The Functional Effect of Repeated Cryopreservation on Transduced CD34⁺ Cells from Patients with Thalassemia

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Stable gene marking and effective engraftment of gene-modified CD34⁺ hematopoietic stem cells is a prerequisite for gene therapy success but may be challenged by the inevitable cryopreservation of the final product prior to extensive quality assurance testing. We investigated the β -globin gene transfer potency in fresh and cryopreserved CD34⁺ cells from mobilized patients with β -thalassemia, as well as the qualitative impact of repeated freeze/thaw cycles on the functionality of cultured and unmanipulated CD34⁺ cells in terms of engrafting capacity in a xenotransplantation model, under partial myeloablation. Cells transduced fresh or after one freeze–thaw cycle yielded similar clonogenic and gene transfer frequencies. Repeated cryopreservation cycles did not affect the transduction rates whereas either one or two freeze–thaw cycles of cultured—but not of unmanipulated—cells significantly reduced their clonogenicity. No differences in the engrafting potential of gene-corrected cells subjected to either none or up to two cryopreservation cycles, were encountered post xenotransplantation. Overall, we assessed the gene transfer efficiency, clonogenicity and engrafting capacity of cryopreserved CD34⁺ cells and the impact of repeated freeze/thaw cycles in their performance. These observations may prove essential in the design of gene therapy trials, considerably facilitating their logistics.

Keywords: thalassemia, cryopreservation, mobilization, gene therapy, engraftment, CD34⁺-cell processing, gene therapy trials

INTRODUCTION

IN ALL CLINICAL APPLICATIONS, the gene-corrected grafts must be mandatorily cryopreserved until the quality criteria are met to allow the product release. For harmonization purposes and wider applicability, multinational gene therapy trials use centralized cell processing, where the leukaphereses products (LPs) are shipped as fresh cells to a central facility for gene modification; the gene therapy product is then cryopreserved for release testing and upon release, is shipped back to the site to be infused to the patient. Cell processing on previously cryopreserved purified CD34⁺ cells, instead of fresh CD34⁺ cells, followed by a second freezing cycle until product release will probably simplify the complex logistics of cell and gene therapy products before reaching the patient.

We investigated the effect of freeze–thaw courses on the transducibility, clonogenicity, and engraftment potential of hematopoietic stem/progenitor cells collected from thalassemia major patients in previous trials^{1,2} that identified Plerixafor+G-CSF (granulocyte-colony stimulating factor)–mobilized cells as an optimal graft source for thalassemia gene therapy.^{3,4}

Currently, several single-center or multinational, phase 2/3 gene therapy clinical trials for hemoglobinopathies are ongoing, providing clear evidence of transfusion-independence in thalassemic patients with non- β^0/β^0 genotypes,^{5,6} whereas the impressive results of gene therapy trials for immunodeficiencies or B-cell leukemias and lymphomas, recently granted marketing authorization for specific gene therapy products (Strimvelis,⁷ Yescarta,⁸ Kymriah⁹). As

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gene therapy is now moving forward to phase 3 national and multinational trials and marketed global products for various target diseases, a smooth transition from single-center, academic manufacturing to controlled processes and coordinated logistics across countries worldwide is required.

We here provide evidence that transduction on previously cryopreserved CD34⁺ cells does not affect gene transfer or the graft's quality characteristics. Shipment of previously cryopreserved CD34⁺ selected grafts to the cell processing facility, will greatly facilitate the orchestration of the supply chain of a gene therapy product before it reaches its end users.

MATERIALS AND METHODS

CD34⁺ cell collection, cryopreservation, and thawing

Two hematopoietic stem cell mobilization clinical trials (EudraCT numbers 2005-000315-10 and 2009-014136-37; ClinicalTrials.gov, NCT00336362 and NCT01206075) were conducted at the George Papanicolaou Hospital (Thessaloniki, Greece) in collaboration with the University of Washington (Seattle, WA) in adults with thalassemia. Patient enrollment, mobilization, and the CD34⁺ selection procedure have previously been described.^{1,2} For this study, we used small aliquots of the final

products, as described in the results section on study design, whereas the bulk of the purified CD34⁺ cells was cryopreserved for future clinical-scale gene transfer trials.

