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Reprogramming of human somatic cells by embryonic stem cell cytoplast

Nick Strelchenko, Valeri Kukharenko, Artem Shkumatov, Oleg Verlinsky, Anver Kuliev[1], Yury Verlinsky

Reproductive Genetics Institute, 2825 North Halsted Street, Chicago, IL 60657, USA

[1]Correspondence: Tel: +1 773 4724900; Fax: +1 773 8715221; e-mail: anverkuliev@hotmail.com

Abstract

Somatic cell nuclear transfer (SCNT) provides the basis for the development of patient-specific stem cell lines. Recent progress in SCNT suggested the presence of reprogramming factors in human embryonic stem (hES) cells, although no method is currently available for replacement of nuclei of hES cells by somatic cell nuclei. An original technique has been developed, involving the fusion of different types of somatic cells with hES cells, which allowed a complete replacement of the nuclei of hES cells by nuclei of somatic cells. The resulting 'cybrids' were demonstrated to have the genotype of the donor somatic cells and 'stemness' of the recipient hES cells. However, the colonies isolated from the resulting fusion contained a mixture of these cybrid cells with the cells with the recipient nuclei, as well as hybrid cells containing both donor and recipient nuclei, so future purification will be necessary before the technique can be considered for future practical application.

Keywords: *cybrids, human embryonic stem cells, patient-specific stem cells, reprogramming of human adult cells, somatic cell nuclear transfer*

Introduction

Somatic cell nuclear transfer (SCNT) offers potential for obtaining patient-specific stem cells. SCNT was initially performed by fusion of karyoplasts and cytoplasts, isolated from parental cell lines prior to fusion, which resulted in the so-called 'reconstituted cells' or 'recon' (Veomett *et al.*, 1974). The enucleation of one of the parental cells was then omitted, so the entire parental cell was fused with the cytoplasts (Wallace *et al.*, 1975). The cell type resulting from this procedure was called a cytoplasm hybrid or 'cybrid' (Bunn and Eisenstadt, 1977).

The cytoplast reprogramming property was observed in nuclear tumourigenicity repression experiments, using the cytoplast of non-tumourigenic cells (Howell *et al.*, 1978). Alternatively, the immortality of cybrids was observed after fusion of lymphocyte nuclei with cytoplasts of the L929 mouse cell line (Abken *et al.*, 1986). The hybrid clones of nullipotent mouse embryonal carcinoma formed solid tumours after injection into syngenic mice.

It was also demonstrated that the fusion of nullipotent embryonal carcinoma F9 and somatic cells of differentiated tissues, such as thymus and lens, resulted in pluripotent hybrid cell lines, exhibiting a wide spectrum of differentiated cell types, including neural tubes, cartilage, skeletal muscle and ciliated epithelium. Again, when injected into syngenic mice they formed solid tumours, cells from which differentiated into different cell types in culture (Atsumi *et al.*, 1982). Several clones were isolated by fusion of mouse pluripotent embryonic teratocarcinoma cells with cytoplasts of rat myoblasts, demonstrating the capability of somatic cell cytoplasm to induce differentiation of teratocarcinoma stem cells (Iwakura *et al.*, 1985).

Successful reprogramming of human somatic cells has recently been achieved by heterologous oocytes from volunteers, showing the feasibility of derivation of patient-specific embryonic stem (ES) cell lines (Hwang *et al.*, 2005). The human ES (hES) cell lines were also obtained by human fibroblast nuclear transfer into enucleated rabbit oocytes (Chen *et al.*, 2003). Finally, nuclear reprogramming of somatic cells has been performed by fusion with ES cells, showing the capability of ES cells to reprogramme adult somatic cells, similar to that shown for oocytes (Tada *et al.*, 1997, 2001; Cowan *et al.*, 2005). However, the resulting hybrid cells contained the nuclei of both somatic and hES cells (Cowan *et al.*, 2005). The reason for this was that the recipient hES cells were not enucleated.

The objective of the present study was to develop a method for obtaining individual-specific hES cells using the reprogramming factors present in cytoplast of hES cells in fusion experiments with different adult somatic cells.

Materials and methods

Experiment design

hES cell lines with female karyotype 46, XX served as a source of the recipient cytoplast for reprogramming of isolated adult lymphocytes, fibroblasts and umbilical cord blood cells with male karyotype 46, XY. The outcome of the reprogramming experiments was evaluated by the appearance of cells with male karyotype and octamer-binding transcription factor-4 (Oct-4) and tumour rejection antigen-2–39 (TRA-2–39) markers in the resulting proliferating colonies.

