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Seminal Report

Diprotin A Infusion into Nonobese Diabetic/Severe Combined Immunodeficiency Mice Markedly Enhances Engraftment of Human Mobilized CD34$^+$ Peripheral Blood Cells

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ABSTRACT

Hematopoietic stem cell (HSC) graft cell dose impacts significantly on allogeneic transplant. Similarly, HSC gene therapy outcome is affected by loss of repopulating cells during culture required for ex vivo retrovirus transduction. Stromal cell-derived factor-1 (SDF-1) and its receptor CXCR4 play a central role in marrow trafficking of HSCs, and maneuvers that enhance CXCR4 activation might positively impact outcome in settings of limiting graft dose. CD26/dipeptidyl peptidase IV (DPP-IV) is an ectoenzyme protease that cleaves SDF-1, thus reducing CXCR4 activation. We show that injection of irradiated nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice with ≥2 μmol Diprotin A (a tripeptide specific inhibitor of CD26 protease activity) at the time of transplant of human granulocyte colony-stimulating factor (G-CSF) mobilized CD34$^+$ peripheral blood cells (CD34$^+$ PBCs) results in a >3.4-fold enhancement of engraftment of human cells. We also show that CD26 on residual stromal cells in the irradiated recipient marrow milieu, and not any CD26 activity in the human CD34$^+$ PBC graft itself, plays the critical role in regulating receptivity of this environment for the incoming graft. Human marrow stromal cells also express CD26, raising the possibility that Diprotin A treatment could significantly enhance engraftment of HSCs in humans in settings of limiting graft dose just as we observed in the NOD/SCID mouse human xenograft model.

INTRODUCTION

TRANSPLANTATION OF ALLOGENEIC HEMATOPOIETIC STEM CELLS (HSCs) has become a central treatment for many hematological diseases that include cancers and dysplasias (1–3), inherited metabolic diseases (4,5), and inherited immunodeficiency diseases (6–8). Complementary to this approach have been the recent demonstrations of efficacy of ex vivo autologous HSC gene therapy in treatment of X-linked severe combined immunodeficiency (X-SCID) (9–11), adenosine deaminase-deficient SCID (12), and X-linked chronic granulomatous disease (X-CGD) (13,14).

It has been shown that the dose of HSCs in the graft can impact on rates of engraftment in the allogeneic setting and on achieving adequate gene marking in the gene therapy setting. In many clinical situations, there is little control over the size of the graft available to the patient. In such settings, knowledge of factors impacting on engraftment efficiency can make a critical difference in the

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clinical outcome. Marrow conditioning and immune suppression regimens are major factors. However, it is likely that other maneuvers are possible to increase engraftment efficiency.

Stromal cell-derived factor-1 (SDF-1) and its receptor CXCR4 play a central role in trafficking of HSCs in the bone marrow (15–17). Gene transfer mediated overexpression of either wild-type CXCR4 or an enhanced-function mutant CXCR4 in human HSCs significantly enhance engraftment in a nonobese diabetic (NOD)/SCID mouse xenograft model (18–20). Preventing degradation of SDF-1 can also enhance engraftment (21) and, conversely, enhancement of proteolysis of SDF-1 can release HSCs to the peripheral blood from bone marrow (22,23). A number of chemokines, including SDF-1, have a proline second from the amino-terminus that appears to be the target for cleavage by a specialized membrane-anchored ectoenzyme, dipeptidyl peptidase IV (CD26/DPP-IV) (24) that cleaves chemokines containing this essential amino-terminal X-Pro-dipeptide motif (25).

Mouse HSCs express CD26/DPP-IV (21,26), and it has been reported that ex vivo treatment of mouse unfractionated bone marrow with Diprotin A (Ile-Pro-Ile), a specific inhibitor of CD26/DPP-IV, enhances engraftment (26). Human blood T cells, B cells, natural killer (NK) cells, and many epithelial, endothelial, stromal, and acinar cells (27–31), as well as a subpopulation of human cord blood $CD34^+$ cells (24) express CD26/DPP-IV. In the current study, we show that ex vivo treatment of human granulocyte colony-stimulating factor (G-CSF)-mobilized $CD34^+$ peripheral blood cells ($CD34^+$ PBC) with Diprotin A does not enhance engraftment in the irradiated NOD.CB17-*Prkdc*^{scid}/J mouse (NOD/SCID mouse) xenograft model, but that in vivo treatment of the mice by injection of Diprotin A at the time of transplant markedly enhances engraftment of these cells, doing so by inhibiting CD26/DPP-IV in the residual marrow stroma. Because human bone marrow stroma also expresses CD26/DPP-IV activity, it is possibly to speculate that our results with Diprotin A could be applicable to transplants in humans.

