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Feasibility of cord blood stem cell manipulation with high-energy shock waves: An in vitro and in vivo study

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Objective. **Cord blood $CD34^+$ cells are more uncommitted than their adult counterparts as they can be more easily maintained and expanded in vitro and transduced with lentiviral vectors. The aim of this study was to evaluate whether pretreatment with high-energy shock waves (HESW) could further enhance the expansion of cord blood progenitors and the transduction efficiency with lentiviral vectors.**

Methods. **Human cord blood $CD34^+$ cells underwent HESW treatment with a wide range of energy and number of shots (from 0.22 mJ/mm^2 to 0.43 mJ/mm^2 and from 200 to 1500 shots). Cells were then evaluated both for their in vitro expansion ability and in vivo engraftment in primary, secondary, and tertiary NOD/SCID mice. The transduction efficiency with a lentiviral vector (LV) was also evaluated in vitro and in vivo.**

Results. **Cell viability following HESW ranged from 75 to 92%. Pretreatment with HESW significantly improved early progenitor cell expansion after short-term suspension culture. Upon transplantation in primary NOD/SCID mice, the HESW treatment enhanced progenitor cell engraftment (total human $CD45^+CD34^+$ cells were 10% in controls and 14.5% following HESW, human $CD45^+CD34^+CD38^-$ cells were 0.87% in controls and 1.8% following HESW). HESW treatment enhanced the transduction of a GFP^+ lentiviral vector (e.g., at day 42 of culture 6.5% GFP^+ cells in LV-treated cell cultures compared to 11.4% of GFP^+ cells in HESW-treated cell cultures). The percentage of human GFP^+ cell engrafting NOD/SCID mice was similar (34% vs 26.4% in controls); however, the total number of human cells engrafted after HESW was higher (39.6% vs 15%).**

Conclusion. **The pretreatment of $CD34^+$ cells with HESW represents a new method to manipulate the $CD34^+$ population without interfering with their ability to both expand and engraft and it might be considered as a tool for genetic approaches.**

Introduction

An essential property of hemopoietic stem cells (HSC) is their ability to undergo multiple divisions without losing their ''stemness'' (i.e., self-renewal) and their ability to proliferate and differentiate in all hemopoietic lineages [1–4]. Among the HSC sources, umbilical cord blood (CB) has been shown to be a rich source of HSCs, attracting attention for both transplantation and gene therapy [5–9]. Piacibello et al. reported a simple method for the in vitro expansion of committed and early hemopoietic progenitors by culturing the $CD34^+$ cells with FLT3 ligand, stem cell factor, thrombopoietin, and IL-6 [10]. The expansion of very immature progenitors was later confirmed by serial transplantation in the immunodeficient animal model (nonobese diabetic-immunodeficient mice) [11]. The engrafted cells have been defined as SCID-repopulating cells (SRCs) or competitive repopulating units (CRUs) [12–15]. The ability of advanced-generation lentiviral vectors to transfer the green fluorescent protein (GFP) gene into human HSCs was studied in culture conditions that allowed the expansion of transplantable human HSCs resulting in multilineage engraftment with GFP^+lineage$^+$ cells [16–20]. Clonality

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analysis performed on murine bone marrow suggested that more than one integrant probably contributed to the engraftment of GFP-expressing cells. However, the percentage of GFP^+ human cells in mice injected with expanded cells was similar to that observed in mice that received transplants directly after transduction, suggesting that transgene integration and expression were not hampered by the ex vivo expansion [21].

In recent years, a few observations have suggested that there is an increase in the plasma membrane permeability of cells exposed to stress waves generated with either a lithotripter [22,23] or a laser [24,25]. Experiments have shown that cells can be loaded with many different types of molecules that were present in the extracellular medium, such as dyes, drugs, and genes (4550-kDa β-galactosidase, which were subsequently expressed in a fraction of the cells). This suggests the possibility of using stress waves to deliver molecules (drugs and genes) into cells for medical applications [26–28]. Shock waves, when administered to cells in vitro, have been shown to cause a transient increase in the permeability of cell membranes by opening micropores of μsec duration (such as electroporation), allowing a higher intracellular drug concentration [29–31], thus opening up new perspectives for both anti-cancer therapy and gene therapy.

The aim of these studies was to evaluate the effect of high-energy shock waves (HESW) on HSCs in terms of expansion, self-renewal, engraftment in NOD/SCID mice, and transduction efficiency with a modified lentiviral vector. This paper shows how HESW treatment significantly enhances both the expansion and the transduction efficiency of HSCs, without interfering with their own engraftment capacity in primary, secondary, and tertiary mice.

Material and methods

Human cells

Human umbilical CB was obtained from full-term deliveries after clamping and cutting the cord. The CB samples were collected at the S. Anna Hospital, Turin, Italy. CB was harvested in sterile tubes containing heparin. Before the deliveries, parents signed the informed consent for cord blood collection.

$CD34^+$ selection

Mononuclear cells were isolated from CB using Ficoll Hypaque 1077 (Nyegaard, Oslo, Norway) density centrifugation. The cells were subjected to two cycles of plastic adherence (60 minutes each) and then washed with Hanks' Balanced Salt Solution (HBSS, GIBCO BRL, Grand Island, NY, USA). The $CD34^+$ fraction was isolated with superparamagnetic microbead selection using a high-gradient magnetic field and miniMACS column (Miltenyi Biotech, Gladbach, Germany). The efficiency of the purification was verified by flow cytometry counterstaining with a CD34-phycoerythrin (PE; HPCA-2; Becton-Dickinson, San Jose, CA, USA) antibody. In the cell fraction containing purified cells, the percentage of $CD34^+$ cells ranged from 92 to 98%. Cells were pooled and frozen in liquid nitrogen. They were then thawed according to the experimental plan. The viability of thawed $CD34^+$ cells ranged from 90 to 92%.

Recombinant human cytokines

The following recombinant purified human cytokines were used in these studies: recombinant human (rh) stem cell factor (rhSCF) and rh megakaryocyte growth and development factor (MGDF) were generous gifts from Kirin (Kirin Brewery, Tokyo, Japan). Recombinant human granulocyte colony-stimulating factor (rhG-CSF) was from Genzyme (Cambridge, MA, USA); recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF), recombinant human interleukin-6 (rhIL-6), and recombinant human interleukin-3 (rhIL-3) were from Sandoz (Basel, Switzerland). Recombinant human erythropoietin (rhEPO; EPREX) was from Cilag (Milan, Italy). Recombinant human FLT3-ligand (rhFL) was from Immunex (Seattle, WA, USA).

Clonogenic assays

Assays for granulopoietic, erythroid, megakaryocytic, and multilineage (granulocyte-erythroid-macrophage-megakaryocyte) colony-forming units (CFU-GM, BFU-E, CFU-Mk, CFU-GEMM, respectively) and long-term culture-initiating cells (LTC-IC) were performed in triplicate or quadruplicate and scored as previously reported [10]. Briefly, aliquots of cell suspension from cultures to be tested were plated in methylcellulose medium (Sigma-Aldrich, Milan, Italy) with appropriate growth factors and then incubated at 37°C for 12 or 14 days. Colony scoring was performed by three independent investigators (F.S., A.P., C.V.), who counted all human colonies (more than 3 cells) grown in each plate on day 12 for CFU-Mk using the immunofluorescent microscope (Carl Zeiss, Milan, Italy) at 4× magnitude after staining with an FITC-conjugated MoAb recognizing human GPIIbIIIa antigen (CD41) and on day 14 for CFU-GM, BFU-E (more than 50 cells), and CFU-GEMM using optical microscope. Several growth factors were added at optimum concentrations to sustain the formation of BFU-E and CFU-GEMM: rhuIL-3 (20 ng/mL), rhuGM-CSF (10 ng/mL), rhuEPO (3 U/mL), and rhuSCF (50 ng/mL). rhuIL-3 (20 ng/mL), rhuSCF (50 ng/mL), and rhuGM-CSF (20 ng/mL) were added for CFU-GM. rhuIL-3 (5 ng/mL) was used as a single growth factor for CFU-Mk [32]. When cells obtained from murine marrows were evaluated for their human hemopoietic progenitor content, the fetal calf serum (FCS, Hyclone, Logan, UT) in the methylcellulose medium was replaced with an equivalent volume of a pretested pool of equivalently supportive normal human serum (HS) and the bovine plasma in the plasma clot assay was replaced with an equivalent volume of human plasma. G-CSF was omitted to minimize the stimulation of murine clonogenic cells [15]. Furthermore, colonies grown in plasma-clot and colonies plucked from methylcellulose cultures were stained with FITC-conjugated anti human CD41, anti-human CD45, anti-human CD13, and anti-human glycophorin-A (Glyco-A) and scored at the immunofluorescence microscope. As a control, BM cells of nontransplanted NOD/SCID mice were plated at identical cell concentrations in the same culture conditions. Under the latter conditions human colonies could not be detected.

The LTC-IC content of the initial $CD34^+$ population and of the long-term liquid cultures at different time points was determined by assaying for secondary colony-forming cell (CFC) in

methylcellulose culture after 6 weeks of stromal coculture, as previously described [10].

Shock wave exposure

The shock wave generator utilized for the in vitro experiments is a piezoelectric device (Piezoson 100, Richard Wolf, Knittlingen, Germany) especially designed for clinical use in orthopedics and traumatology. The instrument, which was kindly provided by Med & Sport 2000 S.r.l. (Turin, Italy), generates focused underwater shock waves at various frequencies (1 to 4 shocks per second) and intensities (0.025 to 1.48 mJ/mm^2). The device has a high-voltage electric current generator and a reflector set in a water-filled container. On the surface of the reflector, piezoelectric elements were arranged to form a part of a sphere and were stimulated with a high-energy electrical pulse. This causes vibration or rapid expansion of the crystals, leading to a shock wave that can be propagated through the water and focused at the center of the sphere. The pressure on the focal area is proportional to the voltage applied. The energy at the focal point is defined as the energy flux density (EFD) per impulse [33], recorded as joules per area (mJ/mm^2). For medical use, in orthopedics, shock waves of approximately 0.01 to 0.6 mJ/mm^2 are applied [34]. The focal area is defined as the area in which 50% of the maximum energy is reached [35]. With Piezoson 100 it has a length of 10 mm in the direction of the axis of the shock wave and a diameter of 2.5 mm perpendicular to this axis.