Aliquots of CD34⁺ cells after CliniMACS separation, were cryopreserved as unmanipulated, transduced or untransduced cells at $1\text{--}2 \times 10^6$ cells/mL in cryopreservation media consisting of 10% dimethyl sulfoxide (Origen, Austin, TX), 5% human serum albumin (LFB Biomedicaments, Courtabœuf Cedex, France) and 2% hydroxyethyl starch (Voluven, Fresenius, Bad Homburg, Germany) in Electrolyte (Fresenius, Bad Homburg, Germany). A 1:1 ratio of cell suspension to cryopreservation media was held at all times. Cells were frozen in a controlled rate freezer and subsequently stored in liquid nitrogen.

Cryopreserved cells were thawed rapidly in a 37°C waterbath. Dimethyl sulfoxide-containing cryopreservation media was removed by washing the cells in 4× volumes of thawing media containing 4% human serum albumin (LFB Biomedicaments, Courtabœuf Cedex, France) and 10 U/mL heparin (Leo Pharma, Ballerup, Denmark) in X-VIVO 10 (Lonza, Basel, Switzerland). A schematic description of the status of cells upon each cryopreservation step and the comparisons applied between the different freeze–thaw cycles is given in Fig. 1.

Figure 1. Experimental scheme depicting transduction on fresh or once-thawed CD34⁺ cells and the single or double freeze–thaw events after transduction. **(A)** Freshly transduced cells manipulated right after CliniMACS immunomagnetic separation. **(B)** Cells cryopreserved as unmanipulated after CliniMACS immunomagnetic separation and thawed for transduction. Pre-stim, pre-stimulation; CFU, colony-forming unit.

***In vitro* characterization of CD34⁺ cells and xenotransplantation**

Fresh or thawed CD34⁺ cells were prestimulated (day 0) and transduced with the TNS9.3.55 lentiviral globin vector (vector plasmids were kindly provided by M. Sadelain, Memorial Sloan Kettering Cancer Center, New York, NY) as described elsewhere.¹⁰ Untransduced cells served as control. After transduction (day 3), cells were harvested and aliquots were seeded for clonogenic assays, erythroid differentiation and equal cell dose transplantation into mice and/or again cryopreserved. Viable cells were enumerated by Trypan blue exclusion at all times.

To assess the gene transfer and clonogenic frequencies, 500 CD34⁺ cells were seeded in duplicate on semisolid media (MethoCult GF H4434; Stem Cell Technologies, Vancouver, British Columbia) and vector copy number (VCN) quantification was conducted in single colonies, as previously described.⁴

The erythroid differentiation culture has been described elsewhere.¹⁰ Cells at various stages of differentiation were analyzed every 4 days for a total of 15 days by flow cytometry. Live cells were selected by gated exclusion of 7-amino-actinomycin D (BD Biosciences, San Jose, CA) and tested for positivity for CD34, CD38 (both from BD Biosciences) and CD235a (Invitrogen, Carlsbad, CA) using antibodies conjugated to allophycocyanin, phycoerythrin, and fluorescein isothiocyanate, respectively, with a FACSCalibur System and the CellQuest analysis software (BD Biosciences). The main bulk of the terminally differentiated cells were used for high-performance liquid chromatography (HPLC) to assess β -globin expression as previously described.⁴

Under an approved protocol by the institutional animal care and use committee, 8-week-old NSG mice (Jackson Laboratories, Bar Harbor, ME) were conditioned for three days with a total of 100 mg/kg Busulfan (Busilvex, Pierre Fabre, Boulogne, France), which corresponds to the human non-myeloablative dose of 8 mg/kg.⁴ One million CD34⁺ cells were transplanted per mouse, via the tail vein. Donor chimerism was measured on a monthly basis in the peripheral blood of mice.⁴

Statistics

Comparisons between groups were determined using the Student's *t*-test. Values of $p < 0.05$ were considered statistically significant. Results are expressed as means $\pm$ standard error of the mean.