Suppression of hES cell nuclei

The lowest concentration of mytomycin C for suppression of hES cell nuclei was experimentally established to be 0.5 ng/ml for 3 min, after which no live colonies were observed. Subsequent fusion of fibroblasts or lymphocytes with these mitotically blocked hES cells resulted in the production of heterokaryons, but no cybrid or hybrid cells were observed.

Preparation of hES cells for enucleation

The hES cell lines were adapted to grow on Matrigel, with the growth medium consisting of Alpha minimum essential medium (Gibco, Grand Island, New York, USA) supplemented 15% of serum replacement medium (SR1) (Gibco), beta-mercaptoethanol, fibroblast growth factor-b (bFGF), 5 microgram/ml and transforming growth factor, 0.4 ng/ml. A monolayer of the hES cells was prepared by overnight plating of the cells at an average density of 200 x 10^3–250 x 10^3 per 110 microlitre of media per 18 mm coverslip.

Enucleation of hES cells

Enucleation of hES cells was performed on round coverslips with the cells inserted upside down into 50 ml centrifuge tubes (Nalgene, cat. no. 3117–0500, PC; Nalge Nunc International, Rochester, NY, USA), containing 6.6% Ficoll-400 solution dissolved in phosphate buffered saline (PBS) and 10% SR1, supplemented with 10 microgram /ml of cytochalasin D and 3 microgram/ml of Nocodazol. To avoid rounding up and, as a consequence, getting hES cells to the bottom of the centrifuge tubes without enucleation, caused by the presence of cytochalasin D in growth medium during centrifugation, Ficoll F400 was added. The nuclei were removed by centrifugation at 25,000 *g* for 50 min, using an RC5C centrifuge with HB4 swinging bucket rotor at 34°C. The coverslips with the resulting hES cytoplasts were then washed and recovered in normal growth medium for 60–90 min.

The enucleation efficiency was evaluated by Hoechst 33342 at a final concentration of 1 microgram/ml.

Fusion of hES cytoplasts with somatic cells

Fusion was performed by 42% PEG (Sigma, Poole, Dorset, UK) and 4% DMSO (Sigma). In experiments with human fibroblasts, a trypsinized cell suspension was layered on top of the hES cytoplasts (~300 x 10^3 units per coverslip). In a separate experiment, fusion was attempted using isolated fibroblast nuclei.

In fusion experiments with lymphocytes and umbilical cord blood cells, the cells were isolated from diluted heparinized whole blood in a one-step ladder density gradient Isopaque 1.083 (Sigma). The isolated 'buffy coats' were washed 3 times with a large volume of PBS (Ca, Mg-free) and layered on the top of hES cytoplasts, with approximately 2 x 10^6 cells in 100 microlitre of normal PBS (sedimentation occurred within 40–60 min). In a separate experiment, phytohaemagglutinin 5 microlitre per 100 microlitre was applied to improve contact between lymphocytes and ES cytoplasts.

After the donor cell nuclei were placed on top of the hES cytoplasts, the cell layers were washed with PBS and 1 ml PEG solution was added for 1 min. Then after dilution with equal amount of normal PBS, followed by mixing and further dilution with 2 ml of normal PBS for 1 min, 250 microlitre of SR1 was added.

Isolation of colonies

The colonies of typical hES cell morphology, appearing on coverslips within 7–10 days of culture, were treated for 5 min with 0.1% EGTA, the action of which was then removed by SR1, and a small cluster of cells was transferred into a separate well of a 48-well dish by an individual micropipette with internal diameter of 80–90 micrometre. Cells grew in wells for 2 weeks and were then passaged and analysed by fluorescence in-situ hybridization (FISH; Verlinsky and Kuliev, 2005).

Study of reprogrammed cybrids

To identify colonies *in situ,* the cells on coverslips were stained by FITC for TRA-2–39 (green fluorescence), which is a pluriopotency marker of alkaline phosphatase (AP), and TRITC for Oct-4 (red fluorescence). This was done 48 h after fusion, followed by FISH analysis at 72–96 h. In a separate experiment, the colonies were isolated and analysed by FISH 2 weeks after fusion.

Results

The results showed, that the hES cell enucleation efficiency improved with increased osmomolarity, from 27% at 160 mOsm to 45% at 170 mOsm, and 98–99% at 180–190 mOsm. The efficiency then decreased to 82%, despite the further increase in osmomolarity to 210 mOsm.

The hES cytoplast fusion efficiency also varied with different donor cell types, as well as with the concentration of PEG (**Table 1**). Only 5.6% of fibroblasts formed heterokaryons using 40% of PEG and 4% of DMSO, the fusion efficacy growing with increasing concentrations of PEG, which, however, also led to an increase in the number of dead cells, the optimal PEG and DMSO concentrations being 42 and 4% respectively.

