Clonal Lines of Transgenic Fibroblast Cells Derived from the Same Fetus Result in Different Development When Used for Nuclear Transfer in Pigs

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ABSTRACT

Different factors are believed to influence the outcome of nuclear transfer (NT) experiments. Besides the cell cycle stage of both recipient cytoplast and donor karyoplast, the origin of the donor cells (embryonic, fetal, and adult) is of interest. We compared in vitro development of NT embryos derived from small serum-starved (G0) or small cycling (G1) porcine fetal fibroblast cells. Serum starvation did not have a positive effect on cleavage rate or the percentage of embryos that developed to the morula and blastocyst stages. Next, we investigated the development of porcine NT embryos derived from different transgenic clonal cell lines that had originated from the same fetus. When different clonal lines of fetal fibroblasts were fused to enucleated metaphase II oocytes, differences in fusion rates as well as in development to the morula and blastocyst stages were observed ($P < 0.05$). When oocytes derived from sow ovaries were used as recipient cytoplasts, significantly better cleavage ($P = 0.03$) and blastocyst formation ($P < 0.014$) was obtained when compared with oocytes derived from gilts. Our data indicate that not only different cell lines, but also different clones derived from the same primary cell line, result in different development when used for NT. In addition, the use of sow oocytes as a cytoplast source also improves the efficiency of NT experiments.

MATERIALS AND METHODS

The subject of this study was to investigate the suitability of different transgenic clones of fetal fibroblasts for use in NT in pigs. One of the factors we discuss as influencing the efficiency of NT is the cell cycle stage of the donor cells. It has been claimed that the use of cells in presumptive G0 [4] or G1 [12] stage is mandatory for correct and complete reprogramming, and was investigated in this study for porcine fibroblast cells. Last, we purchased a limited number of sow-derived oocytes and investigated the feasibility of this cytoplast source for use in NT.

INTRODUCTION

Somatic cell nuclear transfer (NT) in mammalian species has developed and advanced in the last 5 yr and many associated factors have been the subject of investigation (reviewed in [1]). Live animals have been produced from mice [2], sheep [3, 4], goats [5], cattle [6, 7], and pigs [8, 9] using differentiated, embryo-derived fetal or adult cells. However, different efficiencies using different cell types are reported [2, 10]. NT technology was also successfully used to generate transgenic sheep and cattle [11, 12]. A major advance has now been achieved with the production of live transgenic sheep derived from fibroblasts that were genetically modified by homologous recombination [13]. This powerful technique is highly important for developing animal models for various diseases, providing organs for xenotransplantation [14], and many other applications [15, 16].

Source of Oocytes, In Vitro Maturation, and Enucleation

Oocytes were aspirated from ovaries that had been obtained from an abattoir. Oocytes derived from gilts were matured in a defined protein-free medium (TCM199, supplemented with 0.1% polyvinyl alcohol, 0.1 mg/ml cyto- cline, 10 ng/ml epidermal growth factor [EGF], 0.91 mM Na-pyruvate, 3.05 mM D-glucose, 0.5 µg/ml FSH, 0.5 µg/ml LH, 75 µg/ml penicillin, and 50 µg/ml streptomycin [17]). Oocytes from sow ovaries were purchased from BOMED (Madison, WI); they were matured in TCM199-Hepes supplemented with 5 µg/ml insulin, 10 ng/ml EGF; 0.6 mM cysteine, 0.2 mM Na-pyruvate, 3 µg/ml FSH, 25 µg/ml gentamicin, and 10% porcine follicular fluid, and were shipped overnight in maturation medium. In a previous experiment, the development of parthenogenetically activated oocytes (from gilts) matured in both media was compared, and no difference was observed (unpublished data). In addition, there was no difference after in vitro fertilization and culture to the blastocyst stage [18]. For NT experiments, metaphase II (MII) oocytes of both sources were treated the same.