RESULTS

Study design

From the 46 enrolled subjects in both mobilization trials, we obtained LPs from 41 evaluable pa-

tients^{1,2} and proceeded to CD34⁺ cell selection for 32 LPs that contained more than 2.5×10^6 CD34⁺ cells/kg. Ultimately, cell aliquots from 31 CD34⁺-enriched products with $>85\%$ purity were transduced following cryopreservation and thawing (day 0). CD34⁺ cells from a limited number of patients ($n = 5$) were transduced both immediately after CliniMACS purification (fresh cells) and after a single freeze-thaw procedure (once-thawed cells). Following gene transfer (day 3), a part of the transduced cells was plated in clonogenic and erythroid differentiation cultures and another fraction of cells was frozen, thus subjecting the freshly transduced cells to one (Fig. 1A) and the post-thaw transduced cells to overall two cryopreservation cycles (Fig. 1B). Finally, an additional freeze-thaw cycle was applied to the freshly transduced CD34⁺ cells to reach the total of two cryopreservation-cycle treatments (Fig. 1A). CD34⁺ cells in excess on day 3 were transplanted at equal doses into partially myeloablated IL2Rgamma^{null} (NSG) recipients.

A single freeze-thaw cycle does not affect gene transfer and clonogenic potential of mobilized CD34⁺ cells

To determine whether fresh thalassemic CD34⁺ cells differ in gene transfer susceptibility and clonogenic potential from cells that have undergone one freeze-thaw cycle, we used CD34⁺ cells from Plerixafor-mobilized ($n = 3$) and Plerixafor+G-CSF-mobilized ($n = 2$) patients available both as freshly transduced and transduced after one thawing procedure. In addition, samples of unmanipulated CD34⁺ cells plated fresh after ClinMACS purification for colony formation (day 0) were compared with their paired samples after one thawing/freezing procedure, in terms of clonogenic capacity.

Cell viability, fold expansion in culture, colony formation, erythroid differentiation, and β -globin expression were comparable between freshly transduced cells and cells manipulated after one thawing cycle (Table 1). Similarly, the average colony count from cells plated on day 3, the percentage of vector-positive colony-forming units (CFUs) and the average VCN in CFUs did not differ between paired CD34⁺ cells, transduced fresh or after one freeze-thaw cycle ($n = 5$) (Fig. 2A, B), thus suggesting that efficient gene transfer can be achieved both on fresh and previously cryopreserved CD34⁺ target cells. In addition, no significant difference was observed in the clonogenic capacity of paired samples of fresh and once-thawed unmanipulated CD34⁺ cells (day 0), suggesting that a single freeze-thaw cycle does

Table 1. Paired comparison of the functional characteristics of fresh and once-thawed CD34⁺ cells in culture

Culture Parameters		Fresh n = 5	Once-thawed n = 5	p-Value
% Viability (D0)		93.4 ± 2.9	92.1 ± 1.3	0.66
Fold expansion after pre-stimulation (D1)		0.82 ± 0.1	0.74 ± 0.1	0.53
Fold expansion after transduction (D3)	Untransduced	1.61 ± 0.04	1.96 ± 0.3	0.31
	Transduced	1.96 ± 0.2	2.19 ± 0.2	0.39
Cumulative fold expansion (D15)	Untransduced	113 ± 14.2	123.6 ± 36.6	0.8
	Transduced	139.7 ± 22.8	188.9 ± 54.5	0.46
% CD34 ⁺ /CD235a ⁺ (D15)	Untransduced	67.5 ± 2.8	73.3 ± 4.3	0.31
	Transduced	73.1 ± 3.9	78 ± 4	0.41
Average number of CFUs	Untransduced	80.1 ± 27	67.4 ± 8.1	0.64
	Transduced	78.8 ± 21.2	72.2 ± 2.7	0.74
β -globin/ α -globin ratio (D15, HPLC)	Untransduced	0.22 ± 0.05	0.28 ± 0.06	0.47
	Transduced	0.43 ± 0.05	0.49 ± 0.04	0.34

