Donor Cell Lines Considerably Affect the Outcome of Somatic Nuclear Transfer in the Case of Bovines

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Abstract. Since the first successful nuclear transfer (NT) experiments were carried out, various somatic cell types have been used as donor cells for production of cloned animals. In most experiments, fibroblasts are used since they only need to be isolated and cultivated. Recently, some researchers have shown that different cell cultures from different sources possess different capacities to support preimplantation development of NT embryos. The blastocyst rates obtained in our previous studies varied and were as high as 45% in relation to the number of reconstructed embryos. This led us to question whether the origin and culture conditions of the defined male and female fibroblast lines could be responsible for the differences in developmental potency. Taking all our results into consideration, we conclude that different fibroblast lines recovered from the same tissue and cultivated under equal culture conditions could produce dramatically different blastocyst rates. The influence of cell line itself is higher than the influence of passage number. The observed effects of cell cycle stage, chromosomal aberrations, and diminished vitality are important but not sufficient to discriminate well-qualified nuclear donor cells. We speculate that some epigenetically regulated deviations in the gene expression program are responsible for these phenomena. Explanation of the underlying mechanisms should contribute to better understanding of epigenetic reprogramming and may ultimately assist reprogramming in the laboratory.

Key words: Bovine, Donor cell, Somatic cell nuclear transfer

Since the first successful nuclear transfer (NT) experiments were carried out, various somatic cell types, such as fibroblasts [1], mammary gland cells [2], cumulus granulosa cells [3], oviduct cells [4], leukocytes [5], mural granulosa cells [6], liver cells [7] and embryonic stem cells [8], have been used as donor cells for production of cloned animals. It is still unclear which somatic cell type is the most efficient for nuclear transfer into oocytes [4]. There is considerable evidence to suggest that use of embryonic stem cells leads to viable offspring with greater efficiency than many somatic cell types [9, 10]. However, in most experiments, fibroblasts have been used since they only need to be isolated and cultivated. When the efficiencies of various cell types from adult, newborn and fetal male and female donor cells were compared, no significant differences were found in the percentages of blastocysts produced from each cell type [4]. Similar results have been obtained using various cell types derived from mice of different strains, sexes, and ages [11].
al. [12] showed that different cell cultures from different sources possess different capacities to support preimplantation development of horse NT embryos.

Initially, all cloned animals derived from adult somatic cells were produced using female cells [1–4, 6], which raised the question of whether male individuals could be cloned. In subsequent experiments, no significant differences were found in the developmental rates of embryos reconstituted with male or female nuclei in cattle [4] and mice [11]. Although both male and female cell lines have successfully been used to generate cloned offspring, the suitability of these cell lines for NT in terms of their sex (male/female) generates quite a lot of discussion at informal meetings amongst scientists as female cell lines often outweigh their male counterparts in terms of cleavage and blastocyst rates. Astonishingly, not much data is available in the literature to support this argument.

Cell cycle synchrony of donor cells is important for NT. Cloned animals have been obtained consistently from somatic cells in the G0/G1 state. The differences in the distribution patterns of cultivated cells between the cell cycle stages depend on cell cycle activity and cell proliferation. Cell proliferation is influenced by the type of cultivated cell, passage number, and cell culture conditions. Enrichment of cells in the G0/G1 state is possible by cultivating the cells up to the confluence stage and/or by serum starvation [2, 35].

Suboptimal culture conditions or a high number of passages may lead to abnormal histone H3 phosphorylation patterns in somatic cell cultures. During metaphase, these disturbed patterns could lead to abnormal chromosome segregation and chromosome loss during mitosis. The level of chromosomal aberration is different for different cell cultures, cell lines, and passages [13]. These results demonstrate the importance of determining chromosome content in nuclear donor cells in order to decrease the percentage of aneuploid reconstructed embryos and increase the efficiency of NT.

Successful embryonic development after nuclear transfer requires resetting of the gene expression program of the nuclear donor (differentiated somatic cell) to a consistent state (early embryo). The somatic genome presents itself in an extremely different condition compared with the gametes. Epigenetic modification through DNA methylation and histone modification is central to this necessary genome reprogramming [14]. “Reprogramming” of the donor cell after transfer into the oocyte has little influence and is inefficient. The donor nucleus often fails to express early embryonic genes. These defects correlate with the decreased number of cloned embryos that develop into adult animals. First results demonstrate that the differentiation and methylation state of donor nuclei influence cloning efficiency in terms of the blastocyst rate [15]. Assistance in the form of a “helping hand” [14] by partially resetting the epigenetic program of the donor cell should be useful for creation of an embryonic epigenotype.

