animals, fetuses and offspring was extracted by using the QIAamp DNA Mini Kit (Qiagen). Due to the fact that the used wild-type rabbit strains belong to the e15 allotype (accession no. K00752), whereas Ali/Bas rabbits belong to the e14 allotype (Accession no. J00665), it was possible to unequivocally identify fetuses and offspring originating from SCNT. For determination of the 1-bp difference between e14 and e15 allotypes, which maps in the CH2 domain of the IgH constant region, a specialized SNP genotyping kit (Genespector) was developed by Variom (Berlin, Germany). The principle of this oligonucleotide ligation assay is based on the mismatch sensitivity of T4 DNA ligase. In brief, two oligonucleotides specific for the e14 or e15 allele were coated to different wells of a 96-well microtiter plate. A biotinylated signal probe together with a PCR product comprising the e14/15 mismatch were added to the wells. The signal probe was linked to the coated oligonucleotide by T4 DNA ligase if the

corresponding allotype was present in the PCR product. Visualization of the results was possible by addition of a streptavidin-peroxidase conjugate, detecting the linkage of the signal probe and catalyzing the processing of a substrate.

Statistical analysis

The proportions of embryos at each developmental stage were compared by χ^2 analysis. Statistical differences for histone acetylation in donor nuclei were determined by using Student–Newman–Keuls' test. $P < 0.05$ was considered as significant.

Results

Histone acetylation status of donor cells prior to nuclear transfer

Western blotting showed that the levels of acH3K9/14 were higher in RCCs than in RFFs ($P < 0.05$; Fig. 1A

and B). Compared to RCCs and non-treated RFFs, NaBu treatment significantly ($P < 0.01$) increased the level of acH3K9/14 in RFFs. Similar to the results obtained from Western blotting of donor cells, acetylation levels of histone H3K9/14 detected by immunostaining of reconstructed oocytes before artificial activation were higher in the nuclei of NaBu-treated RFFs (Fig. 1C). Fluorescence was not detected in control cells incubated with primary or secondary antibody alone (data not shown).

Development of cloned embryos

The differences in histone acetylation levels between RCC, RFF and RFF-NaBu donor cells suggest that the corresponding cloned embryos may also differ in their developmental potential. Although the cleavage rate of cloned embryos in the RCC group was lower ($P < 0.05$) than that in RFF group, their development to blastocyst was similar (Table 1). Treatment of RFFs with NaBu

Figure 1 Histone H3K9/14 acetylation status of donor cells. (A) Relative levels of H3K9/14 acetylation in various types of cells prior to NT; RCC, cumulus cells; RFF, fetal fibroblasts; RFF-NaBu, fetal fibroblasts treated with NaBu. Data are expressed in relation to the RFF group. The diagram shows means and standard deviations (S.D.) from three independent experiments. Significant differences between donor cell types are indicated by different superscripts: a:b, $P < 0.01$; a:c, b:c, $P < 0.05$. (B) Representative Western blot; 10 μ g protein was loaded per lane; M, molecular weight marker. (C) Immunostaining of donor cells transferred into non-activated oocytes (1–1.5 h after transfer). Cells were immunostained with anti acH3K9/14 (green) and stained with DAPI for chromatin (blue). Images are at magnification $\times 400$.

markedly ($P < 0.05$) improved the development of cloned embryos to blastocyst. Transfer of cloned embryos into recipients resulted in pregnancies in all groups. However, no recipients receiving RFF- or RFF-NaBu-derived embryos maintained pregnancy to term. From two pregnant recipients receiving RCC-derived embryos, one was euthanized on day 16 after embryo transfer and four implantation sites were found. From the other recipient, which suffered from a disease, one live pup was recovered by caesarean section on day 27, and two additional concepti in resorption were found. The prematurely recovered pup died within 3 h. Both the live pup and two concepti were identified to be genetically identical to nuclear donors.

Development of *in vivo* fertilized embryos

After transfer of 77 control *in vivo* fertilized embryos into four recipients, one recipient was euthanized on day 5 and 11 from 30 transferred embryos were flushed (37%). Out of these 11 embryos, six (55%) were growing blastocysts with the size from 750 to 2000 μm . These findings show that about 20% (6/30) of the transferred embryos were viable and could potentially develop further. Two of three recipients became pregnant and gave birth to nine live pups (19%, 9/47).