Fresh: cells cultured immediately after CliniMACS purification. Once-thawed: cells cultured after a single freeze/thaw procedure. Fresh and once-thawed cells are derived from the same patient sample.

Clonogenic assays were conducted with identical numbers of live CD34⁺ cells between groups.

Data are expressed as means ± standard error of the mean (SEM). p-Values were calculated using the Student's *t*-test.

CFUs, colony-forming units; HPLC, high-performance liquid chromatography.

not significantly affect the clonogenicity of unmanipulated cells (Fig. 2C).

Multiple freeze-thaw cycles do not affect gene transfer stability but reduce the clonogenic potential of transduced CD34⁺ cells

The requirement of replication incompetent lentivirus in the genetically engineered graft (replication competent lentivirus assay [RCL]) generates the need for cryopreservation of the gene therapy product until completion of all quality tests. Currently, one freezing/thawing cycle is taking place before cell infusion, however, if transduction is to be performed in previously stored CD34⁺ cells, those will undergo overall two freeze-thaw cycles. We therefore investigated the effect of repeated freeze-thaw cycles on mobilized cells, in terms of gene transfer persistence, clonogenic potential and engraftment in NSG mice.

We first compared CD34⁺ cells transduced fresh, immediately after the enrichment procedure (Plerixafor- and Plerixafor+G-CSF-mobilized) or after one freeze-thaw cycle (G-CSF-, hydroxyurea+G-CSF-, Plerixafor- and Plerixafor+G-CSF-mobilized), to aliquoted cells from the same transduction processes when applicable (*n* = 5 and *n* = 26, respectively), after overall two thawing procedures (Fig. 1).

Gene transfer rates in terms of vector positive colonies and VCN remained stable after one or two freeze-thaw cycles (Table 2; Fig. 3B, D). In contrast, however, the colony yields on semisolid assays of CD34⁺ cells transduced either fresh or post thaw and plated after repeated cycles of freezing/

thawing were considerably less. As displayed in Fig. 3A and C, both untransduced and transduced cells showed an up to 40% drop in colony formation when exposed to either single or double freeze-thaw events (*p* < 0.0001), indicating a strong toxic effect of the freezing cycles on clonogenicity of cultured CD34⁺ cells, which occurs irrespectively of whether transduction took place in fresh or previously cryopreserved cells. The lack of statistical significance in the reduced clonogenicity of freshly transduced cells after one or two freezing cycles (Fig. 3A), most probably reflected the small sample size (*n* = 5). In contrast to the drop in CFUs of untransduced and transduced CD34⁺ cells after a single or double freeze-thaw cycle, unmanipulated CD34⁺ cells were unaffected by the freeze-thaw events in terms of clonogenic capacity (Fig. 2C).

Viability and CD34⁺ cell recovery upon thawing was comparable among freshly or once-thawed transduced cells and the same cells after being subjected to one or two cryopreservation cycles (Table 3). Nevertheless, in line with the known post-thaw CD34⁺ cell loss,^{11–13} there was a ~25% quantitative loss in viable CD34⁺ cells by each cryopreservation cycle resulting in an overall CD34⁺ cell recovery after two cryopreservation procedures of the freshly and once-thawed transduced cells of 53% and 50.5%, respectively (Table 3).