MATERIALS AND METHODS

Human G-CSF-mobilized $CD34^+$ peripheral blood cells

Following informed consent (NIH Institutional Review Board–approved Protocol 94-I-0073), human G-CSF-mobilized $CD34^+$ peripheral blood cells ($CD34^+$ PBCs) were collected by apheresis from healthy volunteers given 5 days of G-CSF (10 μg/kg per day for 5 days). After CD34 antigen-mediated selection with immunomagnetic beads (ISOLEX300i system, Baxter Healthcare, Deerfield, IL) (32), purified $CD34^+$ PBCs were preserved in liquid nitrogen until use.

Culture of human $CD34^+$ PBCs

$CD34^+$ PBCs were cultured in X-VIVO10 (BioWhittaker, Walkersville, MD) supplemented with 1% human serum albumin (HSA), recombinant human stem cell factor (SCF), thrombopoietin (TPO), flt3-ligand, and interleukin-3 (IL-3) at 50, 50, 50, 20 ng/ml, respectively (R&D Systems, Minneapolis, MN) for 4 days after thawing.

Migration assay

$CD34^+$ PBCs, with or without prior Diprotin A treatment, were suspended in 100 μl of X-VIVO 10 (1% HSA) and loaded into upper chambers (5×10^5 cells per well) of a 24-well transwell apparatus (Costar, NY; pore size 5 μm) containing varied concentrations of SDF-1 (PeproTech Inc., Rocky Hill, NJ) in the lower chamber. The number of cells migrating to the lower chamber over 2 h at 37°C was scored visually by light microscopy.

Colony-forming unit assay

Ex vivo-cultured naïve $CD34^+$ PBCs were sorted at day 4 with phycoerythrin (PE)-conjugated anti-human CD26 antibody using flow cytometry. After sorting, the percent of $CD26^+$ cells and $CD26^-$ cells was 99.8% and 0.1%, respectively. Naïve, CD26 sorted, and retroviral green fluorescent protein (GFP)-transduced $CD34^+$ PBCs at 1×10^3 cells/ml were cultured in duplicate in 1% methylcellulose and Iscove's modified Dulbecco medium (IMDM) supplemented with 30% fetal bovine serum (FBS), 1% bovine serum albumin (BSA), 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine, 50 ng/ml recombinant human (rh)SCF, 20 ng/ml rhGM-CSF, 20 ng/ml rhIL-3, 20 ng/ml rhIL-6, and 20 ng/ml rhG-CSF (StemCell Tech-Inc., Vancouver, Canada). All colonies were of myeloid phenotype and most were mixed monocytes/granulocytes in type, but were not further distinguished when counting colonies. For each experiment, at least 90 colonies were scored, where the cultures were scored at 14 days in a blinded manner by inverted fluorescence microscopy as described previously (33).

Plasmid and retroviral vector production and transduction of human $CD34^+$ PBCs

A previously described, a fusion protein sequence consisting of GFP fused to P144K murine mutant homolog of O^6-methylguanine-DNA methyltransferase (mMGMT*) was inserted into the *Nco*I–*Bam*HI cloning site of pMFGS, a Moloney murine leukemia virus replication–incompetent vector (Cell Genesys, Foster City, CA) (13,33).

FLYRD18-packaging cells (34) producing RD114 envelope pseudotyped (35–37) MFGS-GFPmMGMT* retroviral vector (referred to hereafter just as GFP vector) were generated as previously described (32,38). Filtered vector supernatant was collected from confluent FLYRD18 producer cultures and concentrated by centrifugation (18,600 $\times$ *g*, 3 h, 4°C).

CD34$^+$ PBCs were transduced with concentrated GFP vector, with a multiplicity of infection (MOI) of 10 overnight three times (days 1, 2, and 3) in culture medium containing 5 μg/ml protamine in six-well plates precoated with RetroNectin (TaKaRa Bio-Inc., Otsu, Japan). Naïve nontransduced CD34$^+$ PBCs were cultured similarly and served as negative controls for GFP expression.

Flow cytometry

PE- or allophycocyanin (APC)-conjugated mouse monoclonal antibodies (mAbs) were used to perform three-color analysis that also included detection of GFP. Conjugated mAbs were used that were specific for human CD34 antigen, human CD45 antigen, or human CD26 antigen. Mouse nonspecific immunoglobulin G (IgG) mAbs conjugated with PE and APC were used for isotype controls. Data analysis was performed with CellQuest software (Becton-Dickinson, San Jose, CA).