Histone acetylation status of *in vivo* fertilized and cloned embryos

For successful development of cloned embryos, epigenetic status of donor cells must be reprogrammed to that of normal embryos. However, so far, it was unknown whether histone acetylation status of cloned rabbit embryos resembles that of normal embryos. Neither RCC-derived cloned embryos nor embryos cloned from RFF or RFF-NaBu cells were similar to normal embryos in respect to histone acetylation status. *In vivo* fertilized embryos became hypoacetylated at the 2- and 8-cell stages, with increases at the 4-cell stage and after 16-cell stage (Fig. 2). At the morula and blastocyst stages *in vivo* fertilized embryos were hyperacetylated. RCC-derived

cloned embryos displayed low levels of acH3K9/14 at the 1- and 2-cell stages, with increases to moderate levels at the 4- to 16-cell stages and to high levels at the morula and blastocyst stages.

Embryos derived from RFFs were hyperacetylated at all stages except for the 4- and 8-cell stages with moderate and remarkably low acetylation levels respectively. In embryos cloned from RFF-NaBu cells histone acetylation signals were high at the 1- to 4-cell stages, almost undetectable at the 8-cell stage, with increases from low at the 16-cell stage to moderate and high at the morula and blastocyst stages respectively.

Fluorescence was not detected in control embryos incubated with primary or secondary antibody alone (data not shown).

Effect of embryo aggregation

Significantly higher ($P < 0.05$) blastocyst rates were obtained when embryos cloned from RFF-NaBu cells were aggregated with either one (cloned-1FB) or two (cloned-2FB) blastomeres of *in vivo* fertilized embryos as compared with non-aggregated cloned embryos (Table 2). One of six recipients receiving 191 cloned-1FB aggregated embryos became pregnant, but no offspring was obtained. When 201 cloned-2FB aggregated embryos were transferred into five recipients, two of them became pregnant. To assess the origin of the fetuses, the pregnant recipients were euthanized on day 12 and day 13 after embryo transfer respectively. Two and three fetuses respectively, were found in resorption in the two recipients, but none of the fetuses had Ali/Bas genetic background.

When cloned embryos were aggregated with either one (cloned-1PB) or two (cloned-2PB) blastomeres of 6- to 12-cell parthenogenetic embryos, only cloned-2PB aggregated embryos developed to blastocyst significantly better ($P < 0.05$) than non-aggregated cloned embryos. However, after transfer of 109 cloned-1PB aggregated embryos into three recipient mothers, two were diagnosed pregnant on day 14 after embryo transfer. From these two recipients, one lost the

Table 1 Development of rabbit embryos cloned from different types of somatic donor cells.

Nuclear donors	n	Fused (%)	<i>In vitro</i> development		<i>In vivo</i> development		
			Cleaved (%)	Blastocyst (%) ^a	Embryos/recipients	Pregnancy	Offspring
RCC	8	392/427 (92)*	269/341 (79)*	32/95 (34)*	172/5	2/5	1 ^b
RFF	11	704/736 (96)* [†]	611/650 (94) [†]	41/124 (33)*	450/10	2/10	0
RFF-NaBu	6	493/500 (99) [†]	457/469 (97) [†]	86/175 (49) [†]	271/7	1/7	0

RCC, rabbit cumulus cell; RFF, rabbit fetal fibroblast; RFF-NaBu, sodium butyrate-treated RFF; some cleaved 4–10-cell stage embryos were transferred into recipient and some were let to form blastocysts *in vitro*; *[†]Values within the same column with different superscripts differ significantly ($P < 0.05$).

^aBlastocyst rates were calculated based on the numbers of fused embryos. ^bData from two pregnant females: one was killed on day 16, and the other one delivered by operation on day 27 after embryo transfer.