Isolation, Transfection, and Preparation of Donor Cells

For experiment 1, primary cell cultures of porcine fibroblast cells were derived from fetuses on Day 28 of gesta-

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Cytoplast-fibroblast complexes were placed between two contact between the cytoplasm and the donor cell. Reconstituted into the perivitelline space with the same pipette that a smooth membrane [21]). A single cell was then transfected for 45±60 min. Subsequently, 800 was added. The suspension was kept at room temperature for in vitro development of porcine NT embryos derived from four different clones of transgenic fibroblast cells.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Oocyte Embryos</th>
<th>Cleaved</th>
<th>% Mor/ Blast</th>
<th>% Blast</th>
<th>Nuclei/Blast</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1-D1</td>
<td>Gilts</td>
<td>151</td>
<td>24 (15.9)</td>
<td>17 (11.3)</td>
<td>28.5 ± 12.1</td>
</tr>
<tr>
<td>Sow</td>
<td>76</td>
<td>49 (64)</td>
<td>21 (27.6)</td>
<td>16 (21.1)</td>
<td>32.3 ± 15.3</td>
</tr>
<tr>
<td>F1-D7</td>
<td>Gilts</td>
<td>134</td>
<td>12 (9.0)</td>
<td>8 (6.0)</td>
<td>20.6 ± 7.2</td>
</tr>
<tr>
<td>Sow</td>
<td>97</td>
<td>67 (69)</td>
<td>14 (14.4)</td>
<td>11 (11.3)</td>
<td>23.9 ± 7.9</td>
</tr>
<tr>
<td>F1-E5</td>
<td>Gilts</td>
<td>118</td>
<td>51 (43)</td>
<td>7 (5.9)</td>
<td>16.3 ± 7.5</td>
</tr>
<tr>
<td>Sow</td>
<td>114</td>
<td>65 (57)</td>
<td>16 (14.0)</td>
<td>11 (9.6)</td>
<td>23.6 ± 10.1</td>
</tr>
<tr>
<td>F1-F3</td>
<td>Gilts</td>
<td>136</td>
<td>67 (49)</td>
<td>7 (5.1)</td>
<td>31.5 ± 15.9</td>
</tr>
<tr>
<td>Sow</td>
<td>121</td>
<td>80 (66)</td>
<td>18 (14.9)</td>
<td>14 (11.6)</td>
<td>29.3 ± 8.6</td>
</tr>
</tbody>
</table>

* Data are summarized from five replicates. Serum-starved (0.5%, 6 days); % Mor, morulae, cleaved embryos with more than eight nuclei and without a cavity; Blast, blastocysts, cleaved embryos with a clear cavity. Percentages are based on the number of reconstructed embryos cultured on Day 2. Values in the same column differ \( P > 0.1 \).

RESULTS

In the first experiment, the feasibility of small cycling versus small serum-starved fibroblasts (same cell line, same passage number) for the use as donor karyoplasts was investigated. The fusion rate was similar for both groups (62% versus 58% for serum-starved and cycling cells). The use of serum-starved cells (G0) did not show a positive effect on the development of NT embryos in vitro, when compared with cycling cells (G1, Table 1).

For subsequent experiments, cycling fibroblast cells were used as donor karyoplasts. Four different clonal lines of transgenic fetal fibroblasts derived from the same fetus were fused to both sources of oocytes, and their development in vitro was investigated. Results of the in vitro development of NT embryos derived from the four cell lines using both oocyte sources are presented in Table 2.

The main effects of the model for each endpoint were clonal line and oocyte source. The cleavage rate was higher in the sow-derived oocytes.

**Statistical Analyses**

To compare the results using serum-starved versus cycling fibroblasts, we used the Fisher exact test (www.physics.csbsju.edu/stats/fisher.form.html). To compare the development of NT embryos derived from one cell population and oocytes from different sources, the percentage data per replication was arcsin-transformed and entered into SYSTAT, and multivariate general linear hypothesis was performed [23]. Fibroblast-clone and source of oocytes were the main effects. If a difference was detected, then the least significant difference program was invoked to determine where those differences existed.

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for sow oocytes than it was for gilt oocytes ($P = 0.003$). Development to the morula and blastocyst ($P = 0.014$) stages, or just to the blastocyst ($P = 0.011$) stage was also higher for sow oocytes than it was for gilt oocytes. In general, the proportion of embryos that developed to blastocysts was double when oocytes derived from sows were used as opposed to oocytes from gilts. There was no effect of oocyte source on the quality (number of nuclei) of blastocysts.

**DISCUSSION**

For our NT experiments, we decided to use “blind enucleation,” which avoids the exposure of oocytes to bisbenzimide (Hoechst 33342) and UV light. In a previous study [24], it was shown that parthenogenetically activated porcine embryos showed lower quality when the oocytes were stained with Hoechst (without UV exposure). According to the findings in pigs, Dominko et al. [25] and Yassin et al. [26] found negative effects of bisbenzimide in combination with brief UV exposure on the development of bovine oocytes and embryos.