Cell pretreatment

Thawed CD34$^+$ cells separated from CB pools were prestimulated for 72 hours in stroma-free culture containing: Iscove's modified Dulbecco medium (IMDM; Gibco, Grand Island, NY, USA) supplemented with 10% FCS with the following growth factors: SCF (50 ng/mL), FL (50 ng/mL), MGDF (20 ng/mL), and IL-6 (10 ng/mL). The cells were then counted and aliquots of 4×10^6 cells were placed in 45 × 12.5-mm polypropylene tubes (Nunc, Wiesbaden, Germany), which were then completely filled with culture medium. The cells were then slowly pelleted by centrifugation at 250*g* in order to minimize the motion during shock wave treatment.

The experimental set-up was performed as previously described [36]. Briefly, each cell-containing tube was vertically aligned with the focal area and adjusted so that the central point of the focal area corresponded to the center of the tube bottom. The shock wave unit was kept in contact with the cell-containing tube by means of a water-filled cushion. Common ultrasound gel was used as a contact medium between the cushion and the tube. The cell viability following the HESW treatment was analyzed by trypan blue dye exclusion.

Stroma-free long-term expansion cultures

Stroma-free expansion cultures were performed for up to 150 days following 72 hours prestimulations ± HESW treatment as follows. A total of 10,000 to 20,000 CD34$^+$ cells were cultured in triplicate or quadruplicate flat-bottomed 24-well plates in 1 mL of IMDM supplemented with 10% FCS with the following growth factors: SCF (50 ng/mL), FL (50 ng/mL), MGDF (20 ng/mL), and IL-6 (10 ng/mL), which were added to each series of microwells twice a week. The cells were grown at 37°C at 5% CO_2. At culture initiation, the number of CFC (CFU-GM, BFU-E, CFU-GEMM, and CFU-MK) and of LTC-IC present in 1 mL of a single well was determined by triplicate semisolid assays for CFC or seeded onto pre-established irradiated human BM stromal layers (derived by culturing 10^7 fresh BM mononuclear cells in a T_{25} flask for at least 2 weeks in 5 mL stromal medium [12.5% horse serum, 12.5% FCS, IMDM, 2-mercaptoethanol, 10^6 mol/L hydrocortisone, and penicillin/streptomycin] and by plating the irradiated [15 Gy] and trypsinized stroma at 7×10^3/cm^2 in 24-well plates) and maintained at 37°C for 5 to 6 weeks with weekly half media changes, at the end of which all cells were harvested and plated for CFC determination in methylcellulose medium.

All the wells were demidepopulated once a week by removing one half of the culture volume (and cells), which was replaced with fresh medium and growth factors. The harvested cells were counted and suitable aliquots of the cell suspensions were assayed for CFC and for immunophenotype analysis (CD34$^+$, CD34$^+$CD38$^-$). The total number of CFCs generated in culture was calculated as follows: $y = (\text{observed CFU-C} \times 2^n)$, where n is the number of previous demidepopulations and y is the number corresponding to the fraction of 1 mL cell suspension (i.e., 1/62 = 15.6 μL) that was plated in semisolid assays. At different time point, aliquots of cultured cells were dispensed in 500 μL of Trizol reagent (Sigma Chemical Co, Milan, Italy) and stored at −80°C for further RNA extraction as described below.

Shock wave treatment schedule

To assess the role of HESW on expansion and engraftment, the cells were treated as follows: control cells grown for 72 hours received no further treatment, while HESW cells received EFD ranging from 0.22 to 0.43 mJ/mm^2; peak positive pressure ranging 31 to 50 MPa; number of shots ranging 250 to 1000; frequency: 4 shocks per second. Hydrophone measurements showed that peak pressure and pressure profile were only slightly altered inside the tubes (data not shown).

For the study on the engraftment of the HESW cells we decided to apply only EFD = 0.22 mJ/mm^2 per 500 shots and 0.32 mJ/mm^2 per 500 shots according to in vitro studies. For the study on the transduction only EFD = 0.32 mJ/mm^2 per 500 shots condition was studied. After treatment, cell viability was assayed with trypan blue dye exclusion and ranged from 75 to 98% (Table 1).

Production and characterization of the vector

The lentiviral vector was kindly provided by L. Naldini (Scientific Institute S. Raffaele, Milan, Italy). Replication-defective self-inactivating HIV-1 vectors were constructed as described [16,17]. Vectors were produced by transient-3 plasmid transfection of 293T. The 3 plasmids used were the transfer vector pRRLsin.PPT.hPGK.eGFP.Wpre [18], the VSV-G envelope-encoding plasmid pMD.G [16], and the packaging plasmid CMVδR8.74 [19].

Viral supernatants were concentrated by ultracentrifugation. Titers of vector preparations were determined by transduction of HeLA cells with serial dilutions of vector supernatants, followed by cytometric analysis 3 days later. Final vector titers were in the range of 10^9 transducing units (TU) per milliliter.

Transduction of CD34$^+$ cells with the lentiviral vector

One × 10^5 CD34$^+$ cells per milliliter were prestimulated for 72 hours with FL, SCF (both at 50 ng/mL), MGDF (20 ng/mL), and IL-6 (10 ng/mL). Cell transduction was carried out in flat-bottomed 24-well plates (Corning Costar, Cambridge, MA, USA). For transduction, 10^5 prestimulated CD34$^+$ cells were

Table 1. In vitro studies of $CD34^+$ cell expansion following HESW treatment

		Cell viability	Day 0	Day 21	Day 28	Day 35	Day 42	Day 56
Control	Cells	99% ± 2%	1	303 ± 50	650 ± 112	1300 ± 225	1905 ± 365	2251 ± 385
	$CD34^+$		1	9 ± 3	19 ± 10	64 ± 15	70 ± 21	93 ± 33
	$CD34^+CD38^-$		1	9 ± 4	44 ± 22	50 ± 26	210 ± 65	154 ± 121
	CFC		1	9 ± 2	15 ± 4	30 ± 10	52 ± 22	62 ± 31
0.22 mJ/mm^2 × 250 shots	Cells	92% ± 4%	1	340 ± 55	685 ± 148	1209 ± 486	2020 ± 546	2165 ± 365
	$CD34^+$		1	7 ± 4	15 ± 3	59 ± 21	82 ± 51	111 ± 52
	$CD34^+CD38^-$		1	9 ± 2	58 ± 15	58 ± 29	87 ± 38	91 ± 32
	CFC		1	10 ± 3	15 ± 6	35 ± 19	45 ± 15	56 ± 21
0.22 mJ/mm^2 × 500 shots	Cells	90% ± 8%	1	448 ± 96	986 ± 258	1210 ± 350	2381 ± 276	3072 ± 402
	$CD34^+$		1	11 ± 4	20 ± 8	74 ± 22	130 ± 46	143 ± 39
	$CD34^+CD38^-$		1	17 ± 6	88 ± 29	52 ± 26	57 ± 30	56 ± 19
	CFC		1	13 ± 2	21 ± 1	43 ± 6	47 ± 16	37 ± 11
0.22 mJ/mm^2 × 1000 shots	Cells	85% ± 9%	1	480 ± 75	1088 ± 206	1280 ± 259	1882 ± 497	2560 ± 649
	$CD34^+$		1	10 ± 3	23 ± 6	90 ± 17	160 ± 44	217 ± 52
	$CD34^+CD38^-$		1	38 ± 15	138 ± 29	107 ± 27	133 ± 158	100 ± 25
	CFC		1	14 ± 3	25 ± 9	42 ± 9	48 ± 14	47 ± 22
0.32 mJ/mm^2 × 500 shots	Cells	85% ± 7%	1	394 ± 65	669 ± 258	1536 ± 295	2022 ± 149	2816 ± 758
	$CD34^+$		1	11 ± 6	12 ± 4	79 ± 12	148 ± 46	221 ± 61
	$CD34^+CD38^-$		1	27 ± 16	87 ± 20	111 ± 31	142 ± 47	150 ± 47
	CFC		1	13 ± 8	25 ± 3	39 ± 12	51 ± 7	72 ± 41
0.32 mJ/mm^2 × 1000 shots	Cells	80% ± 15%	1	126 ± 53	309 ± 96	361 ± 84	1234 ± 548	1646 ± 419
	$CD34^+$		1	4 ± 2	15 ± 6	17 ± 8	72 ± 17	76 ± 32
	$CD34^+CD38^-$		1	18 ± 10	8 ± 4	6 ± 1	20 ± 5	73 ± 18
	CFC		1	3 ± 1	5 ± 2	7 ± 3	27 ± 9	26 ± 6
0.43 mJ/mm^2 × 1000 shots	Cells	75% ± 21%	1	79 ± 16	270 ± 49	315 ± 85	654 ± 125	814 ± 95
	$CD34^+$		1	2 ± 1	10 ± 4	16 ± 4	56 ± 21	72 ± 15
	$CD34^+CD38^-$		1	49 ± 19	72 ± 41	84 ± 22	452 ± 76	1035 ± 546
	CFC		1	7 ± 2	14 ± 4	7 ± 2	32 ± 11	14 ± 1

Human CB $CD34^+$ cells were cultured in stroma-free suspension medium in Iscove's modified Dulbecco medium (IMDM) with 10% fetal calf serum and FLT3 ligand (50 ng/mL), MGDF (20 ng/mL), SCF (50 ng/mL), and IL-6 (10 ng/mL). On day 3 HESWs were delivered to cells in culture, then cells were maintained in culture for up to 50 days with the biweekly exogenous addition of growth factors. Starting from day 21, aliquots of cultures were removed and analyzed for the progenitor cell content. Values represent the mean ± SD of fold increase in three separate experiments performed in triplicate wells.

resuspended in 100 μL IMDM with 10% FCS: 1) cells were transduced with LVs at a multiplicity of infection (MOI) of 30, corresponding to the transducing units of 30×10^6/mL, for 4 hours in the presence of the same cytokine combination and incubated at 37°C and 5% CO_2. Cells were then harvested, washed twice, and used to initiate both stroma-free expansion cultures, LTC-IC and semisolid cultures, or transplanted in NOD/SCID mice (LV cells); 2) cells were HESW-treated (as explained above) at the start of the transduction procedure, which was carried out as in (1) (HESW-LV cells).

Single-colony reverse trascriptase polymerase chain reaction (RT-PCR) for transgene expression

Single colonies originated from LTC-IC, BFU-E, and CFU-GM at different time points were individually picked up by pipette under optical microscope at 4× magnification and dispensed in 50 μL of double-distilled water treated with dimethyl pyrocarbonate (DEPC) (Ambion Inc., Austin, TX, USA). Samples were resuspended in Trizol reagent (GIBCO BRL, Grand Island, NY, USA) and stored at −80°C for further RNA extraction according to manufacturer's instructions. Briefly, samples were treated with DNAse I (Boeringher Mannheim, Mannheim, Germany) and oligo(dT)-primed first-strand cDNA synthesis using SuperScript II reverse trascriptase (Invitrogen Life Technologies, Gaithersburg, MD, USA). Ten μL of cDNA solution obtained were then used for PCR analysis of the transgene expression using primers and conditions described below.