Figure 2 Histone acH3K9/14 status of *in vivo* fertilized and cloned embryos. Normal: *in vivo* fertilized embryos. RCC, RFF and RFF-NaBu, embryos cloned from cumulus cells, fetal fibroblasts and NaBu-treated fetal fibroblasts respectively. Mor, morulae; BI, blastocysts. *In vivo* fertilized 1-cell embryos (zygotes) were fixed 19–20 h post coitum. Cloned embryos from either group were fixed 3–4 h post activation. Cells were immunostained with anti-acH3K9/14 (green) and stained with DAPI for chromatin (blue). The images represent acH3K9/14 patterns as in the majority of stained embryos (Normal: 1-cell – 9/10, 2-cell – 13/13, 4-cell – 9/9, 8-cell – 15/15, 16-cell – 9/9, morulae – 11/11, blastocysts – 11/11; RCC: 1-cell – 9/9, 2-cell – 8/8, 4-cell – 10/10, 8-cell – 7/8, 16-cell – 8/8, morulae – 6/6, blastocysts – 8/8; RFF, 1-cell – 10/10, 2-cell – 8/8, 4-cell – 9/10, 8-cell – 10/10, 16-cell – 9/9, morulae – 7/7, blastocysts – 6/6; RFF-NaBu: 1-cell – 12/14, 2-cell – 9/9, 4-cell – 12/12, 8-cell – 10/10, 16-cell – 9/11, morulae – 9/9, blastocysts – 7/7). All images except of ‘Normal-BI’ are at magnification $\times 400$. Images of ‘Normal-BI’ are at magnification $\times 200$.

pregnancy at the third week of gestation. The other recipient was operated on day 30, and two live pups as well as one resorbed conceptus were delivered. The body weights of the pups were 96 and 54 g respectively. The placenta of the oversized pup was twofold larger than that of the 54 g-weight pup. The overgrown pup died 2 h after birth; the other appeared clinically healthy but died at the age of 2 weeks by an accident when leaving the nest of a foster mother. Post-mortem analysis showed that the overgrown pup had a number of abnormalities including a ‘bulldog’ phenotype, absence of normal skin pigmentation, swollen limbs and an enlarged liver. The pup, which has died accidentally, had no obvious abnormalities. Genetic analysis revealed that donor cells, tissue samples from placentas and pups were all Ali/Bas origin, but samples from recipient mother and a resorbed fetus showed no Ali/Bas genotype.

Discussion

Epigenetic reprogramming occurs abnormally in a large proportion of cloned embryos (Dean *et al.* 2001, Kang *et al.* 2003, Santos *et al.* 2003, Hiendleder *et al.* 2004, Wee *et al.* 2006) and is apparently species-specific (Dean *et al.* 2001, Beaujean *et al.* 2004a, 2004b, Shi *et al.* 2004) and largely dependent on the type of donor cells (Santos *et al.* 2003). Epigenetic marks, which characterize specific DNA and chromatin modifications, are the main players in nuclear reprogramming and can be used to assess this process and predict SCNT efficiency.

Embryos cloned from RFFs showed similar development to blastocyst as those cloned from RCCs (33 vs 34%, $P > 0.05$). Our data do not confirm results from previous studies, in which cumulus cells were found to be better (Cervera & Garcia-Ximenez 2003) or worse nuclear

Table 2 Development of rabbit embryos cloned from NaBu-RFF after aggregation with blastomeres of *in vitro* fertilized or parthenogenetic embryos.

Embryo type	n	<i>In vitro</i> development				<i>In vivo</i> development		
		Fused (%)	Cleaved (%)	4-cell (%)	Blastocyst (%) ^a	Embryos/recipients	Pregnancy	Offspring
Cloned-2FB	6	354/361 (98)*	331/343 (97)*	252/343 (74)*	44/51 (86)*	201/5	2/5	0
Cloned-1FB	6	317/331 (96)*	269/306 (88) [†]	242/306 (79) [†]	35/51 (69) [†]	191/6	1/6	0
Cloned-2PB	6	277/281 (99)*	237/275 (86) [†]	166/275 (60) [‡]	50/71 (70) [†]	95/3	0/3	0
Cloned-1PB	6	359/367 (98)*	289/346 (84) [†]	216/346 (62) [‡]	62/107 (58) [‡]	109/3	2/3	2
Cloned	6	493/500 (99)*	457/469 (97)*	394/469 (84)*	75/123 (61) [‡]	271/7	1/7	0

Cloned, cloned embryos derived from NaBu-treated RFF; 1FB, 2FB, aggregated with 1 or 2 blastomeres of 6–12-cell fertilized embryos; 1PB, 2PB, aggregated with 1 or 2 blastomeres of 6–12-cell parthenogenetic embryos. *†‡Values within the same column with different superscripts differ significantly ($P < 0.05$). After aggregation with FB or PB, the majority of embryos were transferred after 1 day of culture, but some were cultured up to day 5 and developed to blastocysts.