Table 1. Fusion efficacy of somatic cells and human embryonic stem cells.

DMSO 4% + PEG (%)	40	42	45	50 (no DMSO)
Fusion of fibroblasts (%)	5.6	12.8	15.6	15.3
Dead cells (%)	6.2	8.8	28.5	64.3
Fusion of lymphocytes (%)	12.8	18.4	26.4	18.6
Dead cells (%)	7.3	9.3	31.5	54.8

DMSO = dimethyl sulphoxide; PEG = polyethylene glycol.

As seen from **Figure 1**, the somatic cell nuclei fused with hES cell cytoplast proceeded with cell division, resulting in the establishment of cybrid cells with male karyotype (**Figure 2**) with typical hES cell morphology and stemness, shown by positive Oct-4 and TRA-2–39, confirming the replacement of hES cell nuclei by the nuclei of somatic cells.

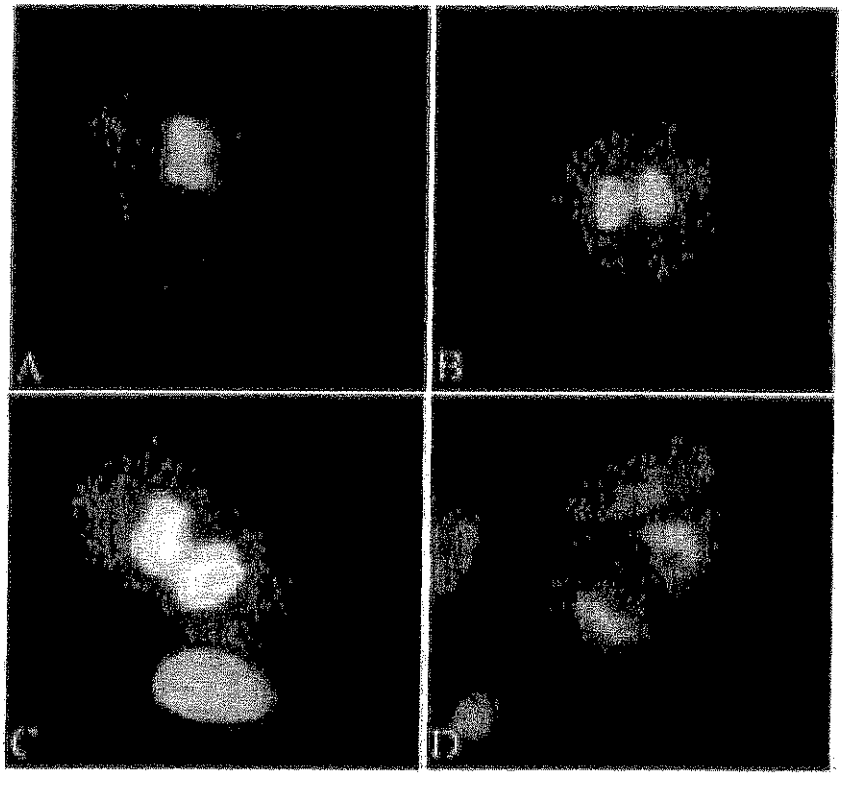

Figure 1. Mitosis in human embryonic stem (hES) cell cytoplast cybrid following fusion. **A**, **B**, **C**, **D**: Increasing appearance in background of red fluorescence [octamer binding transcription factor-4 (Oct-4)] surrounded by ring of green fluorescence [tumour rejection antigen-2-39 (TRA-2-39)], with no nucleoli being present. Blue fluorescence: DAPI, showing the chromosome location. Original magnification x40.

