

Optimisation of transfection conditions of CD34⁺ hematopoietic cells derived from human umbilical cord blood

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Received: 21 May, 2002; revised: 12 August, 2002; accepted: 26 August, 2002

Key words: umbilical cord blood, CD34⁺ cells, transfection, green fluorescent protein

Human umbilical cord blood is frequently used as a source of transplantable hematopoietic cells and more recently as a target of gene therapy – a new approach for treatment of various disorders. The aim of our study was optimisation of the transfection conditions of cord blood-derived CD34⁺ hematopoietic cells. Mononuclear cells fraction was isolated from cord blood samples by density gradient centrifugation. Subsequently, CD34⁺ hematopoietic cells were separated on immunomagnetic MiniMACS columns. Pure population of CD34⁺ cells was incubated in a serum free medium supplemented with thrombopoietin, stem cell factor and Flt-3 ligand for 48 h and then transfected with plasmid DNA carrying the enhanced version of green fluorescent protein (EGFP) as a reporter gene. We studied the influence of various pulse settings and DNA concentrations on the transfection efficiency, measured by flow cytometry as the fluorescence of target cells due to the expression of EGFP. The optimal settings were as follows: 4 mm cuvette, 1600 μ F, 550 V/cm, and 10 μ g of DNA per 500 μ l. With these settings we obtained a high transfection frequency (41.2%) without a marked decrease of cell viability. An increase of the pulse capacitance and/or of DNA concentration resulted in a greater electroporation efficiency, but also in a decrease of cell viability. In conclusion, the results described here allow one to recommend electroporation as an efficient method of gene delivery into CD34⁺ hematopoietic cells derived from human umbilical cord blood.

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Abbreviations: EGFP, enhanced green fluorescent protein; FCS, fetal calf serum; Flt-3L, Flt-3 ligand; FSC, forward scatter; HSA, human serum albumin; HSPC, hematopoietic stem/progenitor cells; IMDM, Iscove's modified Dulbecco's medium; PBS, phosphate-buffered saline; PE, phycoerythrin; PI, propidium iodide; SCF, stem cell factor; SSC, side scatter; TPO, thrombopoietin; UCB, umbilical cord blood.

Since the first clinical application of human umbilical cord blood (UCB) (Gluckman *et al.*, 1989), there has been an increasing use of UCB as an alternative to bone marrow and mobilized peripheral blood source of transplantable hematopoietic stem/progenitor cells (HSPC). More than 1000 cord blood transplantations have been performed to date, in patients with various disorders, including malignant diseases, bone marrow failures, hemoglobinopathies and inborn errors of metabolism (Gluckman, 2000).

The umbilical cord blood-derived HSPC differ from their counterparts present in adult bone marrow or in mobilized peripheral blood. The content of hematopoietic progenitor cells is similar in UCB and in adult bone marrow, however, the former contains a higher proportion of primitive hematopoietic cells, such as the multipotent colony forming cells (Hows *et al.*, 1992; Mayani *et al.*, 1998), long-term culture-initiating cells (Pettengell *et al.*, 1994) and SCID-repopulating cells (Wang *et al.*, 1997). It has been demonstrated that the proliferative potential of UCB-derived HSPC is higher than that of adult bone marrow or peripheral blood cells (Hows *et al.*, 1992; Traycoff *et al.*, 1994; Mayani & Lansdorp, 1998). Due to the low alloreactivity of UCB lymphocytes the incidence and severity of the graft-versus-host disease after UCB transplantation was reduced, when compared to bone marrow grafts even in the case of partially HLA-mismatched patients (Gluckman, 2000).