Transplantation of human CD34$^+$ PBCs into NOD/SCID mice

Two days before transplantation, 8- to 12-week-old NOD/SCID mice were irradiated with 300 cGy of total-body radiation (^{139}Cs gamma source). Because this mouse strain may still retain some NK cells that can interfere with human cell engraftment, all animals were injected intraperitoneally prior to transplant with 1 mg of TM-β1 (anti-murine IL2 receptor β chain) rat mAb, a maneuver that eliminates NK cells during the peritransplant period (39–41). For all experiments, the progeny of 1×10^6 human CD34$^+$ PBCs at the initiation of culture (GFP transduced or naïve nontransduced 4 day-cultured) were injected by tail vein into each mouse, where the average expansion was 3- to 4-fold by day 4 of culture. At the time of transplant, simultaneously with injection of the human CD34$^+$ PBCs, the mice were injected intravenously with 100 μl of phosphate-buffered saline (PBS) containing 0, 0.5, 2, or 4 μmol Diprotin A (concentrations of 0, 5, 20, or 40 mM, respectively).

Assessment of human cell engraftment in the NOD/SCID mouse

Bone marrow (BM) cells were harvested from both femurs of each mouse at 6 weeks after transplantation. After red blood cell lysis, the analysis of human xenografted cells in BM from each mouse was assessed by flow cytometry (FACSort; BD Immunocytometry System, San Jose, CA), using anti human-CD45 APC mAb.

CD26 histochemistry in mouse bone marrow and CD26/DPP-IV activity in mouse serum and BM

For immunohistochemical analyses, the femurs isolated from the NOD/SCID mice at day 2 after 300 cGy of total-body irradiation (^{139}Cs gamma source) were treated with 4% paraformaldehyde for 16 h and then embedded for sectioning. Dewaxed sections of these femurs were stained with anti-mouse CD26 antibody (R&D systems, Minneapolis, MN).

Blood and BM cells were harvested 2 days after irradiation of NOD/SCID mice as per preparation for transplantation with or without treatment intravenously with Diprotin A just before harvest of the blood or BM. The analysis of the DPP-IV activity was performed by DPPIV-Glo Protease assay (Promega, Madison, WI) on the BM cells and on blood serum. Measurements were detected in 96-well microtiter chemiluminescence plates (using 5×10^5 cells/well for marrow cells and using a 10% dilution of the serum with PBS) at 37°C using a Luminoskan Ascent (Thermo Fisher Scientific, Inc., Waltham, MA). The data for enzymatic activity were shown as the peak of Relative Light Units over 30 min.

Statistics

Results of experimental points are reported as mean $\pm$ standard deviation (SD). Significance levels were determined by Student's *t*-test for differences in means.

RESULTS

Effect of culture of human CD34$^+$ PBCs on expression of CD34 and CD26 antigen

Mouse HSCs express CD26/DPP-IV (21,26), but freshly isolated human mobilized CD34$^+$ PBC do not express detectable CD26 (Fig. 1A,B at time 0). When these human CD34$^+$ PBCs are cultured, CD34 expression decreased only slightly during the first 4 days of culture, but rapidly decreased beyond day 5 (Fig. 1A). As noted, the percent of CD34$^+$ cells that expressed CD26 at initiation of culture was essential zero, but increased slowly for the first 3 days, reaching almost 10% by day 4, and then more rapidly beyond day 4 (Fig. 1B). In companion cultures where the human CD34$^+$ PBCs were transduced with GFP vector overnight daily from culture days 1–4, the loss of CD34 antigen and gain of CD26 antigen was equivalent to that seen in the nontransduced (naïve) cultures (not shown). The percent of GFP$^+$ cells at day 4