Assessment of the engrafting efficiency of fresh and cryopreserved gene-corrected CD34⁺ cells

Should previously collected grafts are to be genetically modified and used in gene therapy trials, the maintenance of their engrafting capacity needs to be verified, as those cells will undergo two freeze-thaw cycles before infusion. To comparatively assess the engraftment capacity of fresh and cryopreserved genetically modified CD34⁺ cells, we xenotransplanted CD34⁺ cells from patient samples into NSG mice. Same patient cells were freshly transduced or transduced after one cryopreservation cycle and infused either directly (after 0 and 1 freezing cycle) or after being subjected to two freezing cycles overall.

No differences in the engraftment of CD34⁺ cells transduced as fresh or previously cryopreserved were encountered throughout the 4-month evaluation period, as assessed by flow cytometry for circulating human CD45⁺ cells in the peripheral blood of mice (Fig. 4). These data demonstrate that neither the transduction on previously cryopreserved cells nor the repeated freeze-thaw cycles of transduced cells negatively influence efficient

Figure 2. Paired comparison of gene transfer rates and vector copy number in CD34⁺ cells transduced fresh and after one cryopreservation cycle. **(A)** The average colony count in semisolid assays of fresh and once-thawed cells in transduced ($n=5$) and untransduced ($n=5$) paired groups on day 3 (after pre-stimulation/transduction). **(B)** Gene transfer (left panel) and vector copy number (right panel) in paired cells transduced either fresh ($n=5$) or thawed after cryopreservation ($n=5$). **(C)** The average colony count in semisolid assays of fresh and once-thawed cells on day 0 (before pre-stimulation/transduction). Freshly transduced cells are marked in black and once-thawed cells in grey. All clonogenic assays were conducted with identical numbers of live CD34⁺ cells between groups and experiments. VCN, vector copy number.

engraftment of genetically modified hematopoietic stem cells (HSCs).

DISCUSSION

Gene therapy is now increasingly recognized as a curative option for patients with difficult-to-cure diseases and a number of gene therapy products

have already received a marketing license.^{7–9} As gene therapy for several target diseases now moves into later-phase clinical trials, this requires the transition from single academic manufacturing to controlled processes across countries worldwide.

In multicenter/multinational gene and cell therapy trials, cells are collected at the patient's

Table 2. Transduction efficiency and vector copy number of gene-corrected CD34⁺ cells over repeated freeze/thaw events, sorted by mobilization strategy

	HU+G-CSF		G-CSF		Plerixafor		Plerixafor+G-CSF	
	% Colony TD Efficiency VCN ≥ 0.45	Average Colony VCN ≥ 0.45	% Colony TD Efficiency VCN ≥ 0.45	Average Colony VCN ≥ 0.45	% Colony TD Efficiency VCN ≥ 0.45	Average Colony VCN ≥ 0.45	% Colony TD Efficiency VCN ≥ 0.45	Average Colony VCN ≥ 0.45
Fresh	N/A	N/A	N/A	N/A	n=3	n=3	n=2	n=2
Frozen once	N/A	N/A	N/A	N/A	42.9 $\pm$ 7.7	1.95 $\pm$ 0.27	33.7 $\pm$ 10.1	1.38 $\pm$ 0.23
Frozen twice	N/A	N/A	N/A	N/A	42.7 $\pm$ 4.7	1.58 $\pm$ 0.09	30.1 $\pm$ 5.9	1.65 $\pm$ 0.22
Once-thawed (frozen once)	n=5	n=5	n=6	n=6	50.5 $\pm$ 5.8	1.83 $\pm$ 0.07	35.7 $\pm$ 4.8	1.44 $\pm$ 0.16
Frozen twice	44.8 $\pm$ 11.3	1.95 $\pm$ 0.15	40.1 $\pm$ 6.3	1.61 $\pm$ 0.13	54.8 $\pm$ 6.1	1.92 $\pm$ 0.21	39.3 $\pm$ 5.3	1.35 $\pm$ 0.13
	34.8 $\pm$ 6.1	1.58 $\pm$ 0.14	33.6 $\pm$ 7	1.59 $\pm$ 0.35	50.8 $\pm$ 5.8	1.86 $\pm$ 0.11	36.4 $\pm$ 5.8	1.29 $\pm$ 0.16

Fresh: cells cultured immediately after CliniMACS purification; Once-thawed: cells cultured after a single freeze/thaw procedure.