One important factor that is believed to influence the efficiency of NT is the cell cycle stage of the donor cell. Whereas some authors claim that the use of cells in G0 is required for complete reprogramming [3, 4], others used cycling donor cells in presumptive G1 and obtained offspring [12]. The cell cycle stage is a subject of debate, mainly because there is no system thus far that provides a 100% synchronization of cells in a certain stage of the cell cycle [27]. Therefore, the use of cells in a stage of the cell cycle other than the desired one cannot be excluded. In this study we investigated the development of porcine NT embryos derived from small serum-starved (presumptive G0) and cycling (presumptive G1) fetal fibroblast cells. In contrast to comparative studies in cattle [7, 28], in which the use of serum-starved donor cells resulted in better development, we found no effect of serum starvation on the in vitro development of porcine NT embryos.

Donor cells derived from preimplantation embryos [3, 29], fetal tissues [8, 11, 12], and adult tissues [4, 6, 9, 10, 30] have been used to generate live offspring. However, efficiencies varied with the use of different cell types [2] and also with the use of different passage numbers [30]. In this report we investigated the feasibility of various clones derived from the same primary culture of fetal fibroblast cells for use in NT. Surprisingly, differences in the in vitro development of NT embryos were observed. Differences in the fusion rate between the cell lines used are mainly caused by differences in the size of the cells and also by their surface and membrane stability. Whereas in the cell line that resulted in the lowest fusion rates (F1-E5) the majority of cells showed a slightly rough surface, cells of line F1-D7 were bigger in size (14–16 μm versus 16–18 μm) and therefore resulted in higher fusion rates. It is interesting that no differences in fusion rates were observed when oocytes derived from sows were used. This may be due to differences in the surface structure of the plasma membrane between both oocyte sources. There is a possibility that the surface of sow oocytes is more homogenous, which thereby reduces the influence of the donor cell on the fusion rate.

Unfortunately, the limited number of cells did not allow an extensive characterization of the cell lines. However, a variation in the extent of chromatin methylation or histone acetylation may alter gene expression [31] and could therefore be (in part) responsible for differences in in vitro development after NT. Also, mutations or chromosomal abnormalities that cause reduced developmental ability for a certain clone cannot be excluded. Although it is unlikely in the case of a heterozygous modification, effects due to integration of the vector into the genome cannot be entirely excluded. Although the rate of blastocyst formation and average cell number varied between clonal lines, no characteristic, gross morphological differences were seen in blastocysts of similar cell number derived from different fibroblast clones. In a study in sheep, Wells et al. [29] compared the use of in vivo-matured versus in vitro-matured oocytes for use in NT. However, a factor that sometimes seems to be neglected or underestimated is the age of the cytoplast donor for NT experiments. We investigated the influence of the donor source for oocyte collection. For NT in pigs, the reason for using in vitro matured oocytes varies derived mainly from gilts is primarily one of economics, and because sow ovaries are not available in most areas. However, a limited number of sow oocytes is commercially available. Therefore, we also investigated the development of NT embryos using oocytes from sows. In a preliminary experiment we compared the two maturation systems using the same oocyte source (gilts). After parthenogenetic activation no difference in the development to the blastocyst stage or in the nuclear numbers of blastocysts was found (data not shown). After NT, a significantly higher percentage of embryos developed to blastocysts when sow oocytes were used. In contrast, there was no effect of the oocyte source on the quality of blastocysts. We cannot exclude that sow oocytes may react differently to the maturation systems. There is a slight possibility that development is influenced by the oocyte source and the maturation medium. However, the data indicate that the use of cytoplasts from sow oocytes (in combination with the follicular fluid-containing maturation system) seems to be beneficial for use in NT.

In conclusion, this study suggests 1) that cells in presumptive G0 and cells in presumptive G1 are equally suitable for NT in pigs, and 2) that even within a cell line there are subpopulations of cells that are better suitable as donor karyoplasts for NT than others. To minimize the cost for recipient animals in attempts to produce cloned offspring it may therefore be helpful to culture subpopulations of various cell lines and store aliquots for further use. This will enable subpopulations to be investigated and selected for superior in vitro development before use in an NT embryo transfer scheme.

**REFERENCES**