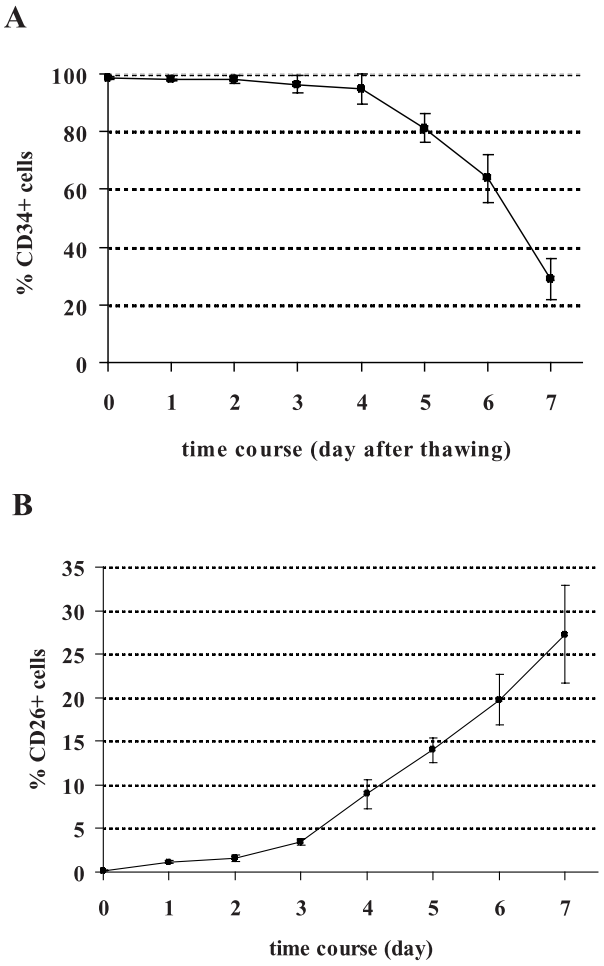

FIG. 1. Ex vivo culture of human $CD34^+$ PBCs decreases CD34 expression and increases CD26 expression. (**A**) Change in CD34 expression over time in culture for human mobilized $CD34^+$ PBCs. (**B**) Change in CD26 expression over time in the same cultures of human mobilized $CD34^+$ PBCs. These purified cells are >98% $CD34^+$ at the start of culture without detectable CD26.

(at the day of transplantation of the human cultured and/or transduced $CD34^+$ PBCs into NOD/SCID mice) was 80 ± 1.3 %. Although the detailed dot plots for each daily analysis are not shown, it should be noted that at each time point after 4 days the loss of CD34 antigen was strongly correlated with gain of expression of CD26 antigen.

Effect of Diprotin A on in vitro migration of $CD34^+$ PBCs

Human $CD34^+$ PBCs express CXCR4 and retain expression of CXCR4 during ex vivo culture (38). Because culture of human $CD34^+$ PBCs induces expression of CD26 in almost 10% of cells by day 4, it was reasonable to ask if this might affect migration responses of the cultured cells to SDF-1. The SDF-1–mediated migration assay was performed using 4-day cultured $CD34^+$ PBCs with or without pretreatment of the cultured human $CD34^+$ PBCs with Diprotin A before placement in the migration assay. With 10 nM SDF-1 as the stimulus in the lower chamber, we observed that of the 5.0×10^5 cultured cells loaded onto the upper chamber, the percent of cells migrating into the lower chamber were $21.6 \pm 3.6\%$ and $26.1 \pm 5.0\%$, respectively, for $CD34^+$ PBCs treated with PBS or 20 mM Diprotin A, but this difference was not statistically significant ($n = 6$). A series of experiments were also performed with lower concentrations of SDF-1 stimulus (0.5, 1, and 5 nM) and higher concentrations of 40 mM Diprotein A pretreatment of $CD34^+$ PBCs ($n = 6$ for all experiments) showing no statistically significant effect of the Diprotin A treatment on migration of the human cultured $CD34^+$ PBCs. We conclude that ex vivo Diprotin A treatment of cultured human $CD34^+$ PBCs had no subsequent effect on migration response to SDF-1.

Effect of Diprotin A on colony formation and the relationship of CD26 expression to colony-forming potential

Colony-forming unit (CFU) assays were performed at day 4 of culture with naïve or GFP-transduced $CD34^+$ PBCs, where each group was treated with PBS or Diprotin A. At 14 days after ex vivo culturing in semisolid medium, there were no differences in the total number of colonies per 1×10^3 cells plated for the naïve $CD34^+$ PBC group: 102 ± 8, 103 ± 9, 104 ± 9, 103 ± 10, and 101 ± 11 colonies following treatment with 0, 5, 10, 20, and 40 mM Diprotin A, respectively (four experiments). There also were no differences in the total number of colonies per 1×10^3 cells plated for the GFP-transduced $CD34^+$ PBC group: 101 ± 11 and 103 ± 12 total colonies ($36.2 \pm 3.9\%$ and $34.7 \pm 4.4\%$ GFP^+ colonies) following treatment with 0 or 20 mM Diprotin A, respectively (four experiments). This confirms both that transduction did not affect total colony number, and that ex vivo treatment with Diprotin A affects neither total colony number nor number of GFP^+ colonies. As another aspect of these experiments, we sorted day-4 cultured $CD34^+$ PBCs for CD26 expression before plating. $CD26^+$ and $CD26^-$ $CD34^+$ PBCs gave rise to 2 ± 0.5 and 101 ± 9.8 colonies per 1×10^3 plated, respectively, indicating that acquisition of CD26 expression was strongly associated with loss of colony-forming potential. This result is consistent with our flow cytometry data, already noted above, which showed a strong correlation between expression of CD26 antigen and loss of CD34 antigen.