Animals

NOD/LtSz scid/scid (NOD/SCID) mice were obtained from Jackson Laboratories through Charles River Italia (Calco, Italy) and maintained in the animal facilities at the Centro di Immunogenetica ed Oncologia Sperimentale (CIOS [Immunogenetic and Experimental Oncology Center], Turin, Italy). All animals were handled under sterile conditions and maintained in microisolator cages. The mice to be transplanted were irradiated at 6 to 8 weeks of age with 350 cGy of total-body irradiation from a ^{137}Cs source. Then, after 24 hours, they were given a single intravenous injection of 1×10^5 human CB $CD34^+$ cells according to the experimental plan. In particular, we performed a first series of four separate experiments in which a total of 12 mice per point were injected with: 1) 1×10^5 human CB $CD34^+$ prestimulated for 72 hours without further treatment (control cells), 2) 1×10^5 CB $CD34^+$ cells prestimulated for 72 hours and then exposed to HESW (HESW cells) to evaluate the effect of HESW on in vivo engraftment. In a second series of three separate experiments, a total of 9 mice per point were injected with: 1) 1×10^5 CB $CD34^+$ cells which were prestimulated for 72 hours and then transduced

with the lentiviral vector (LV cells); 2) 1×10^5 CB $CD34^+$ cells that were prestimulated for 72 hours and then transduced with the lentiviral vector in the presence of HESW (HESW-LV cells) to evaluate the effect of HESW on the long-term expression of the transgene in vivo. For the secondary or tertiary transplantations, 10 to 15×10^6 bone marrow cells of the primary or secondary recipients (corresponding to 1×10^5 human cells) were inoculated in each mouse, after irradiation as detailed above.

Flow cytometric detection of human cells in murine tissues

BM cells were flushed from the femurs and tibias of each mouse to be assessed using a syringe and a 26-gauge needle at 6 to 8 weeks from transplantation. To prepare the cells for flow cytometry, contaminating red blood cells were lysed with 8.3% ammonium chloride and the remaining cells were then washed in HBSS with 0.1% bovine serum albumin (BSA; Sigma Chemical Co, Milan, Italy), 0.01% sodium azide (HFN). The cells were then resuspended at 1 to 2×10^6 cells/mL and incubated with mouse IgG (Fluka Chemika Biochemika, Buchs, Switzerland) and with 5% HS to block nonspecific binding to Fc receptor for 30 minutes at 37°C. Cells were washed and then incubated with monoclonal antibodies (MoAb) specific for human CD45, CD71, and Glyco-A (all from Dako A/S, Glostrup, Denmark) or CD34, CD19, CD14, CD33, CD41, or CD61 (all from Becton-Dickinson, Milan, Italy) directly labeled with fluorescein isothiocyanate (FITC) or PE for 30 minutes at 4°C to assess the human cell engraftment. Cells stained with an anti-CD45 conjugated to an R-phycoerythrin-Cy5 tandem conjugate were simultaneously stained with an anti-human CD34 PE (Becton-Dickinson) and anti-human CD19 FITC to quantify the total human $CD34^+$ and $CD19^+$ cell populations. In some mice, additional aliquots were stained with anti-human CD33 PE, CD61 FITC, or CD41 FITC in combination with anti-human CD45 Cy5 RPE to discriminate subpopulations within the CD45 gate and with anti-human CD71 FITC plus GpA PE. Aliquots of cells from each suspension were similarly incubated with irrelevant (control) MoAbs labeled with FITC and PE. Cells from an unmanipulated NOD/SCID mouse were also stained with each of the MoAbs used to detect positively stained human cells. Only levels of fluorescence which excluded 99.9% of all of these negative controls were considered as specific. After staining, all cells were washed once in sodium azide (HFN) containing 2 μg/mL propidium iodide (PI) to allow dead cells (PI^+) to be excluded from the analyses.

Flow cytometric analysis was performed using a FACScan cytometer (Becton-Dickinson, San Jose, CA, USA). At least 50,000 events were acquired for each analysis. When fluorescent cells represented only a minority of the total population (0.1%) more events (at least 100,000) were analyzed.

DNA extraction and molecular analysis of human cell engraftment

High-molecular-weight DNA was extracted from the BM of mice by the NucleoSpin Blood Kit (Machery-Nagel Inc. Easton, PA, USA). The presence of human-specific DNA within the murine BM of transplanted mice was confirmed by PCR amplifying an 850-bp fragment of the α-satellite region of the human chromosome 17 using forward primer: 5′GGGATAATTTCAGCTGACTAAA and reverse primer: 5′TTCCGTTTAGTTAGGTGCAGTTATC. The reaction was performed in a final volume of 50 μL using 250 nM of each primer, 200 μM each of the respective nucleotides, 2 mM $MgCl_2$,in 1× Taq buffer containing $(NH_4)_2SO_4$. All PCR reaction was performed using 0.5 U of Taq DNA polymerase (Fermentas Inc., Hannover, MD, USA). Following an initial DNA denaturation and at 94°C for 10 minutes, 35 1-minute cycles of denaturation at 94°C, annealing at 60°C, and extension at 72°C were performed followed by a final elongation step at 72°C for 10 minutes. All amplified DNA fragments were electrophoresed through 1% agarose gel and subsequently visualized through ultraviolet light after staining with ethidium bromide.

PCR detection of marker gene green fluorescent protein

The presence of marker gene GFP in NOD/SCID BM cells and in CFU-GM, BFU-E, and LTC-IC generated during stroma-free long-term cultures on day 0, day 28, and day 49 was confirmed by PCR analysis using the specific forward primer 5′-GCTGG ACGGCGACGTAAAC, and the reverse primer 5′CCATGT GATCGCGCTTCTC. The amplification cycle was 4 minutes at 95°C; 35 1-minute cycles of denaturation at 94°C, 1 minute 30 seconds annealing at 54°C, and 1 minute extension at 72°C were performed followed by a final elongation step at 72°C for 10 minutes. Amplified DNA fragments were electrophoresed through 1% agarose gel and the specific 604-bp product visualized through ultraviolet light after staining with ethidium bromide. To enhance signal, PCR products were transfer by Southern blot to nylon membrane (Hybond-N, Amersham Bioscences, Cologno Monzese, Milan, Italy) and hybridized for 1 hour at 37°C in the presence of Rapid-hyb buffer (Amersham Bioscences, Cologno Monzese, Milan, Italy) with GFP forward and reverse primers previously [^{32}P]-labeled using 20 U of T4 polynucleotide kinase (New England BioLabs, Celbio, Italy) and γ[^{32}P]-adenosine-5′-triphosphate.

PCR detection of housekeeping gene gliceraldeide phosphate dehydrogenase (GAPDH)

To verify whether the same amount of DNA was amplified in the above-described PCR, 10 ng of DNA extracted from each murine BM and 10 μL of total cDNA from each plucked colony (CFU-GM, BFU-E, and LTC-IC) was analyzed also for the presence of housekeeping gene GAPDH using forward primer: 5′ACCA CAGTCCATGCCATCCAC and reverse primer: 5′TCCAC CACCCTGTTGCTGTAG that amplified a 555-bp fragment of genomic DNA both of mouse and human origin. The amplification cycle was 5 minutes at 94°C; 35 1-minute cycles of denaturation at 94°C, 1 minute 30 seconds annealing at 60°C, and 1 minute 30 seconds extension at 72°C were performed followed by a final-elongation step at 72°C for 10 minutes.

Statistical analysis

Data are expressed throughout as mean ± standard deviations (SD). Statistical comparisons between control cells and HESW cells and LV cells and HESW-LV cells were performed with the Student's *t*-test per $p < 0.05$.

Results

Table 1 shows the effects of a wide range of HESW energy and number of shots on $CD34^+$ cells in three separate experiments. In brief, based on these initial experiments, considering the day-28 data, in order to evaluate the effects on very long-term cultures, engraftment, and transduction

Table 2. In vitro expansion of CB $CD34^+$ cells

	Controls			0.22 mJ/mm^2 per 500 shots			0.32 mJ/mm^2per 500 shots		
	Cells	$CD34^+$	CFC	Cells	$CD34^+$	CFC	Cells	$CD34^+$	CFC
Day 0	1	1	1	1	1	1	1	1	1
Day 18	51.7 ± 6.3	4.7 ± 0.5	1 ± 0.3	45 ± 5.5	3.4 ± 0.8	0.6 ± 0.1	49.2 ± 17.2	3.7 ± 1.7	209.1 ± 85.8
Day 28	152.5 ± 3.5	2.8 ± 1.5	4 ± 2.2	150 ± 7.9	3.8 ± 1.5	2.9 ± 1	121.6 ± 7.6	3.8 ± 0.2	388.1 ± 167.9
Day 33	244.5 ± 4.9	13.5 ± 0.7	ND	336 ± 22.9	20.7 ± 6.4	ND	244.3 ± 28.1	10.6 ± 3	ND
Day 40	460 ± 14.1	25.5 ± 2.3	10 ± 5.9	642 ± 39	46.8 ± 12.9	5.3 ± 1.4	377.3 ± 108.3	25.9 ± 5	522 ± 69
Day 47	1040 ± 1	ND	ND	800 ± 125	ND	ND	866.6 ± 197.3	ND	619.1 ± 374.6
Day 54	2076 ± 412.9	119.2 ± 18.7	23 ± 3.2	1321 ± 14	70 ± 22	25.1 ± 18	1997.3 ± 32.3	112.1 ± 34	1957.8 ± 778
Day 61	8640 ± 1,357	447.5 ± 7.7	ND	5440 ± 896	238 ± 83	ND	7786.6 ± 2,485	449.3 ± 41	10,136 ± 719
Day 67	14,720 ± 1,267	386.5 ± 142.1	82 ± 17.1	7680 ± 196	159 ± 81	94.2 ± 18	9913.3 ± 2,576	293.7 ± 29	1207.3 ± 675
Day 76	34,560 ± 5430	1188 ± 528	ND	21,760 ± 1687	226 ± 75	ND	31,146.6 ± 9942	1312 ± 153	ND
Day 82	43,136 ± 17,558	656 ± 216	64 ± 24.4	42,752 ± 2597	658 ± 122	250.1 ± 39	36,864 ± 20,704	1428 ± 860	5634.2 ± 675
Day 98	47,608 ± 9580	848.1 ± 326	166 ± 39.1	65,536 ± 3645	1114 ± 321	138.1 ± 29	50,176 ± 5161	1185.8 ± 166	26,679 ± 3872
Day 106	98,816 ± 12,380	ND	253 ± 105.4	55,296 ± 5545	ND	178.5 ± 34	113,664 ± 10,096	ND	31,434 ± 4695
Day 113	176,742.5 ± 21,347	2271.5 ± 997	319 ± 124.6	133,120 ± 12,120	1546 ± 164	478.2 ± 112	184,729 ± 19,449	2576.8 ± 720	23,672 ± 2908
Day 119	285,286.5 ± 31,482	2341.3 ± 300	282 ± 986	108,954 ± 10,255	1240 ± 79	492.9 ± 199	258,185 ± 29,476	3351.1 ± 785	108,078 ± 15,629
Day 132	151,552 ± 25,304	789.3 ± 244	99 ± 24.4	73,728 ± 6456	1111 ± 29	166.2 ± 84	324,949 ± 38,713	12,082 ± 1412	222,495 ± 37,973
Day 142	450,610 ± 54,443	1319.5 ± 179	142 ± 1.8	54,067 ± 4110	1935 ± 134	277.7 ± 85	1,399,740 ± 197,916	5328.5 ± 548	45,558 ± 7890
Day 153	802,816 ± 99,633	1153.4 ± 135	ND	196,608 ± 1987	3283 ± 986	ND	1,419,947 ± 217,786	4792.8 ± 618	ND