In our previous study [16], we found that the method of “handmade cloning” (HMC™) established by Vajta [17] is suitable for reconstructing a large number of embryos by NT. The fact that the obtained blastocyst rates of up to approximately 45% in relation to the number of reconstructed embryos varied greatly between different experiments led us to question whether the origin and culture conditions of the defined male and female fibroblast lines could be responsible for the differences in developmental potency.

**Material and Methods**

Unless otherwise indicated, all plasticware, i.e., culture vessels and dishes, used in our experiments were obtained from Nunc (Wiesbaden, Germany) and all chemicals and media were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA).

**Isolation, cultivation, and passage of bovine fibroblasts**

Four male and two female cell lines of bovine fibroblasts were established using mechanically isolated samples of ear skin from adult animals. The animals, 4 elite bulls (avg. 8 years old) and 2 high performing dairy cows (2nd and 4th lactation periods), were healthy, in good condition, and in commercial use.

A small portion of the ear was shaved carefully and disinfected superficially with 70% ethanol. Samples (10 mm in diameter) were recovered using a punch (disinfected with 70% ethanol), transferred...
into tubes (10 ml PBS, 500 IU/ml penicillin, and 500 µg/ml streptomycin, 4–10 °C), and transported to the laboratory within 1–3 h. After washing them twice (PBS, 300 IU/ml penicillin, and 300 µg/ml streptomycin; RT), small tissue pieces (approximately 0.5 mm in diameter) were prepared using an ultra sharp blade in a Petri dish (TCM-199, 300 IU/ml penicillin, and 300 µg/ml streptomycin); the outer part of the epidermis and cartilage were excluded. The pieces were washed twice and then cultured in cell culture flasks (10 ml of TCM-199, 20% FCS, 1 mM sodium pyruvate, 200 IU/ml penicillin, and 200 µg/ml streptomycin). After one week, fibroblasts from the tissue pieces had spread out onto the bottoms of the culture vessels and the cultures were washed with culture medium (TCM-199, 10% FCS, 1 mM sodium pyruvate, 100 IU/ml penicillin and 100 µg/ml streptomycin). The cells growing in a monolayer were cultivated to confluence. Trypsination (0.02–0.05% trypsin-EDTA) and recultivation were used for passaging (up to passage 11). Aliquots were stored by cryopreservation (20% FCS and 1% DMSO in TCM-199; shock freezing at −96 °C) after each passage.

Cells in the G0/G1 phase of the cell cycle were enriched by serum deprivation in culture medium (0.5% FCS, 2 days).

**Induced demethylation of somatic cells**

To induce nonspecific DNA demethylation of somatic cells, 5-aza-2-deoxycytidine (5-Aza) was added to the cell culture 24 h after passage (fibroblasts) or at the beginning of culture (granulosa cells) to remove the tissue pieces and dead cell material. The cells growing in a monolayer were cultivated to confluence. Trypsination (0.02–0.05% trypsin-EDTA) and recultivation were used for passaging (up to passage 11). Aliquots were stored by cryopreservation (20% FCS and 1% DMSO in TCM-199; shock freezing at −96 °C) after each passage.

Freshly isolated bovine granulosa cells (puncture of ovarian follicles >5 mm in diameter obtained from ovaries collected at commercial slaughterhouse) were cultivated in TCM-199 (10% FCS, 1 mM sodium pyruvate and antibiotics) up to confluence and used for comparison.

**Analysis of cell cycle and vitality**

The adherent cells in confluent cultures were recovered by trypsinization (see above). Cells were fixed drop wise in 70% ethanol at −20 °C, washed, and treated with RNase solution (heat inactivated at 85 °C for 60 min) [100 U/ml in Hank’s buffered saline (HBS) at 37 °C for 30 min]. After staining with propidiumiodide (PI; 70 µM, 30 min) in HBS (5 mM Hepes, pH 7.3, 150 mM NaCl), flow cytometry was performed (Beckman-Coulter EPICS Elite), and the cell cycle was analyzed using the Multicycle Program (Phoenix Flow Systems, San Diego, CA, USA) of the program package of the flow cytometer.