Gene therapy offers a strategy of treatment of various acquired and genetic disorders. The HSPC are an attractive target for gene incorporation, because they can be easily isolated, genetically modified *ex vivo* and, after verifying their functional status, transplanted to the patient. The transplantation should result in long-term repopulation of hematopoietic and immune systems with a sufficient contribution of genetically modified cells (Capel *et al.*, 1990). However, for the two decades since the development of gene delivery technology, for-

eign gene expression in HSPC has been difficult to achieve. A successful attempt of clinical application of gene therapy has to involve (i) optimisation of short-term culture conditions preventing apoptosis and supporting the divisions of cells without a loss of their pluripotency; and (ii) optimisation of gene transfer strategy to increase its efficiency without the reduction of cell viability and alteration of the functional status of the HSPC.

Several approaches have been taken to incorporate foreign DNA into HSPC. The most commonly used are viral and nonviral vectors delivered via different ways. Here we describe transient transfection of HSPC by electroporation with efficiency comparable to that of other DNA delivery methods.

MATERIALS AND METHODS

Cell isolation. Cord blood samples, collected from full-term normal deliveries, were diluted 1:1 with phosphate-buffered saline (PBS) (Gibco). Subsequently, mononuclear cells were isolated by centrifugation on Ficoll (Sigma; 1.077 g/ml) at $400 \times g$ for 40 min. The mononuclear cells were collected, washed twice in IMDM (Gibco) supplemented with 10% FCS (Cytogen) and resuspended in PBS with the addition of 0.5% human serum albumin (HSA) (Biomed).

The CD34⁺ fraction was isolated immunomagnetically using MS⁺ MiniMACS columns and the CD34 Direct Isolation Kit (Miltenyi Biotec) according to the manufacturer recommendations. In brief, after adding FcR Blocking Reagent, cells were labelled with MACS CD34 Microbeads for 30 min at 6–12°C. Subsequently, the labelled cells were enriched by passing the cell suspension through a column placed in a magnetic field. The positively selected cells were removed from the magnetic field and washed out from the column. The purity of the CD34⁺ cell population was determined by flow cytometry immediately after isolation.

Cell stimulation, electroporation, and posttransfection incubation. Target cells were cultured in 25 cm² flasks at a cell density of 1×10^5 /ml in a serum-free medium for hematopoietic stem and progenitor cells (CellGro) supplemented with growth factors: stem cell factor (SCF) (20 ng/ml; PeproTech EC), trombopoietin (TPO) (50 ng/ml; PeproTech EC) and Flt-3 ligand (Flt-3L) (50 ng/ml; PeproTech EC). After 48 hours of incubation at 37°C in a humidified atmosphere of 5% CO₂, cells were resuspended at 1×10^5 /ml in the CellGro medium supplemented with 20% HSA. DNA plasmid (pEGFP-N2; Clontech) carrying EGFP as a reporter gene under control of the CMV promoter, was added to the 400 μ l of cell suspension. The electroporation was performed in a 4 mm cuvette using a BTX ECL600 instrument (Gentronix). The voltage was set at 550 V/cm. The capacitances and the amount of DNA used for electroporation are shown in Table 1 and Table 2, respectively. Cells pulsed without the presence of DNA served as a control. Immediately after the electroporation cells were transferred to fresh CellGro medium containing growth factors and incubated for 48 h at 37°C, 5% CO₂. Subsequently, the transfection

efficiency was measured by flow cytometric analysis of EGFP expression.

Flow cytometric analysis. The assayed cells were acquired using the FACS Calibur flow cytometer (BD) equipped with an argon-ion laser tuned at 488 nm. The analysis was performed by use of the CellQuest software (BD).

For CD34⁺ cell quantitation the samples were labelled with mouse monoclonal anti CD34 antibody (anti HPCA-2, BD) directly conjugated to phycoerythrin (PE). An isotype-matched PE-conjugated antibody (IgG₁) served as a control. The flow cytometric analysis consisted in the exclusion of debris and gating of the low-density cells on the forward scatter (FSC) *versus* side scatter (SSC) dot plot (Fig. 1). To estimate the percentage of CD34⁺ cells, a second plot (SSC *versus* CD34⁺ fluorescence) was drawn and PE-positive cells were gated. The absolute number of CD34⁺ cells was calculated on the basis of the total number of nucleated cells.