Data are expressed as means $\pm$ SEM.

G-CSF, granulocyte-colony stimulating factor; HU, hydroxyurea; TD, transduction; VCN, vector copy number.

Figure 3. Gene transfer rates and clonogenicity over repeated freeze–thaw events. **(A)** Average colony count in day 3 fresh CD34⁺ cells, processed directly after CliniMACS immunomagnetic separation ($n=5$, black bars). The transduced cells ($n=5$) and their untransduced counterparts ($n=5$) were cryopreserved (one cryopreservation cycle, white bars). The cells were later subjected to an additional thawing/freezing cycle (overall two cryopreservation cycles, grey bars). **(B)** Colony transduction efficiency (left panel) and average colony vector copy number (right panel) of freshly transduced CD34⁺ cells ($n=5$) as compared with their once- or twice-cryopreserved freshly transduced counterparts. In each panel, fresh cells are shown on the left side, once-cryopreserved cells in the middle and twice-cryopreserved cells on the right side. **(C)** Average number of colonies in once-thawed manipulated cells. Once-thawed manipulated cells on day 3 and their refrozen counterparts are presented in clear and grey bars, respectively. **(D)** Colony gene transfer rates (left panel) and vector copy number (right panel) in once-thawed transduced cells ($n=26$, clear) that were exposed to a second freeze–thaw cycle ($n=26$, grey). All clonogenic assays were conducted with identical numbers of live CD34⁺ cells between groups and experiments. ** $p=0.001$ and **** $p<0.0001$ (unpaired Student's t -test).

site, transferred for centralized manufacturing of the cellular product, and then shipped back to the site of administration. In gene therapy trials in particular, a time-consuming extensive biosafety screening of the final product before therapeutic infusion is mandatory in order to ensure that patients are not inadvertently exposed to RCL. Despite the suggested reevaluation of biosafety requirements for third generation lentiviral vectors,¹⁴ based on the absence of RCL in hundreds of clinical gene therapy products, RCL remains a *sine qua non* in the biosafety screening of gene-modified CD34⁺ cells. Consequently, manipulated grafts must be cryopreserved until the release criteria are met.

In order to avoid two freeze–thaw cycles, in multicenter stem cell gene therapy trials using centralized cell processing the harvested cells are

shipped fresh for immediate processing. However, if gene modification could be effectively performed in previously frozen CD34⁺ cells, this would greatly facilitate the product logistics and the management of staffing at good manufacturing practice facilities.

We here sought to address by pairwise comparison to what extent the viability, recovery, clonogenicity and engraftability of thalassemic CD34⁺ cells transduced either fresh or post thaw and subjected post transduction to overall 0, 1, or 2 freezing/thawing cycles, are affected.

We first demonstrated that gene transfer was robust and consistent in paired CD34⁺ cell samples from the same patients, irrespectively of whether the transduction process had been performed on fresh HSCs or HSCs after one freeze–thaw cycle. In addition, gene transfer in terms of

Table 3. Viability and recovery of CD34⁺ cells over repeated freeze/thaw events

	% Viability	% Recovery ^a	% Estimated Overall Recovery ^b
Freshly transduced (n=5)			100
Frozen once	91.6 ± 1.1	78.2 ± 4.7	78.2
Frozen twice	83.6 ± 2.1	68.1 ± 5.3	53
Transduced post first thaw (n=26)			100
Frozen once	84.9 ± 2.0	74 ± 6.2	74
Frozen twice	86.9 ± 1.6	68.3 ± 4.8	50.5

Freshly transduced: cells cultured immediately after CliniMACS purification. Transduced post first thaw: cells cultured after a single freeze/thaw procedure.