^aBlastocyst rate was calculated relative to 4-cell stage embryos.

donors (Liu *et al.* 2004) as compared to fibroblasts. These differences might be due to the respective nuclear transfer protocol used in either study. In our study, after transfer into recipients, a higher pregnancy rate was obtained with embryos cloned from RCCs than with RFF-derived embryos. However, *in vivo* development of embryos cloned with either cell type was almost always limited to the middle of gestation, suggesting that other donor cell type-independent factors may impair embryonic development in the second period of gestation. Previous study in mouse showed that cloned embryos derived from cumulus cells developed better both *in vitro* and *in vivo* than those originating from fibroblasts (Wakayama & Yanagimachi 2001). These results indicate that some intrinsic differences (e.g. differences in epigenetic status) may exist between these two cell types but could be species-specific.

Further, we tested whether two types of donor cells differ in the status of histone acetylation. We found significantly higher ($P < 0.05$) levels of acH3K9/14 in RCCs than in RFFs suggesting different organization of the chromatin of these cells. Treatment of RFFs with NaBu prior to nuclear transfer markedly increased the level of acH3K9/14 and this effect was associated with improved development of SCNT embryos *in vitro*. Thus, it is reasonable to suppose that the levels of acetylated histones in donor nuclei may affect their reprogramming after nuclear transfer.

A positive effect of NaBu-treatment of donor fibroblasts was already observed in our previous studies on bovine SCNT, although no change in histone acetylation levels of donor cells could be detected by Western blot analysis (Shi *et al.* 2003a), suggesting that even subtle changes in histone acetylation levels are beneficial or that NaBu has other effects rendering donor cells more suitable for reprogramming. Unlike in bovine, where treatment of fetal fibroblasts with NaBu significantly increased both cleavage and blastocyst rates (Shi *et al.* 2003a), NaBu-treatment of RFFs markedly improved only blastocyst rate corroborating the notion that epigenetic reprogramming occurs late in rabbit compared to other species (Shi *et al.* 2004, Young & Beaujean 2004).

We have demonstrated previously that epigenetic changes to the chromatin of donor cells including DNA methylation and histone methylation/acetylation can be reasonable marks of nuclear reprogramming, which correlate with developmental potential of cloned bovine embryos (Santos *et al.* 2003). In this study, NaBu-treated RFFs and RCCs exhibited higher levels of histone acetylation than non-treated RFFs correlating well with the greatest pre- or postimplantation developmental potential of cloned embryos respectively. Thus, the histone acetylation pattern of donor cells may be a useful mark to predict *in vitro* or *in vivo* development of nuclear transfer embryos in rabbits.

Although NaBu-treatment significantly improved development of embryos cloned from RFF-NaBu cells to blastocyst, their postimplantation development was not better compared to embryos derived from untreated cells. Likewise, embryos cloned from rabbit bone marrow cells, which showed the greatest rate of blastocysts (60%), almost always failed to develop to term giving birth to only one cloned rabbit (Zakhartchenko *et al.* unpublished observations). It appears that development to blastocyst does not indicate proper reprogramming of the genome of a donor cell as does full-term development. Further, an increased level of acetylated histones in donor cells may not necessarily be followed by appropriate reprogramming after nuclear transfer. This suggests that the beneficial effect of enhanced histone acetylation may be overlapped by the defects in other epigenetic marking systems. In our recent study on methylation reprogramming (Shi *et al.* 2004), we showed that *in vivo* fertilized and cloned preimplantation rabbit embryos lack detectable methylation changes suggesting that genome-wide demethylation is not obligatory requirement for epigenetic reprogramming in this species. Moreover, the methylation patterns of embryos cloned from either cumulus or fibroblast cells were similar to those of *in vivo* embryos indicating that genome-wide DNA methylation is probably not a useful mark to assess nuclear reprogramming during cloning in rabbits. The proper reprogramming following nuclear transfer appears to be greatly

dependent on multiple epigenetic modifications, which are affected by a number of crucial factors one of these is the cell cycle stage of a donor nucleus.