CB $CD34^+$ cells were maintained in stroma-free cultures with the beweekly addition of FL and SCF (both at 50 ng/mL), IL-6 (10 ng/mL), and MGDF (20 ng/mL). On day 0 tested cells underwent HESW treatment and finally plated in wells. Results are expressed as mean ± SD fold expansion observed in five separate experiments performed in triplicate wells.

of $CD34^+$ HSC, we opted for the 0.22 mJ/mm^2 and 0.32 mJ/mm^2 per 500 shot conditions and new experiments were carried out.

Cell expansion

In vitro. Results are expressed as mean ± SD fold increases of five experiments (Table 2); a further representative experiment is shown in Figure 1. The cell viability, following HESW, was 88% ± 2% for the 0.22 mJ/mm^2 per 500 shots, and 85% ± 3% for the 0.32 mJ/mm^2 per 500 shots (p = NS). The total cell expansion generated in vitro following HESW at 150 days did not significantly differ from either the control culture or the 0.22 mJ/mm^2 per 500 shots and 0.32 mJ/mm^2 per 500 shots treated cells (p = NS).

The $CD34^+$ cell expansion was higher for the 0.32 mJ/mm^2 per 500 shots treated cells than controls ($p = 0.05$) and for 0.22 mJ/mm^2 per 500 shots treated cells compared to controls ($p = 0.03$). Cumulative 150-day expansion was 1153-fold for controls, 3283-fold for 0.22 mJ/mm^2 per 500 shots, and 4792-fold for 0.32 mJ/mm^2 per 500 shots.

The differences among the groups reached statistical significance after day 98 of culture. For the CFC output we observed a greater and statistically different proliferative stimulus from day 18 following 0.32 mJ/mm^2 per 500 shots ($p = 0.02$) and 0.22 mJ/mm^2 per 500 shots ($p = 0.02$), both compared to controls. This was probably due to an early effect of HESW from day 5 to day 18. After day 18, the expansion of control $CD34^+$ cells and HESW-treated $CD34^+$ cells was similar.

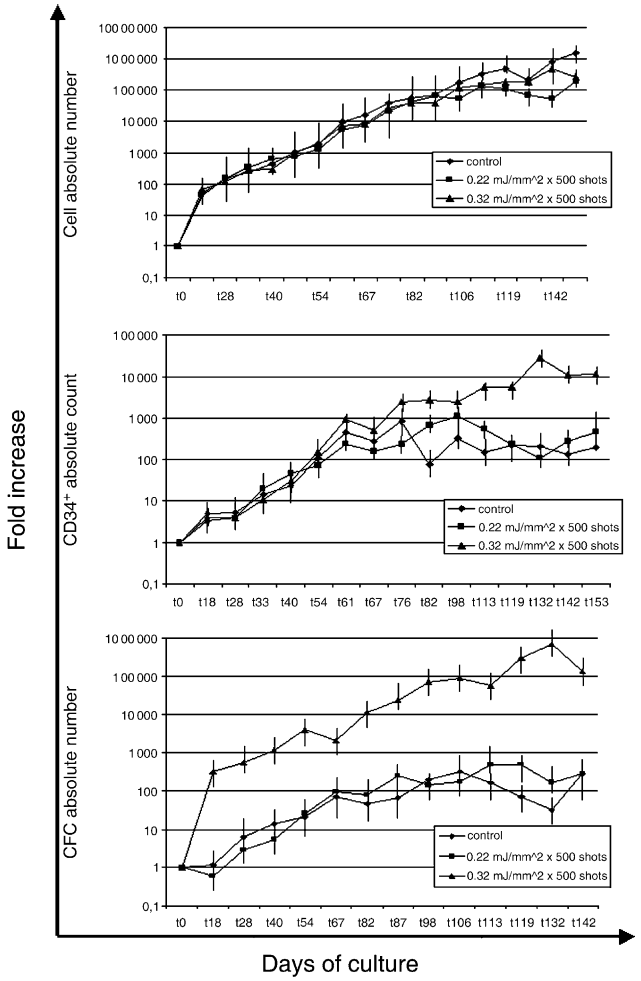

Figure 1. Expansion of cells, $CD34^+$ cells and CFCs following HESW treatment. A representative experiment. 1×10^4 $CD34^+$ cells/well were cultured for 72 hours with FL (50 ng/mL), SCF (50 ng/mL), MGDF (20 ng/mL), and IL-6 (10 ng/mL) and then treated with HESW. Controls: untreated cells. Cells were maintained in cultures as reported in material and methods. Each week's aliquots were analyzed for the $CD34^+$, $CD34^+CD38^-$, and CFC content. Results are expressed as a mean value of three wells per experimental point of a representative experiment out of five (summarized in Table 2).

Engraftment of human HESW-treated cells in NOD/SCID mice. The long-term engraftment ability of HESW-treated $CD34^+$ cells was determined following transplantation in NOD/SCID mice. Results are outlined in Table 3, which depicts the mean engraftment values ± SD of 12 animals. While no differences were found for the human $CD45^+$ cell engraftment, the HESW treatment did permit a greater engraftment of human $CD45^+CD34^+$ cells (10% for controls, 12.2% for 0.22 mJ/mm^2 per 500 shots [$p = 0.05$], and 14.5% for 0.32 mJ/mm^2 per 500 shots [$p = 0.05$], respectively) and human $CD45^+CD34^+CD38^-$ cells (0.87% for controls and 1.4% for 0.22 mJ/mm^2 per 500 shots [$p = 0.05$] and 1.8% for 0.32 mJ/mm^2 per 500 shots [$p = 0.05$], respectively) (Fig. 2). Moreover, the B cell and the erythroid progenitor engraftment was greater in HESW cell–transplanted mice. Five hundred thousand mononuclear cells harvested from primary mice were cultured in triplicate in semisolid media, which only allowed the growth of human cells, to assess the human hemopoietic progenitor engraftment. The HESW treatment enhanced the human CFC engraftment in primary mice (13 ± 5.4 vs 103 ± 28) (Table 4). For the secondary and the tertiary transplantations, 10 to 15 $\times$ 10^6 bone marrow cells of the primary or secondary recipients (corresponding to 1 $\times$ 10^5 human cells) were inoculated in each recipient. Results show how the secondary and tertiary repopulating abilities were not lost following HESW treatment. The finding of human cells in the marrow of secondary and tertiary recipients may suggest that self-renewal properties of the SRC cells is not lost in HESW-treated cells. Moreover, similar data (not shown) were observed from the spleen of the same NOD/SCID mice.

The results of flow cytometry were confirmed by qualitative PCR analysis of the human α-satellite gene in primary, secondary, and tertiary mice (a representative experiment is shown in Fig. 3).

Table 3. Engraftment of human CB CD34$^+$ cells in NOD/SCID mice

	Control	p	0.22 mJ/mm^2 per 500 shots	p	0.32 mJ/mm^2 per 500 shots
			Primary recipients		
BM huCD45$^+$	10.19% ± 4.2%	NS	11.3% ± 7.9%	NS	11.3% ± 8.2%
BM huCD34$^+$	10.2% ± 2.8%	NS	12.2% ± 1.22%	0.05	14.5% ± 5.4%
BM huCD34$^+$CD38$^-$	8.7% ± 3.4%	NS	11.7% ± 4.6%	0.05	12.5% ± 2.7%
BM huCD19$^+$	18.7% ± 3%	0.03	34.2% ± 8.7%	0.04	29.2% ± 2.1%
BM huCD33$^+$	49.8% ± 5.9%	NS	57.8% ± 9.7%	NS	57.8% ± 3.3%
BM huCD61$^+$	4.2% ± 1.7%	NS	2% ± 0.5%	NS	2.6% ± 1.1%
BM huCD71$^+$Glyco-A$^+$	0.7% ± 0.9%	0.05	3% ± 2.4%	0.02	5.8% ± 3.3%
			Secondary recipients		
BM huCD45$^+$	2.6% ± 1.4%	NS	1.1% ± 1.3%	NS	1.5% ± 1.7%
			Tertiary recipients		
BM huCD45$^+$	0.3% ± 0.2%	NS	0.3% ± 0.2%	NS	0.7% ± 0.7%

The primary recipients were transplanted with 1×10^5 expanded CD34$^+$ cells. The secondary and tertiary mice were grafted with 10 to 15 $\times$ 10^6 murine marrow cells (corresponding to 1 $\times$ 10^5 human cells) of the primary or secondary animals, respectively. The animals were sacrificed 6 weeks following transplantation. The percentage of human lineage-committed cells were calculated within the humanCD45$^+$ gate. p values refer to each type of HESW treatment compared to controls (no treatment). Values are the mean ± SD of the levels of engraftment evaluated in three animals in four experiments each.