To measure the percentage of viable cells PI staining (30 µM, 10 min) was performed without fixation and RNase treatment of the cells. The percentage of stained cells was analyzed by flow cytometer.

**Cytogenetic analysis**

Fibroblasts were used for metaphase preparation using standard procedures. Cells were grown in MEM (10% FCS, antibiotics) until confluence. After passaging, the cells were treated with 10⁻⁷ M MTX to block mitotic activity for 18 h. The block was released by washing in PBS and cultivating in medium containing 1.6 mM thymidine. Five hours later, the cells were treated with 0.0001% colchicine for 30 min and then harvested. After hypotonic treatment in 7.5 mM KCl for 15 min, the cells were fixed in 3:1 methanol-glacial acetic acid for 20 min. The fixative was changed twice, and the cells were then dropped to ice-cold wet slides and air-dried. For overview analysis, the slides were stained with 4% Giemsa. At least 30 metaphase spreads were examined at 1,600× magnification. Analysis of chromosomal abnormalities was conducted, and abnormalities were identified as deletions, breaks, fragments, or gaps as defined by Bergsma [19].

For G-banding, 4-day-old slides were treated with 0.2 N HCl for 10 min followed by a short rinse in 0.9% NaCl. After digestion in 0.025% trypsin (Gibco BRL, Invitrogen, Karlsruhe, Germany) in 0.9% NaCl at 37 °C for 25–45 sec, the slides were incubated in 2 × SSC at 60 °C for 10–15 min and stained with 5% Giemsa (Azur Eosin, Merck, VWR, Darmstadt, Germany) in Sorensen buffer (pH 6.8) for 7–8 min. Karyotyping was performed according to the method of Cribiu et al. [20] using the McKitype image analysis system (Perceptive Scientific Instruments). Ten kg were examined for visible abnormalities.

**Semi-quantitative detection of DNA methylation**

For the measurement of DNA methylation level,
aliquots of cells were cultivated on glass cover slides in the wells of a 24-well cell culture plate in medium and with and without 5-Aza treatment as described above. The cover slides were washed twice in HBS with Ca$^{2+}$ and Mg$^{2+}$ and fixed in pure methanol at −20 °C for 20 min for immunocytochemical 5-methyl cytosine staining. After washing with Tris buffered saline (TBS; 20 mM Tris and 150 mM NaCl), the monolayers were treated with 7 µM NaOH (10–15 sec). The cells were then washed with TBS and permeabilized with 4 mM deoxycholic acid for 10 min. They were subsequently washed with TBS containing 0.025% Triton X-100, and then nonspecific binding of antibodies was blocked with Roti-Immunoblock (Roth T144) for 2 h at RT. Incubation with the first antibody (sheep polyclonal; 5-methyl cytosine antibody, Abcam 1884) was carried out overnight (dilution 1:500) at 4 °C. Subsequently, the cells were washed and the secondary antibody (Alexa Fluor 488 donkey anti-sheep IgG; Molecular Probes A-11015) was added for 2 h (dilution 1:1,000) at RT. The probes were gently washed, fixed with 2% paraformaldehyde for 15 min, and covered with gelatine and a second cover slide.

Semi-quantitative examination of the probes was conducted using a confocal laser scanning microscope (Carl Zeiss, Jena, Germany) and image analyzing software (AnalySIS 3.3; SIS, Muenster, Germany). The mean fluorescence intensity of single nuclei (20 per image, 4 images per slide and 3 slides per group) was measured after correcting nonspecific fluorescence. Two classes (unstained and stained) were established using control images. The percentage of stained nuclei and mean intensity of the stained cells were calculated.