Directly prior to flow cytometric acquisition, the electroporated cells were stained for cell viability assay with propidium iodide (PI) (50 μ g/ml; Sigma) according to the method described by Wu *et al.* (2001b). The analysis

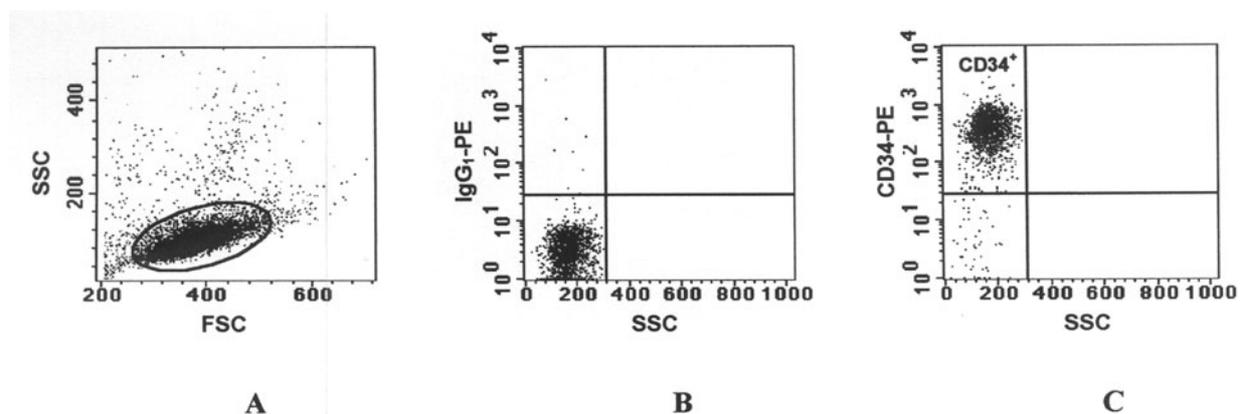


Figure 1. Flow cytometric analysis of UCB-derived CD34⁺ cells.

(A) Gating of cells and debris exclusion on a FSC/SSC dot plot; (B) evaluation of the frequency of non-specific antibody binding on a SSC *versus* IgG₁-PE fluorescence dot plot of cells stained with control antibody; (C) evaluation of the frequency of CD34⁺ cells on a SSC *versus* CD34-PE dot plot of cells stained with anti-CD34 monoclonal antibody.

(Fig. 2) consisted in gating of the cells on the FSC/SSC dot plot for exclusion of contaminating debris and cell doublets. The gated cells were subsequently evaluated by drawing the second plot (EGFP fluorescence *versus* PI fluorescence) for EGFP expression in the viable cells. Representative flow cytometric analyses of the CD34⁺ cells electropulsed with or without plasmid DNA, showing their viability

2400 μ F. However, application of the latter capacitance resulted in a significant decrease of cell viability (Table 1).

We also examined the effect of DNA concentration on the effectiveness of gene delivery. CD34⁺ cells were electroporated at the optimal capacitance (1600 mF) and in the presence of various concentrations of plasmid DNA. As it was clearly shown (Table 2), the in-