Effect of in vivo versus ex vivo treatment with Diprotin A on human CD34$^+$ PBC engraftment in the NOD/SCID xenograft model

Because ex vivo treatment with Diprotin A had no effect on cultured or cultured/transduced human CD34$^+$ PBCs with respect to colony formation or migration response to SDF-1, we performed transplant experiments in which NOD/SCID mice were injected with Diprotin A at the time of transplant to determine if that affected engraftment. When 1×10^6 4-day-cultured human CD34$^+$ PBCs were transplanted together with injection of 0, 0.5, 2, and 4 μmol of Diprotin A in a 100-μl volume of PBS (corresponding to concentrations of 0, 5, 20, and 40 mM Diprotin A) into NOD/SCID mice, there was a profound enhancement of engraftment. Specifically, the percent of human cells in the BM of these mice at 6 weeks after transplant was 17.4 ± 7.4%, 22.5 ± 5.2%, 62.0 ± 12.5%, and 60.4 ± 8.4%, respectively (Fig. 2A; $p < 0.001$ when comparing the 2 and 4 μmol Diprotin A/mouse groups versus the PBS-only/mouse group).

In a similar experiment, the human CD34$^+$ PBCs were incubated ex vivo in either 5 or 20 mM Diprotin A for 30 min, washed twice by centrifugation to remove Diprotin A, and immediately transplanted into NOD/SCID mice. The result as shown in Fig. 2B (left bar versus middle bar) indicates that there is no effect on engraftment from ex vivo exposure of cultured human CD34$^+$ PBCs to Diprotin A. However, there was still a profound enhancement of engraftment mediated by in vivo administration of Diprotin A when the cells exposed to ex vivo Diprotin A were transplanted (middle bar versus right bar in Fig. 2B; $p < 0.01$). These experiments confirm that only the Diprotin A actually injected into the mice significantly enhanced the outcome of the human CD34$^+$ PBC xenograft and that ex vivo exposure of the cells to the Diprotin A before transplant did not affect the outcome.

In another series of experiments, we transduced human CD34$^+$ PBCs with the GFP retrovirus vector to >80% transduction ex vivo over 4 days and transplanted these cells into the 300-cGy-conditioned NOD/SCID mice