Cell transduction

In vitro. In these experiments CD34$^+$ cells prestimulated for 72 hours were: 1) HESW-treated at 0.32 mJ/mm^2 per 500 shots and incubated for 4 hours in the presence of the lentiviral vector (HESW-LV cells); or 2) prestimulated for 72 hours and then transduced in the presence of the lentiviral vector (LV cells). Cells were then maintained in vitro in long-term culture and the GFP$^+$ expression was monitored weekly. On day 7 GFP$^+$ cell expression was comparable between the groups (7.8% ± 2.3% for the LV cells vs 8.3% ± 2% for the HESW-LV cells, p = NS). After 42 days the LV cells had 6.5% ± 2.4% of GFP$^+$ cells in culture, while HESW-LV cells had 11.4% ± 8.4% of GFP$^+$ cells (p = 0.04). After 56 days of culture the LV cells had 5.3% ± 2.6% of GFP$^+$ cells while the HESW-LV cells had 25.2% ± 7.5% of GFP$^+$ cells (p = 0.003). Moreover, after 70 days of culture the GFP$^+$ cells were 5.8% ± 2.5% for the LV cells, while the HESW-LV cells were 65% ± 22% (p = 0.00001) (Table 5). A representative experiment is outlined in Figure 4, which shows that the absolute number of GFP$^+$ cells increases with time. This and Table 6 indicate that the GFP$^+$ cells are present in different subpopulations, including the more immature CD33$^+$CD14$^-$ cells. GFP$^+$ cells were also found in the Glyco-A$^+$ cells and in the CD19$^+$ cells. Moreover, the absolute number of GFP$^+$ cells increases with time; thus the increased number of GFP$^+$ cells in cultures was probably due to a GFP trasduction of more immature cells and not to the macrophages phagocytosing the GFP protein released in the cultures. Results are expressed as a cumulative fold increase compared to day 3 after transductions. The RT-PCR analysis of CD34$^+$ cultured cells also showed the presence of the GFP mRNA (data not shown). The CFU-GM, BFU-E, and LTC-IC were also GFP transduced (Table 7). FACS dot plots are provided in Figure 5. GFP$^+$ expression was also monitored in the CD34$^+$, CD14$^+$, CD19$^+$, and Glyco-A$^+$ populations (Table 5 for CD34$^+$ transduced cells, Table 6 for committed myeloid cells, B lymphocyte, and erythroid progenitor transduction) and also in a clonogenic compartment (Table 7; images of GFP$^+$ colonies are also provided in Fig. 6). RT-PCR analysis for mRNA of the GFP marker was performed in plucked CFC generated in stroma-free long-term cultures and showed the presence of the mRNA of GFP marker in CFU-GM, BFU-E, and LTC-IC on day 0, day 28, and day 49 (Fig. 7).

Engraftment of human GFP$^+$ human CD45$^+$ HESW-treated cells in NOD/SCID mice. Following HESW treatment and transduction, cells were immediately transplanted in NOD/SCID mice which had received 350 cGy 24 hours before. Results are expressed as the mean values ± SD of 9 animals. Mice were sacrificed after 6 to 8 weeks from transplantation. All injected animals were engrafted. The percentage of GFP$^+$ cells found in the HESW-LV cells was not significantly different from LV cells. Furthermore, since in this series of experiments greater levels of human cell were found in the murine marrows (39.6% vs 15%), it is reasonable to argue that a greater number of human GFP$^+$ cells home into the xenogenic recipients. Once again, also in these series of NOD/SCID transplants, there was a higher engraftment level of CD34$^+$ cells following HESW treatment compared to the control (33.8% compared to 27.3% for LV-treated cells, respectively; p = 0.005). Also, the HESW treatment favorably affected the megakaryocyte engraftment (4.2% compared to 2.5% for LV-treated cells, respectively; p = 0.05). The percentage of GFP$^+$ human cells in mice injected with HESW-LV cells was greater than, but not statistically significant to, that observed in mice injected with LV cells (34% and 26.4%, respectively, p = NS),

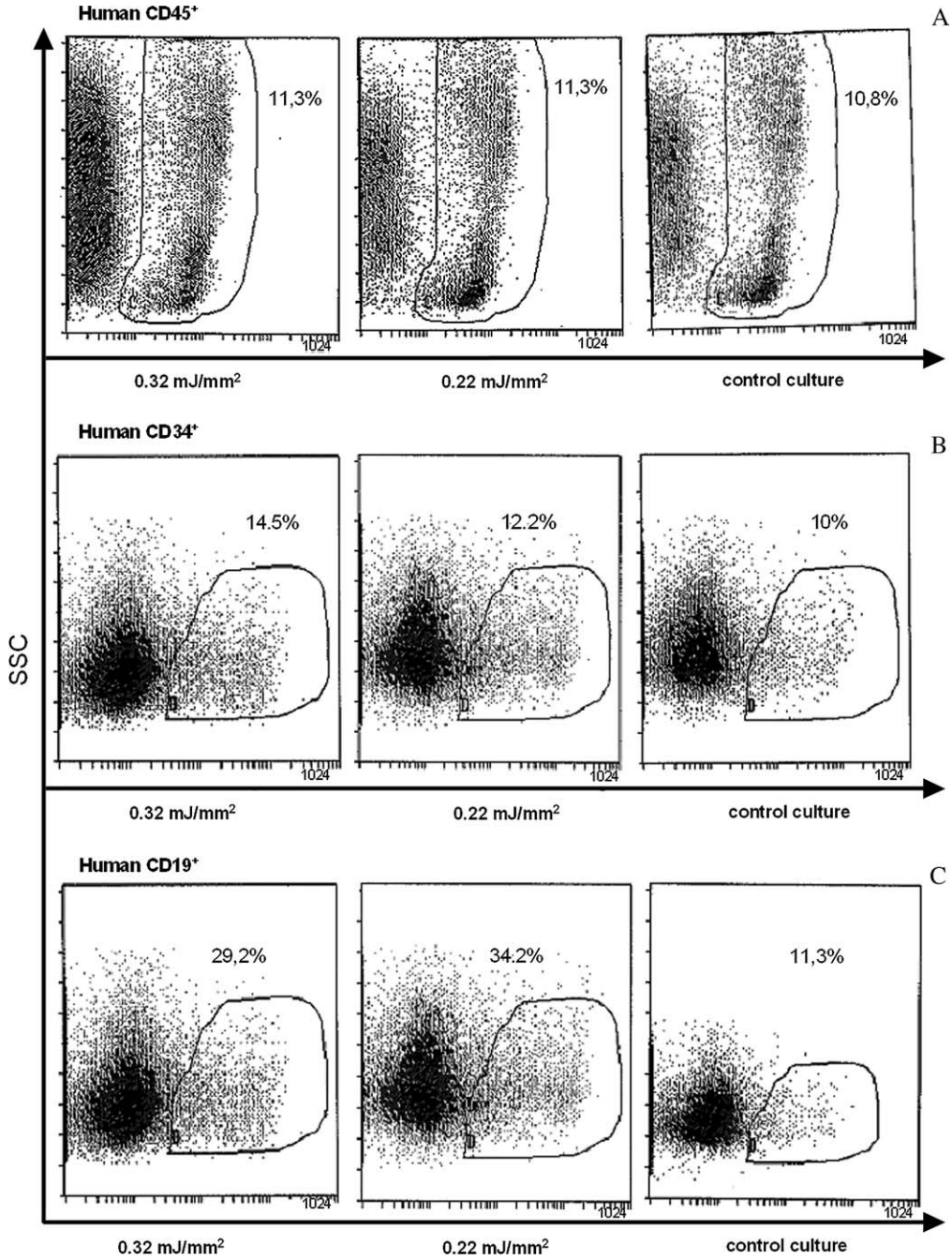

Figure 2. FACS dot-plots prints of human CD34$^+$ CB cells engraftment in NOD/SCID mice. Representative experiment of human cell engraftment in NOD/SCID mice. Cells were stained with FITC or PercP anti-human CD45, PE anti-human CD34, and PE anti-human CD19.

suggesting that the transgene integration and expression were not hampered by HESW manipulation. Moreover, all animals that received transplants showed multilineage engraftment (Table 8). Each subpopulation contained similar proportions of GFP$^+$ cells, and 15% to 29% of the human engrafted CD34$^+$ cells were GFP$^+$, indicating that the HESW application was not detrimental to the maintenance of the transduced CD34$^+$ population. The engraftment of human GFP$^+$ cells in the murine marrow was also confirmed by a qualitative PCR analysis of the human α-satellite and the GFP genes (a representative experiment is shown in Fig. 8).

Discussion

The effect of mechanical forces on cells and tissues is of great interest because living organisms undergo constant

Table 4. Human colonies generated from bone marrow of NOD/SCID mice

	Controls	*p*	0.22 mJ/mm^2× 500 shots	*p*	0.32 mJ/mm^2 × 500 shots
CFU-GM	9±3.4	0.002	60±21.5	0.0001	74±19.7
BFU-E	4±1.5	0.004	20±4.9	0.003	29±14.8

The results are expressed as mean ± SD of colony numbers generated from triplicate cultures of five individual mice per point. 5×10^5 mononuclear cells harvested from primary mice were cultured in plasma clot with an addition of specific human growth factors. Colonies were counted 14 days later. Statistical analysis was performed comparing controls and the 0.22 mJ/mm^2 per 500 shots condition and controls and the 0.32 mJ/mm^2 per 500 shots condition.

changes during stress. A shock wave–induced acoustic streaming effect may facilitate cell growth or enzymatic activities. Furthermore, HESW-promoted cavitation and cell permeability may result in vascular and bone regeneration [37–43].