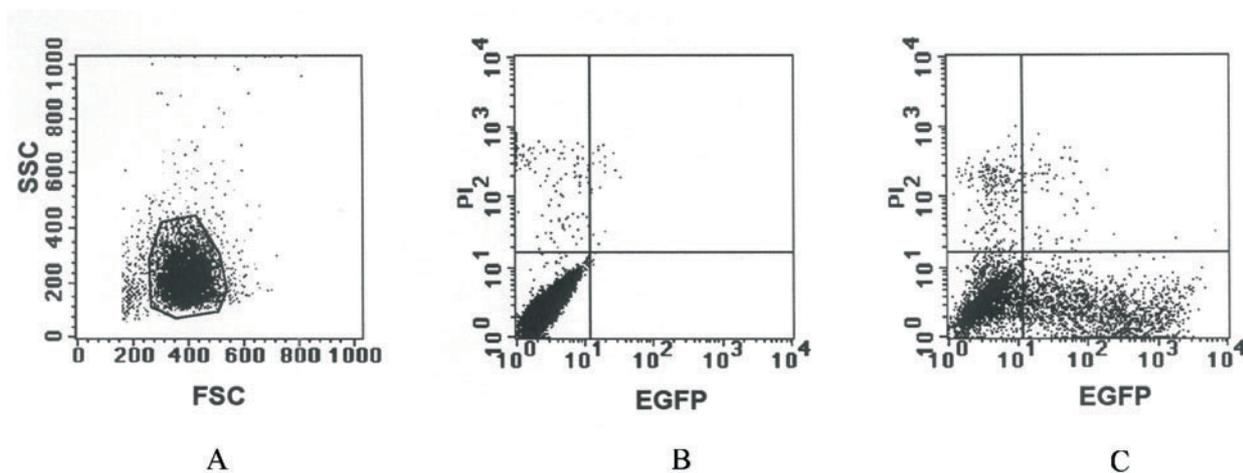


Figure 2. Flow cytometric analysis of electroporated CD34⁺ cells isolated from umbilical cord blood.

Gating of cells and debris exclusion on a FSC/SSC dot plot (A); evaluation of the transfection efficiency on the EGFP expression *versus* PI fluorescence (cell viability) dot plot of cells electroporated without (B) or with (C) plasmid DNA.

and the expression of EGFP in the electroporated cells, are demonstrated in Figs. 2B and 2C, respectively.

RESULTS

In our study we evaluated the effect of electroporation conditions (pulse capacitance and DNA concentration) on the transfection efficiency and the viability of the UCB-derived CD34⁺ cells. As demonstrated in Fig. 2C, cells pulsed in the presence of the DNA and incubated in the CellGro medium supplemented with TPO/SCF/Flt-3L for 48 h showed expression of EGFP. The highest (over 40%) percentage of cells expressing the reporter gene was observed among the CD34⁺ cells electroporated with the capacitances of 1600 μ F and

crease of transfection efficiency due to the application of high plasmid DNA concentration was paralleled by a decrease of cell viability.

DISCUSSION

Clinical application of genetically modified CD34⁺ hematopoietic stem/progenitor cells transplantation rapidly becomes an area of intensive investigations. Gene therapy requires an efficient and safe procedure of gene delivery into HSPC. The first attempt at gene delivery into HSPC was the use of adenoviral or retroviral vectors (Baum *et al.*, 1996; Frey *et al.*, 1998). The main disadvantage of application of viral vectors is the possibility of viral infection and their immunogenicity. Moreover, adenoviral vectors are not suitable for

Table 1. The effect of electroporation capacitance on transfection efficiency and cell viability

Capacitance	DNA	Pulse length	Viability	Efficiency
μF	μg	ms	%	%
1600	0	38.4	83.1	0.0
1000	10	26.0	93.9	20.3
1200	10	31.8	89.3	23.4
1600	10	39.1	82.1	41.2
2400	10	62.0	68.8	43.3

the treatment of disorders requiring stable gene integration into the genome of target cells and the efficiency of retroviral vectors is usually poor (Corrias *et al.*, 1998). Virus-mediated gene incorporation typically includes the long-lasting stimulation with growth factors (von Kalle *et al.*, 1994), which causes HSPC differentiation and loss of their self-renewing capacity (Corrias *et al.*, 1998). Moreover, a number of diseases (i.e. some leukemias and lymphomas) are resistant to virus-mediated gene delivery (Brenner, 1997).