Recently, Li *et al.* (2006) reported a higher blastocyst rate with serum-starved than with confluent rabbit fibroblasts (46 vs 21%) attributing this effect to synchronizing the cells in an inactive G0/G1 cell cycle phase. Moreover, after transfer of embryos cloned from serum-starved fibroblasts into recipients, 14 pups have been obtained and three of them survived. This study contrasts to our observations when we obtained the same proportions of embryos developing to blastocysts using serum-starved or confluent fetal fibroblasts (33 and 33%, Zakhartchenko *et al.* unpublished observations). Although it is not more likely, but the two major differences, outbreed Zika vs inbreed New Zealand White rabbits, and fetal vs adult fibroblasts, could contribute to the discrepancy in the results. As to the effect of cell cycle synchronization, previous studies in several species demonstrated that, after culture to confluence or serum starvation, the majority of the cells are in G0/G1 phase of the cell cycle (Wilmot *et al.* 1997, Cibelli *et al.* 1998, Golzio *et al.* 2002, Shi *et al.* 2003a, Liu *et al.* 2004). For example, Dinnyes *et al.* (2001) showed that both starved and confluent rabbit fibroblasts have very similar proportions of G0/G1 and S phase cells (89 and 91%, and 2 and 2% respectively), which differ significantly from those of cycling cells (60 and 11% respectively). In the present study, we have omitted cycling RFFs cells because of an increased proportion of such cells is in S-phase and do not work for NT. Previously we demonstrated that efficiency of NT was significantly lower with cycling than with serum-starved (30 vs 68% morulae/blastocysts, Galat *et al.* 2002) or confluent fibroblast cells (10 vs 33% blastocysts, Zakhartchenko *et al.* unpublished observations).

NaBu-treatment also influences the cell cycle by increasing the proportions of G0/G1 cells (cycling bovine epithelial cells, non-treated vs NaBu-treated cells, 48 and 65%, Shi *et al.* 2003a; human carcinoma cells, non-treated vs NaBu-treated cells, 59 and 72%, Bartova *et al.* 2005; mouse fibroblasts, non-treated vs NaBu-treated cells, 47 and 62%, Matheu *et al.* 2005) but this effect is not so great as with serum starvation or culture to confluence. This suggests that the major effect of NaBu, as an inhibitor of histone deacetylases, is dynamic reorganization of chromatin with changes in its epigenetic modifications.

Considering the fact that embryos cloned from RFF-NaBu cells and RCCs showed the greatest pre- or postimplantation developmental potential, it was reasonable to expect that these embryos would be more similar to *in vivo* fertilized embryos in respect to the patterns of acH3K9/14 than those cloned from non-treated RFFs. The distribution of signals for acH3K9/14 in either group of cloned embryos did not resemble that in *in vivo* fertilized embryos. Although both *in vivo* fertilized and cloned

embryos in either group showed a reduction of acH3K9/14 intensity at the 8-cell stage, most dramatical reduction of acH3K9/14 intensity was observed at the 8-cell stage in embryos cloned from RFF and RFF-NaBu cells. Nevertheless, the RCC group exhibited more similarities in acH3K9/14 patterns to the *in vivo* fertilized group than other two groups of cloned embryos. The fact that only NT with RCCs directly resulted in production of a cloned rabbit suggests that reprogramming occurs more easily with this type of cells. Surprisingly, acH3K9/14 signals of *in vivo* fertilized rabbit embryos were enhanced at the 4-cell stage but not at the 8-cell stage at which the rabbit embryonic genome activation occurs (Kanka *et al.* 1996, Henrion *et al.* 2000, Brunet-Simon *et al.* 2001). However, initiation of mRNA synthesis determined by *in situ* hybridization with a poly (U) probe was first detectable at the late 2-cell stage (Kanka *et al.* 1993) and autoradiographically detectable initiation of transcription from an embryonic rabbit genome occurs at the 4-cell stage (Christians *et al.* 1994). An intense transcription then starts after the 8-cell stage and includes the onset of rRNA transcription (Kanka *et al.* 1996). Thus, enhanced levels of acH3K9/14 observed in our study at the 4-cell stage of *in vivo* fertilized and cloned embryos could be due to initiation of embryonic genome activation.