Experiments described here were carried out to study the effects of HESW treatment on human CD34$^+$ stem cells. Two main points were investigated. The first was aimed at understanding whether (as also reported for osteoprogenitors [41,42]) the HESW might induce a proliferation stimulus on hemopoietic stem cells. Although we found that middle- to high-energy shock wave treatment did give a growth boost to CD34$^+$ and CFC proliferation, the reason as to why this effect was elicited remains unclear. In all the experiments we performed, 0.22 or 0.32 mJ/mm^2 and a low to intermediate number of shots enhanced the cytokine-induced cell proliferation compared to control cells. This was clearly evident in long-term suspension cultures, while in the short-term cultures it was not. For osteoprogenitors acoustic energy and pulsed pressure released by HESW is translated into biological signals that promote recruitment and osteogenic differentiation of mesenchymal stem cells [43]. These HESW events may be mediated by trasforming growth factor β1 (TGF-β1) and vascular endothelial growth factor-A (VEGF-A) [44], in which HESW-mediated VEGF-A release was mediated by Ras-induced superoxide and ERK-dependent HIF-1α activation [45]. Moreover, studies on in vitro expansion of human CB CD34$^+$ cells with MGDF, SCF, FL may benefit from the addition of VEGF-A [46]. On the other hand, previous studies have shown that the most primitive engrafting HSC resides mainly in the TGF-β-dependent quiescent phase of the cell cycle and the addition of anti-TGF-β1 in culture medium, containing TPO, FL, SCF, and IL-6, did cause an early loss of multilineage potential while a low proportion of HSC maintain quiescent behavior [47]. Other recent reports have shed some light on the role of TGF-β1 on the cell-cycle regulation. In particular it has been shown that the addition of anti-TGF-β1 to cultures of CD34$^+$CD38$^-$c-kit^{high} cells increase the percentage of the S/G2/M phase of the cell cycle, while no effects were documented on CD34$^+$CD38$^-$c-kit $^{low/neg}$ cells [48]. These published data could suggest the possibility that in the hemopoietic system the biological effects mediated by HESW might be due to the modulation of TGF-β1 and VEGF. In this study we adopted the ELISA method to assess if HESW treatment did enhance the release of VEGF-A and TGF-β1 early after treatment. No differences were observed among HESW cells and controls (data not shown).

In our study, the major effect of HESW treatment seems to be directed on the early progenitors. If the CD34$^+$CD38$^-$ population survives the treatment and more of the committed progenitors and mature cells die, the HSC must undergo multiple mitosis in order to continuously expand. In our system, it is possible to argue that we obtain an early progenitor cell expansion while the terminally committed cells die.

The second point we analyzed was the transductional effect throughout the cavitational effect mediated by HESW. Results are provided by two types of experiments: the in vitro cultures and the in vivo transplantation of transduced

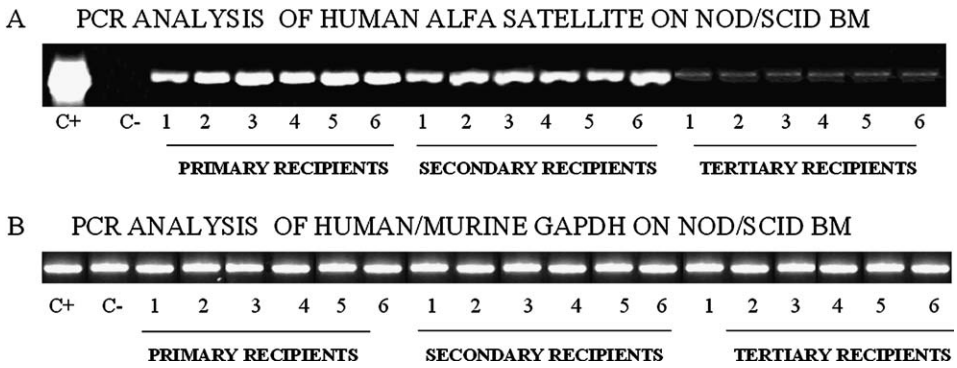

Figure 3. PCR analysis of human α-satellite in bone marrow cells of NOD/SCID mice following serial transplantation. A representative experiment showing human engraftment in NOD/SCID mice injected with control cells (mice 1 to 3) or with HESW-treated cells (mice 4 to 6). Secondary and tertiary recipients received 10–15 × 10^6 cells derived from BM of primary and secondary mice respectively. C+ = human CD34$^+$ cells, C− = nontranplanted NOD/SCID mouse marrow.

Table 5. Total GFP^+ cells and $CD34^+$ /GFP cells during in vitro expansion cultures

		GFP^+ cells In stroma-free culture	*p*	$CD34^+$ GFP^+ cells	*p*
Day 7	LV cells	7.8% ± 2.3%	NS	1.8% ± 1.5%	NS
	HESW-LV cells	8.3% ± 2%		2.2% ± 1.17%	
Day 14	LV cells	10.83% ± 1.17%	0.03	3.23% ± 0.07%	NS
	HESW-LV cells	21.88% ± 0.57%		4.25% ± 0.66%	
Day 21	LV cells	8.44% ± 0.96%	0.001	1.2% ± 0.25%	NS
	HESW-LV cells	14.1% ± 4.7%		1.55% ± 0.54%	
Day 28	LV cells	7% ± 1.48%	0.05	0.98% ± 0.16%	NS
	HESW-LV cells	12.61% ± 2.7%		1.23% ± 0.33%	
Day 35	LV cells	6.2% ± 0.6%	NS	0.84% ± 0.32%	NS
	HESW-LV cells	10.83% ± 4.5%		1.14% ± 0.71%	
Day 42	LV cells	6.5% ± 2.4%	0.04	1.5% ± 0.77%	0.05
	HESW-LV cells	11.45% ± 8.4%		2.7% ± 2.06%	
Day 49	LV cells	3.9% ± 2.5%	0.004	0.86% ± 0.2%	0.04
	HESW-LV cells	20.1% ± 8.5%		1.39% ± 0.97%	
Day 56	LV cells	5.3% ± 2.6%	0.003	2.05% ± 0.98%	NS
	HESW-LV cells	25.2% ± 7.5%		1.82% ± 0.53%	
Day 63	LV cells	5.6% ± 3.9%	0.001	2.17% ± 0.19%	NS
	HESW-LV cells	29.3% ± 18.4%		2.54% ± 1.02%	
Day 70	LV cells	5.8% ± 2.5%	0.00001	2.9% ± 1.5%	0.002
	HESW-LV cells	65% ± 22.5%		7.5% ± 4.5%	

Results are expressed as mean ± SD of the percent values of five separate experiments each performed in triplicate wells.

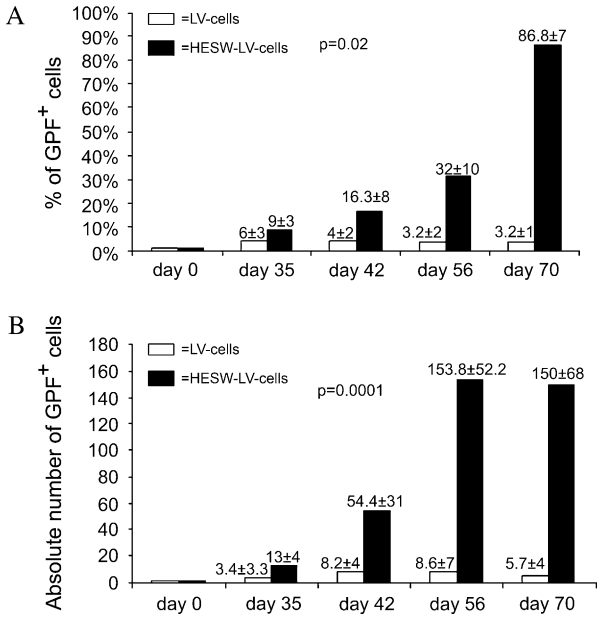

Figure 4. Percentage and absolute number of cells expressing the GFP marker. Cells were cultured for 72 hours with FL (50 ng/mL), SCF (50 ng/mL), MGDF (20 ng/mL), and IL-6 (10 ng/mL) and then were transduced with a lentiviral vector (30 MOI) (controls), or were treated with HESW at 0.32 mJ/mm^2 per 500 shots and then transduced with a lentiviral vector (30 MOI). Cells were cultured in a stroma-free medium and the percentage of cells expressing the green fluorescent protein (GFP) were monitored by flow cytometry. Results are expressed as the mean ± SD of three wells of a representative experiment (see also Table 5, which summarizes the results of five separate experiments).

HSCs. In vitro cell cultures following HESW treatment and transduction with LVs (30 MOI corresponding to 30×10^6 transducing units/mL) gave rise in vitro to a greater production of GFP^+ cells compared to LV cells. Experimental conditions with HESW treatment after 70 days of cell culture produced 65% ± 22.5% of GFP^+ cells compared to 5.8% ± 2.5% of control cells. This was matched by an absolute 150-fold increase of HESW-treated cells compared to a 7.7-fold increase of GFP^+ cells of controls (LV cells). This may be due to a clonal selection that sometimes occurs in long-lasting cultures. However, the clonal selection might also occur in the control cultures, but this is not the case. Also, a larger production of GFP^+ colonies was observed following HESW treatment, indicating that the transduction occurred at the level of very early progenitor stem cells. In fact, a large proportion of both myeloid and erythroid colonies and also more primitive LTC-IC were GFP^+ (on day 56 40% of CFU-GM were GFP^+ in the HESW-LV cell cultures compared to 3.8% in the LV cell cultures; on day 28 24% of LTC-IC were GFP^+ in the HESW-LV cell cultures compared to 3.8% in the LV cell cultures; no difference could be detected in the BFU-E transduction following HESW treatment). However, the PCR analysis showed the presence of mRNA GFP marker also in the BFU-E plucked from methylcellulose medium at days 28 and 49, whereas the GFP protein could not be detected. The RT-PCR analysis of $CD34^+$ cultured cells showed also the presence of GFP

Table 6. Monitoring of GFP expression in different cell populations during in vitro expansion cultures