Electroporation is an attractive method of gene transfer into HSPC for clinical applications because it is free from biological contamination and possibility of immune reaction.

cells (Fig. 3). The plasmid carrying an enhanced version of green fluorescent protein (EGFP) used in our experiment is a frequently applied marker of gene incorporation (Chalfie *et al.*, 1994). Cells expressing this molecule become fluorescent and detectable by flow cytometry or fluorescent microscopy (Bierhuizen *et al.*, 1997; Limon *et al.*, 1997; Persons *et al.*, 1997). To assess proliferating cells, a condition necessary for non-retroviral plasmid incorporation into the target genome, we stimulated cell divisions by the addition of TPO, SCF and Flt-3L to a serum-free medium. As reported previously, such medium promotes rapid exit from G₀ phase, but prevents cell differentiation (Li *et al.*, 2001). It has been

Table 2. The effect of DNA amount on transfection efficiency and cell viability

Capacitance	DNA	Pulse length	Viability	Efficiency
μF	μg	ms	%	%
1600	0	40.2	86.5	0.0
1600	5	41.6	83.9	28.9
1600	10	42.7	76.7	38.7
1600	20	43.6	71.1	46.5

Electrotransfection results in sustained foreign gene expression in CD34⁺ cells (Toneguzzo & Keating, 1986; Van Tendeloo *et al.*, 2000; Wu *et al.*, 2001a). In this paper we report the optimisation of gene incorporation parameters into CD34⁺ cells derived from UCB. Enrichment of CD34⁺ cells on immunomagnetic columns allowed us to perform the experiments with a pure population of these

demonstrated that short-term culture of CD34⁺ cells in a TPO/SCF/Flt-3L serum-free medium resulted in an increase of the cycling CD34⁺ cells fraction and in augmenting the efficiency of reporter gene transfer. Supplementation of the serum-free medium with other growth factor (IL-3) had no further effect on cell expansion and the efficiency of gene delivery (Wu *et al.*, 2001b). At the opti-

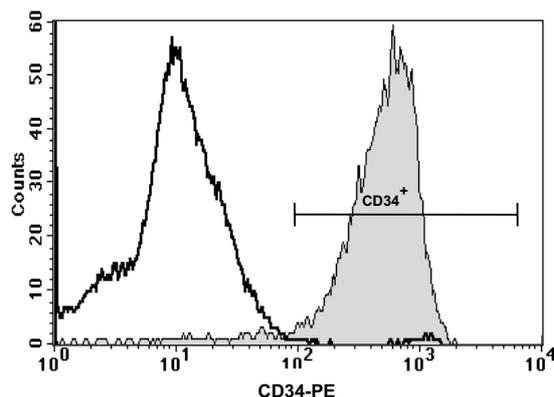


Figure 3. Comparative flow cytometric analysis of the CD34⁺ cell population purity, measured by CD34-PE fluorescence intensity in a fresh UCB sample (transparent histogram) and after the immunomagnetic isolation on MiniMACS columns (gray histogram).

mised conditions of electroporation (1600 μ F, 550 V/cm, 10 μ g of plasmid DNA per 400 μ l of cell culture) we obtained expression of the reporter gene (EGFP) in more than 40% of treated cells (Fig. 4) with high cell viability. Al-

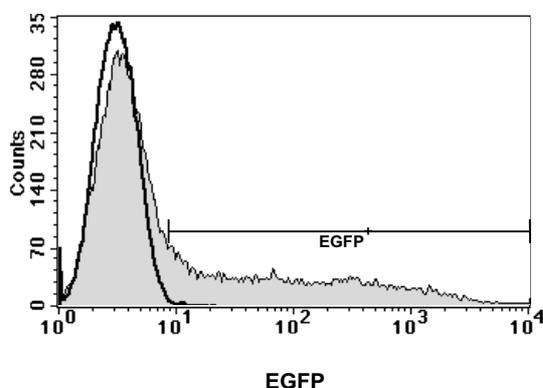


Figure 4. Comparative flow cytometric analysis of EGFP expression in UCB-derived CD34⁺ cells electroporated without (transparent histogram) or with plasmid DNA (gray histogram).