In the present study, we tested different types of blastomeres for aggregation with embryos cloned from NaBu-treated RFF, which exhibited the greatest potential to develop to blastocyst *in vitro*. When cloned embryos were aggregated with blastomeres from *in vivo* fertilized embryos, none of the resulting fetuses and concepti were genetically identical to the nuclear donor cells. Blastomeres of cloned embryos were obviously not competitive with blastomeres of *in vivo* fertilized embryos corroborating the observations of Chrenek & Makarevich (2005) when only 14% of SCNT blastomeres were present in the ICM of chimeric blastocysts obtained after their aggregation with blastomeres of *in vivo* embryos. Thus, we used a less competitive partner for aggregation that might nevertheless support implantation and postimplantation development of cloned embryos. It is well documented that mammalian parthenogenetic embryos do not develop to term because of deficient expression of maternal imprinted genes and are characterized by poor development of their extraembryonic membranes (Surani *et al.* 1986, 1990). Parthenogenetic rabbit embryos have the potential to implant but die near midgestation (Ozil 1990), and mouse parthenogenotes were used to assist the development to term of single blastomeres from 4-cell mouse embryos (Pinyopummin *et al.* 1994). Taking into account these facts, we performed aggregation of cloned embryos with parthenogenetic blastomeres. Although the development of these cloned embryos, aggregated with a single parthenogenetic blastomere, to blastocyst was similar to that of non-aggregated cloned embryos (58 vs 61%), two clone-parthenote aggregates developed to term.

Genotypic analyses confirmed that both pups were genetically identical with the Ali/Bas RFF donor cells. It is worth noting that we were unable to produce offspring either after transfer of embryos cloned from RFF-NaBu cells or RFF-derived embryos aggregated with parthenogenetic blastomeres (data not shown). This may indicate a positive effect of combination of NaBu-treatment and chimeric embryo complementation.

It could be suggested that the two rabbits produced after complementation of cloned embryos with one parthenogenetic blastomere are chimeric. Even though parthenogenetic embryos die shortly after implantation, their cells are capable of participating in normal development of chimeras when aggregated with fertilized embryos (Fundele *et al.* 1989, Nagy *et al.* 1989, Allen *et al.* 1995, Boediono *et al.* 1999). Parthenogenetic cells were detected in some organs and tissues of adult chimeras. However, these cells were under severe selective pressure between days 13 and 15 of mouse development compared with cells from normal mouse fertilized embryos (Fundele *et al.* 1990). In the majority of chimeras, parthenogenetic cells in individual animals were observed in a limited number of tissues and organs and, even in these instances, their contribution was substantially reduced. Detection of any chimerism would require a genetic marker in the parthenogenetic cells, which was unfortunately not the case in this study. However, the use of genetically labeled nuclear donor cells clearly demonstrated that both cloned rabbit pups originated from these cells.

The overall efficiency of SCNT in our study based on the specific experiment that resulted in one live kit is 0.9% (one kit from 109 cloned embryos transferred) and similar or slightly lower compared to that reported by Chesne *et al.* (1%, 4/371; 2002) and Li *et al.* (3%, 14/467; 2006) respectively. We obtained a slightly higher efficiency of rabbit cloning with embryonic donor cells (3%, 8/284; Zakhartchenko *et al.* unpublished observations). This efficiency is similar to those obtained by Stice & Robl (4%, 1988) or by Yang *et al.* (3%, 1992) providing evidence that the basic rabbit cloning procedure does work in our laboratory. Our control experiment with transfer of *in vivo* fertilized embryos proves the reliability of the applied embryo transfer technique.

In summary, our study demonstrates that although rabbit cumulus and fibroblast cells differ in the levels of acH3K9/14, the embryos cloned from either type of these cells show similar developmental potential *in vitro*. The levels of histone acetylation in donor cells can be modified by treatment with NaBu resulting in improved development of cloned embryos to blastocyst but not to term. Neither embryos cloned from RCC nor from RFF or RFF-NaBu cells entirely resembled the patterns of acH3K9/14 of *in vivo* fertilized embryos, suggesting that reprogramming of this epigenetic mark is aberrant in cloned rabbit embryos and cannot be corrected by

treatment of donor cells with a HDAC inhibitor. However, RCCs seem to be better nuclear donors because of RCC-derived cloned embryos, which are more similar to *in vivo* fertilized embryos in respect to acH3K9/14 patterns than other two groups of cloned embryos, gave rise to a cloned pup without any assistant approaches. Production of live clones from RFFs required treatment of these cells with NaBu and chimeric embryo complementation with parthenogenetic blastomeres. This suggests that reprogramming occurs more abnormally with RFFs than with RCCs. Levels of acetylated histones, at least acH3K9/14, in donor cells and cloned embryos correlate with their developmental potential and may be a useful epigenetic mark to predict efficiency of NT. Cloning of rabbits from cultured donor cells provides the basis for further development of SCNT in this species, which would have a plethora of important applications in biomedicine and biotechnology.

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