		% of positive cells							
		$CD34^+33^+14^-$	$CD34^+33^+14^-$ GFP^+	$CD14^+$	$CD14^+$ GFP^+	$CD19^+$	$CD19^+$ GFP^+	Glyco-A^+	Glyco-A^+ GFP^+
Day 7	LV cells	ND	ND	ND	ND	ND	ND	ND	ND
	HESW-LV cells	ND	ND	ND	ND	ND	ND	ND	ND
Day 14	LV cells	18 ± 2.8	2.2 ± 0.05	ND	ND	ND	ND	ND	ND
	HESW-LV cells	16.7 ± 2.2	4 ± 0.5	ND	ND	ND	ND	ND	ND
Day 21	LV cells	10.4 ± 0.9	1.1 ± 0.3	26.9 ± 2.9	2.8 ± 0.3	1.2 ± 0.5	0.5 ± 0.04	0.7 ± 0.3	0.48 ± 0.16
	HESW-LV cells	6.5 ± 1.8	1.5 ± 0.5	26.9 ± 7.1	6.2 ± 2.8	2.5 ± 0.89	1 ± 0.5	2.1 ± 0.9	1.1 ± 0.8
Day 28	LV cells	8.4 ± 0.8	0.8 ± 0.3	35.2 ± 3.2	3.3 ± 0.3	1.1 ± 0.1	0.6 ± 0.06	1 ± 0.5	0.5 ± 0.14
	HESW-LV cells	5.6 ± 2.6	1.1 ± 0.5	27.9 ± 5.3	4.1 ± 1.4	1.9 ± 0.6	1 ± 0.1	2.3 ± 1.6	0.9 ± 0.5
Day 35	LV cells	5.7 ± 2.6	0.7 ± 0.2	41.5 ± 3.9	2.9 ± 0.5	1.7 ± 0.4	0.6 ± 0.1	1 ± 2.2	0.8 ± 0.1
	HESW-LV cells	4.2 ± 1.7	1 ± 0.3	42.4 ± 7.8	5.7 ± 2.2	2.3 ± 1.1	1.4 ± 0.4	2.1 ± 1.1	1.3 ± 0.5
Day 42	LV cells	6.6 ± 1.7	1.4 ± 0.4	20.1 ± 3.9	3.4 ± 0.7	1.2 ± 0.5	0.7 ± 0.14	1.8 ± 0.5	0.9 ± 0.4
	HESW-LV cells	4.3 ± 1.6	2.5 ± 0.6	25 ± 7.8	5.5 ± 1.8	2.1 ± 0.9	1.3 ± 0.9	1.5 ± 0.9	1.3 ± 1
Day 49	LV cells	1.6 ± 0.5	0.7 ± 0.4	ND	ND	ND	ND	ND	ND
	HESW-LV cells	1.5 ± 0.6	1.3 ± 0.6	ND	ND	ND	ND	ND	ND
Day 56	LV cells	2.8 ± 0.04	2 ± 0.2	25.9 ± 2.6	3.2 ± 1.4	2 ± 0.7	1.2 ± 0.19	1.2 ± 0.05	1.1 ± 0.1
	HESW-LV cells	1.6 ± 0.8	1.5 ± 0.6	22.7 ± 3.5	11.6 ± 8.4	3 ± 1	1.4 ± 0.15	0.9 ± 1.9	0.7 ± 0.5
Day 63	LV cells	2 ± 0.4	1.7 ± 0.5	27.7 ± 6.2	4.4 ± 1.6	0.19 ± 0.1	0.1 ± 0.2	0.2 ± 0.1	1.9 ± 0.2
	HESW-LV cells	1.8 ± 1.2	1.3 ± 0.9	30.2 ± 4.6	18.6 ± 5	0.4 ± 0.14	0.2 ± 0.4	1.5 ± 0.9	1.5 ± 3.3
Day 70	LV cells	2 ± 0.5	1.8 ± 1	25.4 ± 5.4	4.7 ± 2.4	0.7 ± 0.5	0.5 ± 0.6	0.3 ± 0.2	2 ± 0.9
	HESW-LV cells	2.4 ± 1	1.9 ± 3.4	22.7 ± 5.3	7.5 ± 3.8	1.4 ± 0.3	1.2 ± 0.9	1.9 ± 1	1.4 ± 0.8

Results are expressed as mean ± SD of five experiments each performed in triplicate wells.

mRNA marker, allowing us to hypothesize that the colonies generated in semisolid medium expressing the GFP mRNA and GFP protein were derived from those GFP^+CD34^+ cells.

Lastly, we investigated the repopulating ability of NOD/SCID mice by the HESW-treated cells. The human engraftment in serial transplantations of $CD34^+$ expanded cells has been shown to be the most instrumental in defining and characterizing the most primitive cells of the hemopoietic system [12–15]. In a first series of NOD/SCID mice we demonstrated that the engraftment of human early progenitors is enhanced following the application of HESW (human

Table 7. Monitoring of progenitor cell transduction (CFU-GM, BFU-E, LTC-IC) during in vitro expansion cultures

		CFU-GM	CFU-GM/GFP^+	BFU-E	BFU-E/GFP^+	LTC-IC	LTC-IC/GFP^+
Day 0	LV cells	197.5 ± 67.1	23 ± 2.8 (11.6%)	264.5 ± 72.4	9 ± 1.4 (3.4%)	3 ± 1.4	0
	HESW-LV cells	168.2 ± 58	23.5 ± 2.3 (13.9%)	335 ± 139.2	17 ± 6.1 (5%)	13 ± 5.6	3 ± 0.9 (23%)
Day 7	LV cells	318 ± 34.1	64.6 ± 4.7 (20%)	82 ± 17.5	5.6 ± 4 (6.8%)	Nd	Nd
	HESW-LV cells	156 ± 44.1	40.8 ± 7 (26.1%)	53 ± 26.5	3.2 ± 3.1 (6%)	Nd	Nd
Day 14	LV cells	368 ± 32	67.1 ± 11.4 (18.2%)	29 ± 1.5	1 ± 0.8 (3.4%)	Nd	Nd
	HESW-LV cells	183.4 ± 44.5	72 ± 19.3 (39.2%)	17.6 ± 7.8	0.6 ± 0.2 (3.4%)	Nd	Nd
Day 21	LV cells	81.5 ± 16.2	21.5 ± 6.2 (26.4%)	2.6 ± 2	0	Nd	Nd
	HESW-LV cells	99.6 ± 63.4	25.4 ± 15.7 (25.5%)	1.8 ± 1.5	0	Nd	Nd
Day 28	LV cells	132 ± 17	29.6 ± 2 (22.4%)	14 ± 12.6	0	2.4 ± 1.7	0
	HESW-LV cells	88.2 ± 47.4	26.7 ± 15.5 (30.2%)	7.2 ± 5	0	11.9 ± 2.2	2.9 ± 0.8 (24.3%)
Day 35	LV cells	131 ± 20.7	10.8 ± 5.7 (8.2%)	15 ± 14	0	Nd	Nd
	HESW-LV cells	72.5 ± 17	15 ± 10.4 (20.7%)	2.8 ± 2.3	0	Nd	Nd
Day 42	LV cells	59 ± 29.9	4 ± 3.6 (6.7%)	2 ± 2	0	Nd	Nd
	HESW-LV cells	56 ± 13	20.5 ± 15.8 (36.6%)	1.6 ± 1.5	0	Nd	Nd
Day 49	LV cells	71 ± 16.1	3.1 ± 2 (4.3%)	2.5 ± 2.2	0	Nd	Nd
	HESW-LV cells	43 ± 1.7	18.1 ± 15.7 (42%)	1 ± 0.5	0	Nd	Nd
Day 56	LV cells	70 ± 21.5	2.7 ± 0.9 (3.8%)	3 ± 2.8	0	Nd	Nd
	HESW-LV cells	55 ± 5.4	22.4 ± 13.9 (40.7%)	3.4 ± 2.9	0	Nd	Nd

Results are expressed as mean ± SD of colony numbers performed in five separate experiments in triplicate cultures each.

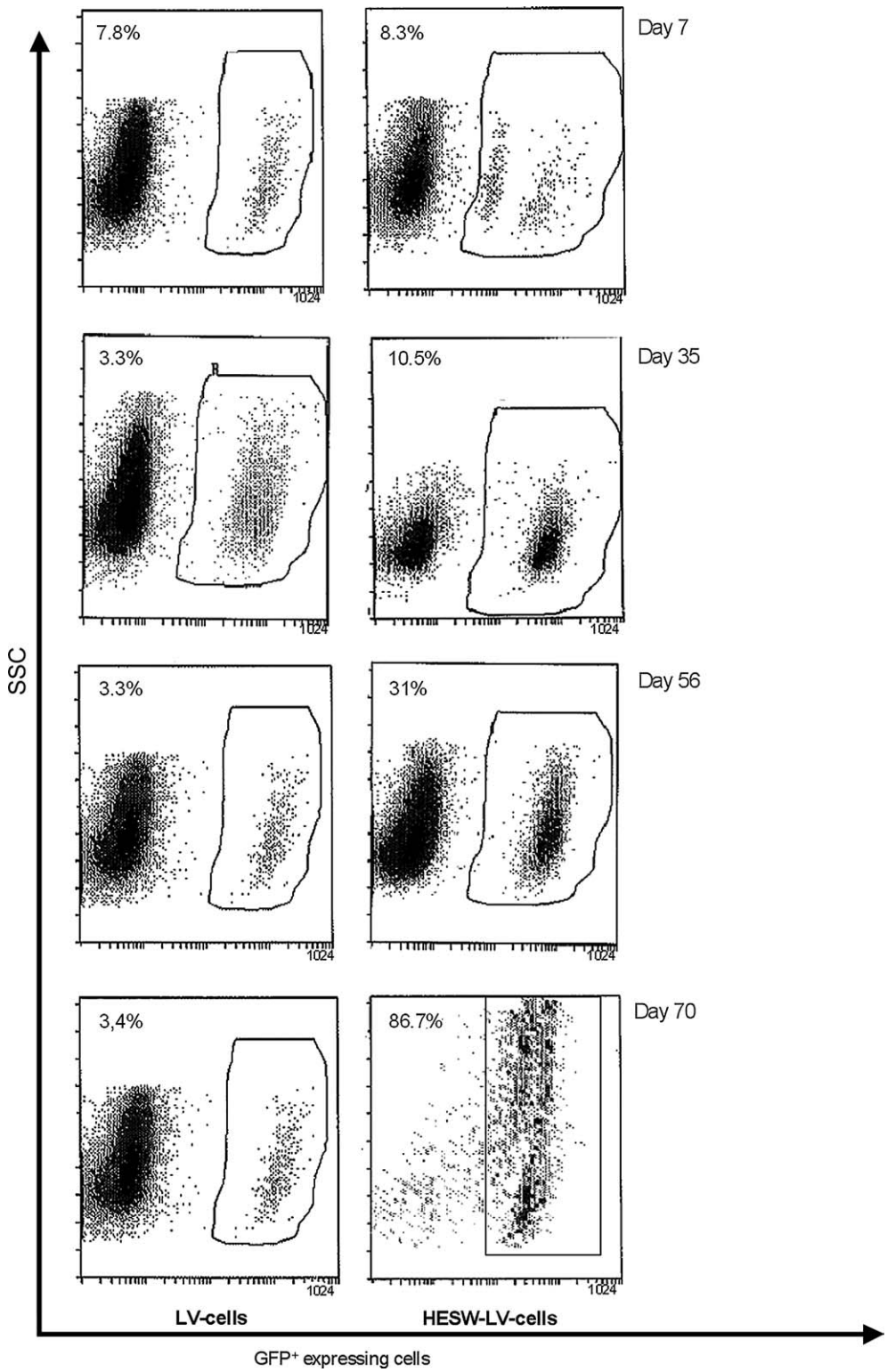

Figure 5. FACS dot-plot prints of GFP^+ cells in stroma-free cultures. A representative experiment showing GFP^+ cells during in vitro cultures following HESW treatment.