though further increase of the pulse capacitance and DNA concentration resulted in higher transfection efficiency, it caused greater cell death due to the “electroporation toxicity” (Li *et al.*, 2001). The obtained efficiency of transfection of CD34⁺ cells isolated

from umbilical cord blood was comparable to previous studies on UCB- (Wu *et al.*, 2001c) and peripheral blood-derived (Wu *et al.*, 2001a) HSPC and was higher than that determined for adult bone marrow cells (Van Tendeloo *et al.*, 2000). This high gene transfer efficiency might be due to the immature status of umbilical cord blood CD34⁺ cells. Some authors suggest that transfection capacity of more differentiated progenitors is lower than with the primitive cells (Wu *et al.*, 2001c). Further investigations are necessary to evaluate the correlation between the hematopoietic cell maturation and the efficiency of their transfection. It is also of interest whether the transfected CD34⁺ cells remain capable of foreign gene expression after their differentiation into fully matured blood cells.

In conclusion, the high frequency of transfection without a marked viability decrease justifies recommendation of electroporation as an efficient method of gene incorporation into UCB derived CD34⁺ cells.

REFERENCES

- Baum C, Eckert HG, Stockschrader M, Just U, Hegewisch-Becker S, Hildinger M, Uhde A, John J, Ostertag W. (1996) Improved retroviral vectors for hematopoietic stem cell protection and *in vivo* selection. *J Hematother.*; **5**: 323–9.
- Bierhuizen MFA, Westerman Y, Visser TP, Dimjati W, Wognum AW, Wagemaker G. (1997) Enhanced green fluorescent protein as selectable marker of retroviral-mediated gene transfer in immature hematopoietic bone marrow cells. *Blood.*; **90**: 3304–15.
- Brenner MK. (1997) Emerging application of gene transfer in the hematopoietic cancers. *J Pediatr Hematol Oncol.*; **19**: 1–6.
- Capel B, Hawley RG, Mintz B. (1990) Long- and short-lived murine haematopoietic stem cell clones individually identified with retroviral integration markers. *Blood.*; **75**: 2267–82.

- Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC. (1994) Green fluorescent protein as a marker of gene expression. *Science*; **263**: 802–5.
- Corrias MV, Scuderi F, Pasino M, Biglino P, Bocca P, Marotta F, Figini E, Pistoia V, Mori PG. (1998) Growth factors increase retroviral transduction but decrease clonogenic potential of umbilical cord blood CD34⁺ cells. *Haematologica*; **83**: 580–6.
- Frey BM, Hackett NR, Bergelson JM, Finberg R, Crystal RG, Moore MA, Rafii S. (1998) High-efficiency gene transfer into *ex vivo* expanded human hematopoietic progenitor and precursor cells by adenovirus vectors. *Blood*; **91**: 2781–92.
- Gluckman E. (2000) Current status of umbilical cord blood hematopoietic stem cell transplantation. *Exp Hematol*; **28**: 1197–205.
- Gluckman E, Broxmeyer HE, Auerbach AD, Friedman HS, Douglas GW, Devergie A, Esperou H, Thierry D, Socie G, Lehn P, Cooper S, English D, Kurtzberg J, Bard J, Boyse EA. (1989) Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical cord blood from an HLA-identical sibling. *N Engl J Med*; **321**: 1174–82.
- Hows JM, Bradley BA, Marsh JC, Luft T, Coutinho L, Testa NG, Dexter TM. (1992) Growth of human umbilical cord blood in long-term haematopoietic cultures. *Lancet*; **340**: 73–6.
- von Kalle C, Kiem HP, Goehle S, Darovsky B, Heimfeld S, Torok-Storb B, Storb R, Schuening FG. (1994) Increased gene transfer into human hematopoietic progenitor cells by extended *in vitro* exposure to a pseudotyped retroviral vector. *Blood*; **84**: 2890–7.
- Li LH, McCarthy P, Hui SW. (2001) High-efficiency electrotransfection of human primary hematopoietic stem cells. *FASEB J*; **15**: 586–8.
- Limon A, Briones J, Puig T, Carmona M, Fornas O, Cancelas JA, Nadal M, Garcia J, Rueda F, Barquinero J. (1997) High-titer retroviral vectors containing enhanced green fluorescent protein (EGFP) for efficient expression in hematopoietic cells. *Blood*; **90**: 3316–21.
- Mayani H, Lansdorp PM. (1998) Biology of human umbilical cord blood-derived hematopoietic stem/progenitor cells. *Stem Cells*; **16**: 153–65.
- Mayani H, Gutierrez-Rodriguez M, Espinoza L, Lopez-Chalini E, Huerta-Zepeda A, Flores E, Sanchez-Valle E, Luna-Bautista F, Valencia I, Ramirez OT. (1998) Kinetics of hematopoiesis in Dexter-type long-term cultures established from human umbilical cord blood cells. *Stem Cells*; **16**: 127–35.
- Persons DA, Allay JA, Allay ER, Smeyne RJ, Ashmun RA, Sorrentino BP, Nienhuis AW. (1997) Retroviral-mediated transfer of the green fluorescent protein gene into murine hematopoietic cells facilitates scoring and selection of transduced progenitors *in vitro* and identification of genetically modified cells *in vivo*. *Blood*; **90**: 1777–86.
- Pettengell R, Luft T, Henschler R, Hows JM, Dexter TM, Ryder D, Testa NG. (1994) Direct comparison by limiting dilutions analysis of long-term culture-initiating cells in human bone marrow, umbilical cord blood and blood stem cells. *Blood*; **84**: 3653–9.
- Toneguzzo F, Keating A. (1986) Stable expression of selectable genes introduced into human hematopoietic stem cells by electric field-mediated DNA transfer. *Proc Natl Acad Sci U S A*; **83**: 3496–9.
- Traycoff CM, Abboud MR, Laver J, Clapp DW, Srour EF. (1994) Rapid exit from G₀/G₁ phases of cell cycle in response to stem cell factor confers on umbilical cord blood CD34⁺ cells an enhanced *ex vivo* expansion potential. *Exp Hematol*; **22**: 1264–72.
- Van Tendeloo VF, Willems R, Ponsaerts P, Lenjou M, Nijs G, Vanhove M, Muylaert P, Van Cauwalaert P, Van Broeckhoven C, Van Bockstaele DR, Berneman ZN. (2000) High-level transgene expression on primary human T lymphocytes and adult bone marrow CD34⁺ cells *via* electroporation-mediated gene delivery. *Gene Ther*; **7**: 1431–7.
- Wang JCY, Doedens M, Dick JE. (1997) Primitive human hematopoietic cells are enriched

- in cord blood compared with adult bone marrow or mobilized peripheral blood as measured by the quantitative *in vivo* SCID-repopulating cell assay. *Blood.*; **89**: 3919-24.
- Wu MH, Liebowitz DN, Smith SL, Williams SF, Dolan ME. (2001a) Efficient expression of foreign genes in human CD34⁺ hematopoietic precursor cells using electroporation. *Gene Ther.*; **8**: 384-90.
- Wu MH, Smith SL, Danet GH, Lin AM, Williams SF, Liebowitz DN, Dolan ME. (2001b): Optimization of culture conditions to enhance transfection of human CD34⁺ cells by electroporation. *Bone Marrow Transplant.*; **27**: 1201-9.
- Wu MH, Smith SL, Dolan ME. (2001c): High efficiency electroporation of human umbilical cord blood CD34⁺ hematopoietic precursor cells. *Stem Cells.*; **19**: 492-9.