Nuclear transfer and embryo culture

Nuclear transfer experiments were conducted using the HMC™ method established by Vajta et al. [17] and described in detail by Bhojwani et al. [16, 18]. Briefly, oocytes were collected using the ovarian slicing method [21]. Prior to slicing, each ovary was placed in a Petri dish and covered with TCM-199 (with Hank's salts and 25 mM HEPES buffer) containing 0.5 IU/ml heparin. A stereomicroscope was used to collect the oocytes into a 4-well culture dish containing 500 µl TCM-199. Oocytes with a compact cumulus were selected and incubated in maturation medium [TCM-199 containing 20% FCS and 10 µg/ml FSH (Ovagen, icp, New Zealand)] for 21 h at 38.5 °C in 5% CO$_2$ in air. Matured oocyte-cumulus-complexes were vortexed for 3 min in 0.5 mg/ml hyaluronidase dissolved in 500 µl tissue culture medium (TCM-199) to remove the cumulus cells. The oocytes were then transferred for 8 min into 1.5 mg/ml pronase (Sigma) dissolved in 600 µl TCM-199 containing 10% FCS to remove the zona pellucidae and manually bisected under stereomicroscopic control with Ultra Sharp Splitting Blades (AB Technology, Pullman, WA, USA). Ultraviolet light was used to sort out the cytoplasts from the 10 µg/ml Hoechst 33342-stained karyoplasts. Meanwhile, the somatic cells were prepared by trypsinization (see above). Cytoplasts were immersed in PHA and then individually dropped onto a single somatic cell for attachment. The cytoplasm-somatic cell pair was then transferred to a fusion chamber (0.5-mm fusion chamber, CFA 500; Krüss, Hamburg, Germany) in such a way that the somatic cell was positioned furthest from the wire. Fusion was first performed with half the total quantity of cytoplasts using a double DC pulse of 65 V; each pulse lasted 20 µsec, and the pulses were 0.1 sec apart. The pairs were then carefully collected. Fusion was then performed with all the remaining cytoplasts and the fused pairs; they were transferred to the fusion chamber, and one fused pair was then attached to each cytoplast. This second fusion was performed with the same parameters as previously used except for use of a DC pulse of 45 V. The reconstructed embryos were then transferred to culture medium, covered in oil, and incubated (5% CO$_2$ in air at 39 °C).

Two-step activation was carried out approximately 4 h after second fusion; the first step was conducted with 2 µM Ca ionophore (A23187) for 5 min at RT, and the second was conducted with 2 mM 6-dimethylaminopurine (6-DMAP; 5% CO$_2$ in air) at 39 °C for 6 h. The embryos were then washed twice in culture medium, cultured individually in a well of the wells (WOWs) system [22] in synthetic oviductal fluid (SOF) medium (Minitüb, Germany) supplemented with 10% (v/v) estrous cow serum, and covered with mineral oil at 39 °C in 5% CO$_2$, 5% O$_2$, and 90% N$_2$. 
Results

Somatic fibroblast cell lines were established from all 6 animals (lines B1-B4 for bulls; lines C1 and C2 for cows). The lines were cultivated up to passage 11, and aliquots were deep-frozen from each passage. The vitalities of the cells checked before and after deep-freezing at passages 6 and 11 were 94 ± 12% before deep-freezing and 80 ± 16% after thawing, respectively, and were not significantly different between lines and number of passages.

The cells from passages 6 and 11 were used for the NT experiments. In total, 650–850 (passage 6) and 1000–1200 (passage 11) embryos were generated per cell line for these two passages, respectively. The results for the male cell lines (Fig. 1) demonstrated no significant differences in cleavage rate (60 to 72%) between the lines and / or passages. In contrast, the percentage of embryos that developed to the 8-cell stage and especially to the blastocyst stage were significantly different between the cell lines but not between the passages within the same lines. Line B1 had the worst results (30–32% 8-cell rate, 2% blastocyst rate), and line B4 had the best developmental capability (58–65% 8-cell rate, 19–22% blastocyst rate).

The two female fibroblast lines led to significantly increased developmental rates. There was no difference between the two female cell lines in 8-cell rate (72–73%; cleavage rate, 72–85%); however, there 8-cell rates were higher than that of the best male line (B4). The blastocyst rates of the two female lines, C1 (59%) and C2 (45%), were different but were significantly increased compared with that of the best male line (B4). There were no significant differences between the passages (6 and 11) within the same female lines. Based on these results, we concentrated further study on two of the female lines (C1 and C2) and

Fig. 1. Developmental capability of NT embryos to the blastocyst stage depending on fibroblast cell line and passage number used as the nuclear donor. Different letters in the same column (embryo stage) indicate significant differences (P≤0.05).
the two extremely different male lines (B1 and B4).

Cell cycle analyses of these 4 cell lines and primary culture of granulosa cells to confluence showed high percentages of cells (approximately 70%) in the G0/G1 phase (Table 1). There were no significant differences between the fibroblast lines. There was a clear tendency towards increased proportions at higher passage numbers and in the granulosa cell groups. After serum deprivation (2% FCS, 2 days), the number of cells in the G0/G1 phase increased to 80–90%. No differences were discovered between the passage numbers but an increased proportion of G0/G1 phase cells was detected for line B4 in relation to B1.