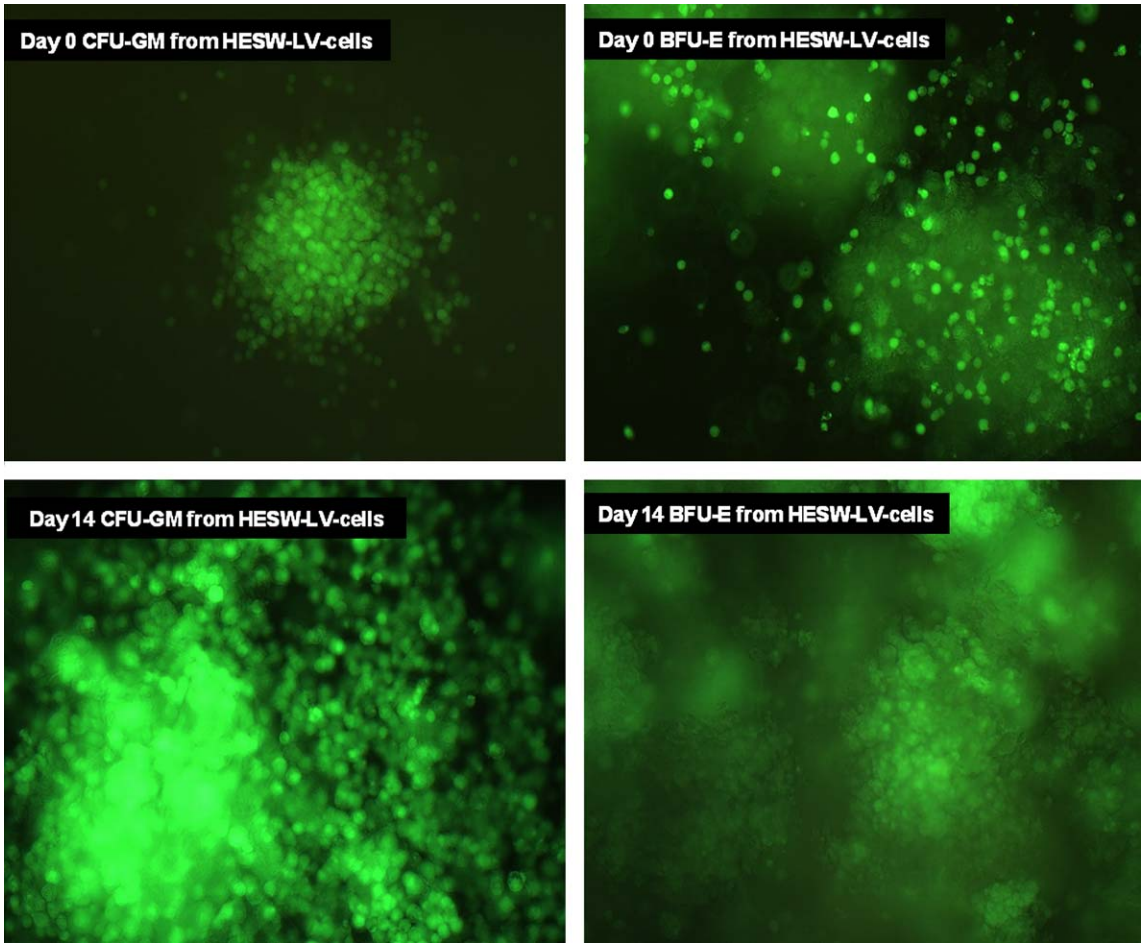

Figure 6. GFP$^+$ colonies generated in vitro during stroma-free cultures following HESW treatment.

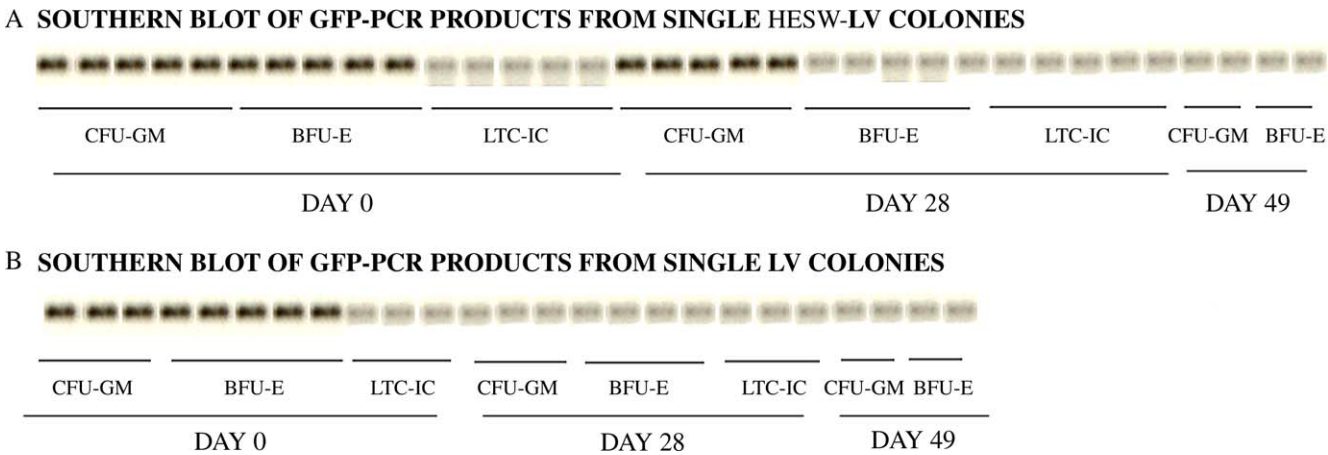

Figure 7. Southern blot of GFP RT-PCR products in colonies generated from stroma-free long-term cultured cells at different time points. A representative analysis showing the mRNA GFP marker in CFU-GM, BFU-E, and LTC-IC in single colonies plucked from semisolid medium generated from HESW-LV cell cultures and LV cell cultures at days 0, 28, and 49.

Table 8. NOD/SCID mice engraftment of human $CD34^+$ transduced cells

		HESW-LV cells	LVcells	*p*
$huCD45^+$		39.6% ± 24.6%	15% ± 8.9%	NS
$huCD34^+$	Human $CD45^+$ gate	33.8% ± 20.4%	27.3% ± 19.7%	0.005
	Entire mouse marrow	13.3% ± 7.2%	4% ± 3.8%	
$huCD19^+$	Human $CD45^+$ gate	22.8% ± 18.9%	29.1% ± 21.7%	NS
	Entire mouse marrow	9% ± 5.4%	4.3% ± 4.2%	
$huCD33^+$	Human $CD45^+$ gate	27.5% ± 22.4%	38.7% ± 20.9%	0.003
	Entire mouse marrow	10.9% ± 4.9%	5.8 ± 4.9%	
$GFP^+/huCD45^+$	Human $CD45^+$ gate	34% ± 11.4%	26.4% ± 10.8%	NS
	Entire mouse marrow	13.4% ± 8.4%	4% ± 3.7%	
$GFP^+/huCD34^+$	Human $CD45^+$ gate	6.6% ± 4.4%	5.9% ± 4.8%	NS
	Entire mouse marrow	2.6% ± 1.9%	1% ± 0.9%	
huGlyco-A^+	Human $CD45^+$ gate	10% ± 8.7%	2% ± 1.7%	0.004
	Entire mouse marrow	4% ± 3.1%	0.3% ± 0.3%	
$huCD41^+$	Human $CD45^+$ gate	4.2% ± 1.9%	2.5% ± 1.8%	0.05
	Entire mouse marrow	2% ± 1.5%	0.3% ± 0.3%	

Following HESW treatment and transduction with a lentiviral vector (30 MOI), $CD34^+$ were immediately transplanted in NOD/SCID mice. Six weeks later, murine marrow was harvested and the content of human GFP+ cells was determined by flow cytometry. Results are expressed as mean ± SD values of 9 animals divided in three separate experiments each. The percentage of lineage-committed human cells was calculated within the gate of human $CD45^+$ cells or the entire mouse marrow cell population.

$CD45^+CD34^+$ from 10% to 14.5% and human $CD45^+CD34^+CD38^-$ from 0.87% to 1.8%). Recent reports indicate that human expanded $CD34^+$ cells possessing long-term repopulating ability in NOD/SCID mice can be efficiently transduced with a lentiviral vector, without losing their repopulating ability [21]. HESW-treated cells were incubated for only 4 hours with a low MOI (30×10^6/mL of transfection units). In this series of mice, human engraftment was 15% ± 8.9% for controls, while following HESW treatment human cell engraftment was 39.6% ± 24.6%. The percentage of GFP^+ was greater but did not reach statistical significance (34% vs 26.4%). However, taken together, greater numbers of human GFP^+ cells were found in murine marrow, thus suggesting that this may be the way to obtain a stable and durable expression of the transgene in the recipient.

In conclusion, this is the first study conducted to investigate the role of HESW in the expansion, transduction, and transplantation of CB $CD34^+$ cells. The pretreatment of $CD34^+$ cells with HESW may represent a new method to manipulate the $CD34^+$ population without interfering with their ability both to expand and long-term engraft and thus be considered as a tool for genetic approaches. These data represent another encouraging step towards the identification of the optimal condition for the implementation of preclinical protocols for both expansion and gene transfer.

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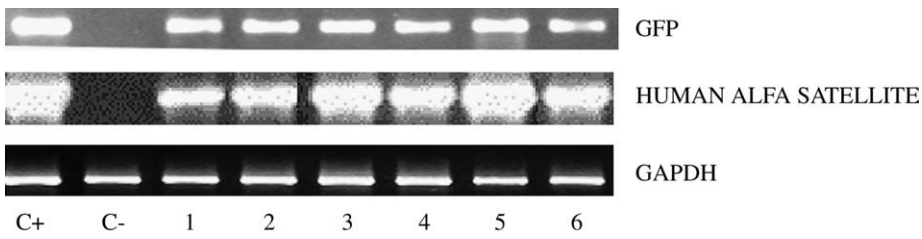

Figure 8. PCR analysis of GFP marker gene and human α-satellite in bone marrow cells of NOD/SCID mice. A representative experiment showing human cell and GFP^+ cell engraftment. Mice were injected with LV cells (mouse 1 to 3) or with HESW-LV cells (mice 4 to 6). C+ = positive control (it was LV-transduced HeLa cells for GFP, human CB $CD34^+$ cells for human α-satellite and house-keeping gene GAPDH). C− = negative control (it was untransduced $CD34^+$ cells for GFP, nontranplanted NOD/SCID mouse marrow for human α-satellite and housekeeping gene GAPDH).

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