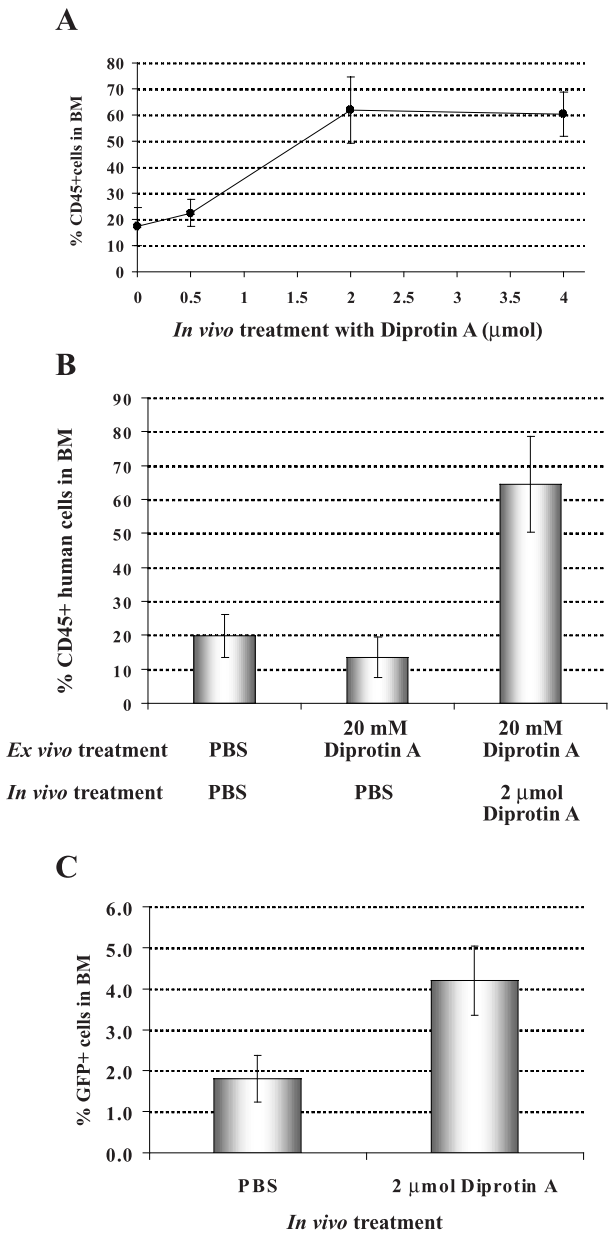

FIG. 2. In vivo injection treatment of the NOD/SCID mice but not ex vivo treatment of the human CD34$^+$ PBC graft with Diprotin A improves the engraftment of human cells in the NOD/SCID mouse xenograft. (**A**) Measurement of the percent of human CD45$^+$ cells in the NOD/SCID BM xenograft at 6 weeks after transplant, following in vivo injection treatment of the mice at the time of transplant with the amounts of Diprotin A shown on the horizontal axis. There is significant enhancement of engraftment by Diprotin A and statistics are provided in the text (three separate experiments; $n = 6$–9 mice in each dose group for the graph in **A**). (**B**) Measurement of the percent of human CD45$^+$ cells in the NOD/SCID BM xenograft at 6 weeks after transplant, following either ex vivo treatment alone (middle bar) or together with in vivo injection treatment (right bar) of the mice at the time of transplant with the amounts of Diprotin A shown. Only if in vivo treatment is given is there an enhancement of engraftment. **B** shows the results of experiments separate from those shown in **A** (two separate experiments; $n = 5$–7 mice for each treatment shown in **B**). (**C**) Percent of GFP$^+$ cells relative to the whole-mouse BM cell count following transplant of GFP retrovirus-transduced human CD34$^+$ PBCs into NOD/SCID mice without (*left bar*) or with (*right bar*) injection of 2 μmol Diprotin A. (**C**) Results of experiments separate from those shown in **A** or **B** (two separate experiments; $n = 5$–7 mice for each treatment shown in **C**).

without or with infusion of 2 μmol Diprotin A. The xenograft of human CD45$^+$ cells in the BM of the NOD/SCID mice comprised 12.9 $\pm$ 6.0% and 33.5 $\pm$ 7.3% for the mice without Diprotin A or with Diprotin A injection, respectively (not shown graphically; two experiments of 5–7 mice per group, $n = 7$, comparing PBS to Diprotin A, $p < 0.01$). As expected, the percent of the human cells in the xenograft that were GFP$^+$ were the same at 12.6 $\pm$ 1.7% and 14.6 $\pm$ 2.2%, respectively. However, as shown in Fig. 2C, because there were significantly more human cells in the Diprotin A-treated mice, this resulted in a higher percent of GFP-marked cells overall in that group (1.8 $\pm$ 0.6% GFP$^+$ for PBS versus 4.2 $\pm$ 0.8% GFP$^+$ for Diprotin A; $p < 0.01$). This demonstrates that transduction does not disadvantage NOD/SCID repopulating cells from participating in the enhancement in engraftment induced by Diprotin A, but neither are the percent of transduced cells enhanced in engraftment relative to the nontransduced fraction of the incoming human xenograft.

DPP-IV activity measured in the graft before transplant and in mouse serum and marrow over 24 h after injection with Diprotin A

DPP-IV activity of 4-day cultured human CD34$^+$ PBCs was measured without or with a 30-min incubation in 20 mM Diprotin A. Cells were washed by centrifugation and 0.5×10^6 cells per well loaded in a 96-well chemiluminescence assay plate demonstrated 81 $\pm$ 4.7 and 12.4 $\pm$ 2.2 activity units for the untreated and ex vivo Diprotin A-treated cells ($n = 4$; $p < 0.01$). This demonstrates the presence of Diprotin A-inhibitable CD26/DPP-IV protease activity in the human CD34$^+$ PBC 4-day cultured cells. Thus, the lack of demonstrable Diprotin A effect on human CD34$^+$ PBC migration in response to SDF-1 or on engraftment was not because the Diprotin A treatment of the cultured cells ex vivo failed to reduce DPP-IV activity significantly.

We next evaluated the DPP-IV activity of serum and BM cells from NOD/SCID mice irradiated 2 days previously, in the standard way as if for transplant, to assess the effect of intravenous in vivo treatment with different amounts of Diprotin A. At 5 min after injection of 0, 0.5, 2, and 4 μmol Diprotin A (consisting of 100 μl of PBS containing a concentration of 0, 5, 20, and 40 mM Diprotin A, respectively), the mice were sacrificed and blood and BM collected for assays performed as quickly as possible after harvest. The DPP-IV activity for 10% mouse serum diluted in PBS was 552 $\pm$ 47, 550 $\pm$ 49, 555 $\pm$ 46, and 546 $\pm$ 43 activity units, respectively ($n = 4$), indicating no effect on this type of protease activity in mouse serum. However, in the same mice sacrificed at the time of blood sampling, the DPP-IV activity of marrow cells harvested and assayed as quickly as possible (about an hour after sacrifice) was 76 $\pm$ 8.8, 79 $\pm$ 8.6, 43 $\pm$ 7.8, and 43 $\pm$ 9.5 activity units for the same groups, respectively, indicating a significant early inhibition of BM DPP-IV activity for the two highest doses of Diprotin A compared to the PBS control ($p < 0.001$, Fig. 3). However, 24 h after injection of either 2 or 4 μmol of Diprotin A, the DPP-IV activity appeared fully restored ($n = 4$, 79 $\pm$ 10.3, 80 $\pm$ 11.7, and 78 $\pm$ 9.9 activity units for treatment with PBS, 2 μmol, and 4 μmol Diprotin A, respectively; Fig. 3).