No chromosomal abnormalities were detectable in cytogenetic analyses of cell lines B1, B4, C1 and C2 at passage 6 and 11. Two examples are provided in Figs. 2a and b.

Immunofluorescent staining of 5-methyl-cytosine is shown in Figs. 2c and d. Specific distribution patterns with “hot spots” and regions with low or no staining intensity were visible in the nuclei. The frequency of hot spots and their mean fluorescence intensity were different between the nuclei and were quantified by image analysis. A granulosa cell culture was included for comparison with the 4 tested fibroblast lines. The results (Fig. 3) demonstrated that DNA methylation decreased at higher passage numbers in all cell cultures. There was a tendency towards lower methylation levels in conjunction with strong reductions depending on passage number. The differences between the fibroblast lines were marked and partly significant at the beginning of cultivation (passage 1). In passage 6 and 11, the differences between the fibroblast cells were not significant. Nevertheless, it is evident that line B1 (lowest blastocyst rate) had a tendency to present the highest methylation level.

Treatment with 5-Aza led to both a decrease in

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Passage number</th>
<th>With serum (%) mean ± SD</th>
<th>Serum starvation (%) mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>6</td>
<td>60.0 ± 13.2^a</td>
<td>80.7 ± 7.8^a</td>
</tr>
<tr>
<td>B1</td>
<td>11</td>
<td>69.0 ± 9.4^d</td>
<td>79.9 ± 6.0^d</td>
</tr>
<tr>
<td>B4</td>
<td>6</td>
<td>73.4 ± 14.4^b</td>
<td>91.3 ± 5.6^c</td>
</tr>
<tr>
<td>B4</td>
<td>11</td>
<td>79.1 ± 7.5^b</td>
<td>90.5 ± 5.1^b</td>
</tr>
<tr>
<td>C1</td>
<td>6</td>
<td>64.2 ± 16.4^a</td>
<td>83.4 ± 7.8^a</td>
</tr>
<tr>
<td>C1</td>
<td>11</td>
<td>73.5 ± 15.7^b</td>
<td>86.8 ± 6.9^b</td>
</tr>
<tr>
<td>C2</td>
<td>6</td>
<td>72.5 ± 14.3^b</td>
<td>87.2 ± 7.1^b</td>
</tr>
<tr>
<td>C2</td>
<td>11</td>
<td>78.8 ± 12.3^b</td>
<td>87.4 ± 6.6^b</td>
</tr>
<tr>
<td>GZ</td>
<td>6</td>
<td>84.8 ± 11.0^b</td>
<td>91.2 ± 4.1^b</td>
</tr>
<tr>
<td>GZ</td>
<td>11</td>
<td>83.2 ± 16.1^b</td>
<td>88.7 ± 10.1^b</td>
</tr>
</tbody>
</table>

The cells were cultivated as a confluent monolayer (GZ, granulosa cells; different letters in the same column indicate significant differences, P <0.05)
methylation level and a demonstrable cytotoxic effect at higher concentrations (>0.8 \( \mu M \); Fig. 4). Granulosa cells were more sensitive (approximately 40% vs. 65–80% viable cells after 72 h treatment with 0.8 \( \mu M \) 5-Aza, respectively) than fibroblasts. There were no significant differences between the male fibroblast lines and passages. The two female fibroblast lines were more resistant (no differences between the two lines), and no influence of passage number was detected.

The cell cycle distribution pattern was influenced by the 5-Aza treatments. There was a decreased tendency of cells in the S phase, while there was a significant increase in cells in the G2/M phase (Fig. 5). These effects appeared at relatively low doses of 5-Aza (0.04 \( \mu M \)). We found some variation between the cell lines and/or the different passages. An example is provided in Fig. 6. It is clear that the variation is mainly attributable to the different reactions in regard to the S phase. In some cases, the S phase was reduced (Fig. 6, left side), uninfluenced (upper right), or increased (bottom right). In contrast, the G2/M-phase was increased in all cases. It was not possible to recognize a pattern between the cell lines and/or the different passages.