Data are expressed as means ± SEM.

^aRecovery refers to the % CD34⁺ cell recovery "post each thawing" as compared with "before each freezing" condition.

^bEstimated overall recovery refers to the CD34⁺ cell recovery "post each freezing" as compared with the "before any freezing" condition. Recovery before any freezing was considered as 100%. Estimation was done according to the formula: Estimated overall recovery = (% recovery of twice frozen cells × % recovery of once frozen cells) / 100.

vector positive CFUs or VCN as well as erythroid differentiation and transgene expression was stable throughout the freeze–thaw events in those cells.

However, exposure of cultured cells to either one or two cryopreservation cycles decreased the clonogenic capacity up to 40%, not only in transduced but also in untransduced cells, thus excluding vector-mediated toxicity in transduced HSCs.

Figure 4. Engraftment kinetics of genetically modified thalassemic CD34⁺ cells in partially myeloablated NSG mice. Grey line (n=3): The infused cells were transduced fresh, right after CliniMACS purification without previous cryopreservation. Dashed line (n=9): The infused cells were cryopreserved after CliniMACS separation and transduced after thawing. Black line (n=6): The infused cells were cryopreserved after CliniMACS separation, transduced after thawing and underwent an extra freeze–thaw course before infusion into the animals. All transplantations were conducted with identical numbers of live CD34⁺ cells.

Of interest, unmanipulated cells subjected to one freeze–thaw cycle produced similar CFU numbers with their fresh counterparts, in line with previously published data on the maintenance of clonogenic capacity in long-term cryopreserved hematopoietic grafts.^{15,16} Our data imply that the *ex vivo* cell culture *per se* renders CD34⁺ cells susceptible to a freezing/thawing-associated partial loss of graft potency.

The negative impact of the *ex vivo* culture and/or retroviral transduction on the performance of gene-engineered HSCs over unmanipulated HSCs has been acknowledged in early studies.^{17–19} Alterations in cell cycle, apoptosis, and adhesion molecules that ultimately lead to differentiation and loss of the primitive phenotype of transduced HSCs²⁰ have been suggested to reduce their engraftability after transplantation. However, our data support that gene-modified HSCs subjected to two freeze–thaw cycles do not suffer a greater loss of clonogenic potential than transduced HSCs undergoing the currently compulsory in clinical applications, one freezing procedure. Moreover, the engrafting capacity of transduced CD34⁺ cells after 0, 1, or 2 freezing cycles in partially myeloablated and xenografted mice, assessed by the circulating human CD45⁺ cells in peripheral blood, was highly similar.

Both one freezing cycle of CD34⁺ cells before or after transduction or a second freezing cycle of transduced CD34⁺ cells before release have a similar qualitative effect on clonogenicity, differentiation, and engraftability. However, the decision on performing transduction on fresh or previously cryopreserved cells may ultimately rely on the magnitude of CD34⁺ cell dose post leukapheresis and cell selection, given that cell loss is induced by each freeze–thaw cycle, as shown here and in other reports.²¹

In summary, our data warrant both stable gene transfer and engraftment throughout two freeze–thaw events of gene-corrected CD34⁺ cells and for large CD34⁺ cell harvests support the possibility for double CD34⁺ cell freezing before product release, without affecting graft quality. Should these data be validated in large scale and under good manufacturing practice conditions, ultimately, the decision for *ex vivo* transduction on high dose, fresh or previously cryopreserved, CD34⁺ cell grafts may be driven by logistical considerations, such as location of cell processing facility and trial's site, staffing, time to transportation, etc., thus facilitating centralized gene therapy manufacturing in multicenter/multinational gene therapy trials.

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AUTHOR DISCLOSURE

The authors declare no conflicts of interest.

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