Pattern of CD26 expression in pre- and post-irradiated NOD/SCID bone marrow

Bone marrow of NOD/SCID mouse femur from untreated or 2-day post 300-cGy-irradiated mice was fixed for histochemical and immunohistochemical staining to detect mouse CD26 expression in paraffin sections. The upper panels in Fig. 4 compare the normal cellularity of nonirradiated animals (left) to the hypocellular pattern seen on the right in the irradiated animals where the remaining stromal cells predominate. In the bottom panels, shown as increased magnification compared to the top panels, it is possible to note the wide distribution of the immunoperoxidase-positive brown precipitate in many cell types on the lower left, but it is possible in the post-irradiation marrow on the right lower panel to see stellate cells that are presumably stromal are staining pos-

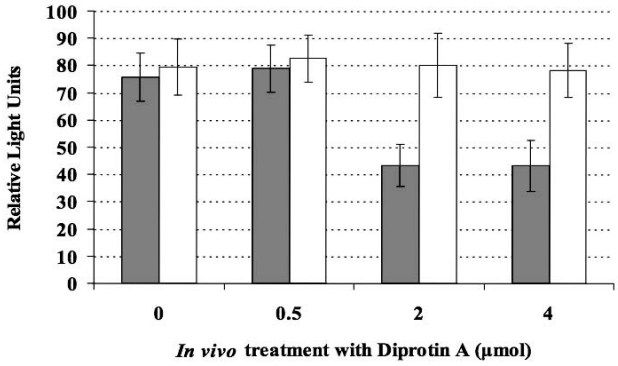

FIG. 3. In vivo treatment of Diprotin A reduced the CD26/DDP-IV activity of irradiated NOD/SCID mouse BM. The graph shows CD26/DDP-IV protease activity measured in a chemiluminescence assay and expressed in relative light units in the marrow cells of irradiated NOD/SCID mice treated with PBS, 0.5, 2, and 4 μmol Diprotin A. For the filled bars, the mice are sacrificed at 5 min after intravenous injection, but it took about an hour to harvest and measure marrow activity. For the open bars, the mice are sacrificed at 24 h after intravenous injection. The inhibition of CD26/DDP-IV at 5 min after treatment with 2 and 4 μmol Diprotin A compared to the PBS control group is highly significant ($n = 4$, $p < 0.01$). However, this early inhibition of CD26/DDP-IV by treatment with Diprotin A seen at 5 min (*filled bars*) is no longer evident at 24 h (*open bars*).

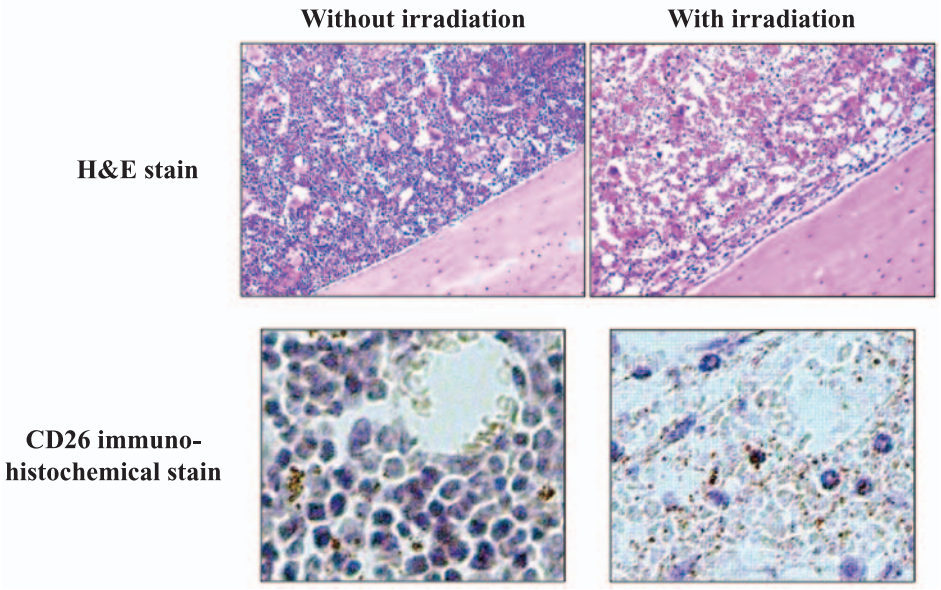

FIG. 4. CD26 expression in the marrow of naïve and 2 days post 300-cGy-irradiated NOD/SCID mice. Low magnification of Hematoxylin & Eosin (H&E)-stained sections of BM from NOD/SCID mice before (*left upper panel*) or 2 days after 300 cGy of irradiation (*right upper panel*). With radiation, there is significant loss of cellularity with retention of mostly stellate cells likely representing stroma. (*Lower panels*) Higher magnification of immunohistochemical brown peroxidase staining of $CD26^+$ cells. Before radiation, there are significant numbers of cells of various types that express CD26 (*lower left panel*), but 2 days after irradiation the positive cells are mostly the remaining stellate cells that may be stroma.