The main function of 5-Aza is nonspecific demethylation of DNA. This effect is dependent on the 5-Aza concentration and duration of influence. Both phenomena are demonstrated in Fig. 7.
Demethylation reached a maximum after 72 h (Fig. 7c). Prolonged cultivation (96 and 120 h) under the influence of 5-Aza did not lead to a further reduction of DNA methylation (data not shown). On the other hand, the concentration necessary to induce a considerable effect is high (≥0.2–0.4 µM). From these results and in combination with the data shown in Fig. 4, it can be concluded that the dose range that can be used for physiological DNA demethylation is very small (0.4 µM for granulosa cells and 0.4–0.8 µM for fibroblasts). It was not possible to reduce the methylation to the assay limit within this dose range. In our experiments, the minimum methylation level in relation to the initial rate was about 20–35% (Fig. 7). There were no significant differences between the cell lines and passages used. The effect of 5-Aza in the non-cytotoxic dose range (0.2–0.8 µM, 72h) was reversible within 10–15 days of culture in 5-Aza-free medium (data not shown).

The three best cell lines, B4 (based on the blastocyst rate in Fig. 1), C1 and C2, were incubated for 72 h with 0 µM (control) and 0.8 µM 5-Aza (a dose with considerable effect on DNA methylation and acceptably low cytotoxicity) in order to test the effect of 5-Aza on the suitability of somatic cells for...
nuclear transfer. Nuclei pretreated in this manner were used for the nuclear transfer experiments. The results in Table 2 show a decreased developmental capacity for embryos reconstructed from 5-Aza pretreated nuclei. Notably, the blastocyst rates were dramatically decreased after induction of demethylation. It is evident that the blastocyst rates of the different cell lines did not drop by equal amounts and that they did not show any similarities in regard to their low values. The cell lines with higher blastocyst rates in the control group (C1>C2>B4) also had higher rates in the treatment groups.

### Discussion

Increasingly, laboratories worldwide are beginning to work in the field of somatic nuclear transfer. Both the classic procedure [2] and the still relatively new method of handmade cloning (HMCTM) [23, 16] have led to live offspring. However, both techniques are problematic because they require resetting of the gene expression program of somatic cells to that consistent with early embryonic development. Epigenetic modification is of central importance for genome reprogramming in the process of nuclear transfer [14]. It is quite understandable that the suitability of different types of nuclear donor cells to successfully and completely pass through the process of epigenetic reprogramming strongly influences the success of the whole method of cloning. Therefore, some of the work presented in the literature deals with the question of whether certain cell types are more suitable for somatic cloning than the others [4, 7, 14]. Recently, some papers have hinted at the possibility that different lines of the same cell type could result in different success rates after NT (horse [12], pig [24, 25]).

Results obtained from NT using four primary cell lines of adult bovine somatic cells indicate that primary donor cell culture has a significant impact on in vitro development, initial pregnancy rate, and percentage of live births [26, 27].

In our previous studies [16, 18], we demonstrated successful application of the HMCTM method in our laboratory. In spite of our continued success in carrying out NT experiments, we frequently encounter huge variations in blastocyst rates. This led us to the question whether the different fibroblast cell lines we have used over the course of several years in our lab could be responsible for this situation. Therefore, we analyzed our male and female bovine fibroblasts in detail.

The procedures for recovering and cultivating fibroblasts described above were suitable and efficient. In all cases, fibroblast lines could be established and passaged at least 12 times. The vitality of the cells and the cytogenetic situation described in the results section indicate the high quality and intactness of the cells in all the tested passages. Cultivation up to the stage of confluence resulted in a high proportion of cells in the G0/G1 phase of the cell cycle due to contact inhibition of proliferation. Coupled with this, serum starvation led to a small but nevertheless significant increase in the proportion of G0/G1 cells. Therefore, we used only cells recovered from confluent monolayers and subjected them to serum starvation for our NT experiments.