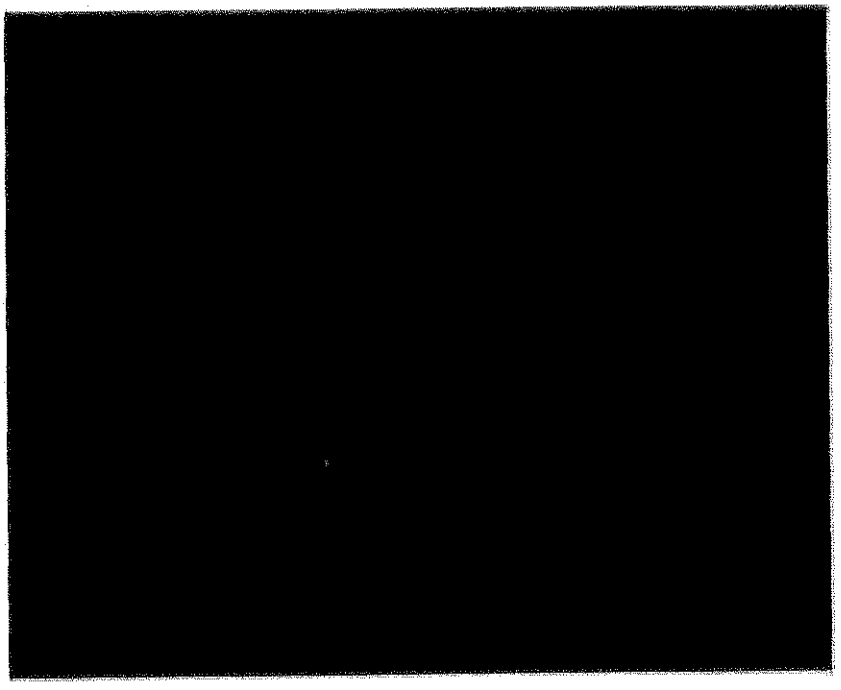

Figure 2. Colony of proliferated cybrid cells with 46,XY nuclei detected by FISH probes. Y chromosome – aqua; X chromosome – green; chromosome 18 – red. Original magnification x40.

From 10 to 40 colonies were obtained following 1-week culture of the resulting hybrid cells on 18 mm coverslips (**Table 2**). The resulting colonies contained cells with different karyotypes, including the 46,XY cybrid cells, representing a complete replacement of hES cell nuclei by the nuclei of donor somatic cells, as well as 46,XXY and 92, XXYY cells, representing hybrids between the donor and non-enucleated hES-cells. As seen from **Figure 3**, the hES cell markers were positive in cybrid colonies, which were cultured for many passages.

Table 2. Analysis of 41 isolated cybrid clones.

Fusion	*No. of isolated colonies*	*XY/XX*	*XXYY/XX*	*XXY/XX*	*XX*
A	8				8
B	8				8
C	3				3
D	7		1	1	5
E	7	1			6
F	8	1		1	6

Figure 3. Proliferating cybrid colony growing on human feeder layer (passage 2). Green fluorescence is TRA-2-39, red fluorescence is Oct-4. Cells in colony have a typical morphology of embryonic stem cells, which were split for FISH analysis (see **Figure 2**) and for freezing. Rest of cells continued proliferating until passage 8. Original magnification x20.

Discussion

Presented results provide the first evidence of complete replacement of hES cell nuclei by the nuclei of somatic cells. The typical ES cell morphology and the presence of Oct-4 and TRA-2–39 confirm the 'stemness' of the resulting cybrids. The hES cell cytoplasm was clearly responsible for reprogramming of lymphocytes or fibroblast nuclei as a result of fusion. The efficacy of the method may be further improved by synchronizing the recipient hES cell cytoplasts and donor somatic cell nuclei cell cycle at G0/G1.

As shown in **Figure 1**, mitotic donor (lymphocytes) nuclei in cytoplast (green fluorescence) start synthesizing Oct-4, not typical for the donor nuclei, with its gradual increase despite origination from differentiated cell. Similarly, the colonies deriving from the differentiated cell nuclei fusion with cytoplasts of hES cells also expressed Oct-4/TRA-2–39 (**Figure 1**). FISH analysis provided the evidence that the resulting colonies contained cells with XY karyotype (**Figure 2**), as well as mixed with recipient XX nuclei. All isolated colonies were also positively tested for SSEA3, SSEA4, TRA-1–60, TRA-1–80 (data not shown) as well as Oct-4/TRA-2–39.

Table 2 shows that only a small proportion of cybrid cells were derived from donor lymphocytes or fibroblasts, with no pure population of cybrid stem cells isolated at the present time. In a similar mouse experiment, reprogramming was demonstrated in only a single colony of hybrid cells expressing Oct-4 (Do and Scholer, 2004). This may be explained by the smaller size of mouse ES cells (10–15 mm) compared with human ES cells (18–22 mm), containing only about half the amount of cytoplast in comparison with human ES cells.

Although the presented data provide evidence for obtaining hES cells with the required somatic cell nuclei, the problem still remains of how to isolate the pure population of hES cell cybrid colonies, which will require the development of special selective media systems or other alternative methods. Further studies will be also necessary to investigate the extent to which the hES cell cybrids can be reprogrammed, in comparison with those created by SCNT (Hwang *et al.*, 2005; Stojkovic *et al.*, 2005). Although the majority of markers are present in the resulting reprogrammed cells, the experience of cloning animals (Pace *et al.*, 2002) has

shown that some groups of genes were not expressed in cloned animals, the reprogramming being incomplete even after birth. The research efforts in the above directions will allow more efficient production of pure cybrid hES cell colonies required for the development of cellular therapy.

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