There were significant differences in blastocyst rates after NT between the donor cell lines even though all the cells were recovered and cultivated under the same conditions. The differences occurred between the cell lines themselves and not between the different passages within the lines. Furthermore, they were not attributed to differences in cell vitality, chromosomal aberrations, or cell cycle stage. Appearance of

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Pretreatment</th>
<th>Cleavage rate</th>
<th>8-cell rate</th>
<th>Blastocyst rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>B4</td>
<td>Control</td>
<td>84%</td>
<td>77%</td>
<td>32%</td>
</tr>
<tr>
<td>B4</td>
<td>0.8 µM 5-Aza</td>
<td>68%</td>
<td>52%</td>
<td>5%</td>
</tr>
<tr>
<td>C1</td>
<td>Control</td>
<td>72%</td>
<td>72%</td>
<td>59%</td>
</tr>
<tr>
<td>C1</td>
<td>0.8 µM 5-Aza</td>
<td>57%</td>
<td>49%</td>
<td>18%</td>
</tr>
<tr>
<td>C2</td>
<td>Control</td>
<td>85%</td>
<td>73%</td>
<td>45%</td>
</tr>
<tr>
<td>C2</td>
<td>0.8 µM 5-Aza</td>
<td>69%</td>
<td>59%</td>
<td>16%</td>
</tr>
</tbody>
</table>
genetic disturbances could possibly lead to changes in the blastocyst rate of higher passages. The somewhat contradictory information in the literature [28–31] concerning the influence of passage number on the blastocyst rate is possibly the result of genetic disturbances resulting from the culture conditions.

The small differences in total DNA methylation at the beginning of cultivation (passage 1) were not detectable for long and were undetectable in passages 6 and 11, which were used for the NT experiments. In this context, it is important to note that we measured whole DNA methylation and not the methylation level of single relevant genes. It is possible that the small differences discovered in passage 1 reflect more important epigenetic deviations than are assumed [25]. This could be a reason for the better suitability for NT of the appropriate line in increasing passages. This speculation should be examined in future experiments. In particular, the methylation of specific genes relevant to epigenetic modification should be investigated.

It was striking that the female lines always led to better blastocyst rates. However, it is not possible to suggest a general effect of sex because the number of cell lines was too small for statistical evaluation.

Cell cultures were treated with 5-Aza to induce partial nonspecific DNA demethylation as an attempt to provide a “helping hand” [14] for epigenetic reprogramming after NT. Our results demonstrate the problems related to this idea. The usable dose range of 5-Aza is very small and must be determined for specific conditions. Its strong collateral effects include cytotoxicity and influence on cell cycle stage. The DNA demethylation it produces is nonspecific and, at least within non-cytotoxic dose ranges, incomplete. The reaction to treatment of all the tested cells was similar, with a tendency to higher sensitivity in the male cell lines. Reversibility in the form of spontaneous remethylation after 5-Aza withdrawal and the mechanism or principle of molecular action indicate an “epigenetic memory” that is uninfluenced by 5-Aza.

Nevertheless, partially DNA demethylated fibroblasts were used as donor cells in our NT experiments. The results of the cloning experiments showed decreased embryonic development, especially a very low blastocyst rate in relation to the untreated control groups. Therefore, only the three best fibroblast lines (according to blastocyst rate) were used for the experiments. The blastocyst rates were 3–6 times lower after the 5-Aza treatments. Our findings are in accordance with the data published in the literature [32–34]. However, the 5-Aza concentrations used by the previous authors were higher (≥1 µM). These concentrations proved to be cytotoxic in our tests. Two aspects were striking in our experiments. The first was that the blastocyst rate did not drop to a value near 0%. In some cases, blastocyst rates of 18% were obtained. On the other hand, the second was that the relationships between the cell lines were conserved. This means that the line with the best blastocyst rate in the control group also showed the highest percentage of blastocysts after the 5-Aza treatments.

Summarizing our findings, we conclude that different somatic cell lines of the same cell type (fibroblasts) recovered identically from the same tissue and cultivated under equal conditions can result in dramatically different blastocyst rates. In our experiments, the influence of cell line itself on NT efficiency was higher than the influence of passage number. The reason for this variability remains unclear. The commonly discussed effects of cell cycle stage, chromosomal aberrations, and diminished vitality are important but are not sufficient to discriminate well-qualified nuclear donor cells. We speculate that some epigenetic regulated deviations in the gene expression program are responsible for the variations in NT results. Explanation of the underlying mechanisms should contribute to better understanding of epigenetic reprogramming and ultimately should assist reprogramming in the laboratory. Until this is possible, our only option is to select cell lines that are suitable for NT through the use of cell lines in NT experiments. Nuclear donor cells that lead to low blastocyst rates should be excluded and replaced with a different cell line from the same or different animal. Finally, a high blastocyst rate is necessary to establish pregnancy and birth of healthy offspring after NT. Nevertheless, a high proportion of blastocysts in vitro is no guarantee of the developmental competence of embryos after transfer to recipients.
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