itive. In a separate experiment not shown, we confirmed previous reports that human marrow stromal cells express high levels of CD26, suggesting the possibility that our findings in NOD/SCID mice with human $CD34^+$ PBC xenografts presumably would be translatable to clinical human transplantation.

DISCUSSION

There are a number of clinical transplant settings where the size of the HSC graft may be limiting and where it would be useful to enhance engraftment without increasing intensity of conditioning. There is much interest in the mechanisms by which the chemokine receptor CXCR4 and its ligand, SDF-1 (CXCL12) regulate trafficking of HSCs (15–17). Activity of SDF-1 may be regulated by the DPP-IV proline dipeptide protease activity associated with CD26 (24,42) and Diprotin A, a specific tripeptide inhibitor of the CD26 protease, can significantly enhance engraftment of mouse bone marrow (21,26,43). Many human cells express CD26, which is associated with the same DPP-IV activity in human cells. Mouse HSCs have been reported to express CD26, as do a subset of human cord blood $CD34^+$ cells (24).

We find in our current study that human mobilized $CD34^+$ PBCs do not express detectable CD26, although CD26 expression did appear during ex vivo culture. However, acquisition of expression of CD26 in cultured human $CD34^+$ PBCs correlated strongly with both loss of CD34 antigen expression and loss of colony formation potential. Thus, we confirm that most or all colony-forming human $CD34^+$ PBCs do not express CD26 and that this may be the basis for the failure of ex vivo treatment with Diprotin A to have a significant effect on human $CD34^+$ PBC engraftment as it clearly does with mouse HSCs. We did not study BM-derived $CD34^+$ cells, and it remains possible that there is a subpopulation of engraftable BM-derived adult human HSCs that express CD26 that would be affected by ex vivo treatment with Diprotin A. Our studies with human $CD34^+$ PBCs emphasize the importance of delineating both source-specific and species-specific differences of hematopoietic progenitors and stem cells in biological processes.

Following our inability to enhance engraftment of human $CD34^+$ PBCs by pretreatment ex vivo with Diprotin A, we tried injecting the NOD/SCID mice with Diprotin A simultaneous with injection of the cells and in our initial experiments found a profound enhancement of engraftment of human $CD34^+$ PBCs. Both because of our interest in the potential of enhancing engraftment of gene-marked human $CD34^+$ PBCs, and because short-term culture clearly enhanced expression of CD26 in the non-colony-forming portion of the cultured graft, we focused our attention on this type of graft in our subsequent studies. Despite expression of CD26 by a portion of the cul-

tured human CD34$^+$ PBC graft, inhibition of that DPP-IV activity by ex vivo exposure to Diprotin A still had no effect on engraftment. However, injecting the NOD/SCID mice with Diprotin A simultaneously with injection of the graft of cultured or cultured/gene transduced human CD34$^+$ PBCs reliably resulted in enhancement of both the nontransduced and transduced human CD34$^+$ PBCs in the NOD/SCID xenograft.

The mechanism by which this occurs likely involves inhibition of the NOD/SCID host marrow cells. Our studies of CD26 in nonablative irradiated (300 cGy) NOD/SCID marrow demonstrates that at the time of engraftment most of the remaining cellular component in the marrow appears to be stroma and this marrow stroma expresses high levels of CD26 by histochemical staining. We also confirm the presence of DPP-IV enzymatic activity in marrow harvested from irradiated NOD/SCID mice, also demonstrating that injection of Diprotin A greatly inhibits the DPP-IV activity in irradiated NOD/SCID mouse BM.

Short of performing Diprotin A injection studies in human subjects, we still lack direct evidence that our xenograft studies of human CD34$^+$ PBCs in Diprotin A-treated NOD/SCID mice would be reproduced in transplanted humans. However, we and others do find that human marrow stroma and human stroma cell lines highly express CD26 (44), strongly suggesting, but not yet proving, that Diprotin A injection in human subjects would enhance engraftment of human CD34$^+$ PBCs.

In summary we demonstrate for the first time that Diprotin A can greatly enhance the engraftment of human CD34$^+$ PBCs into the NOD/SCID mouse xenograft, but that this effect requires injection of Diprotin A into the mouse and likely acts on the mouse marrow stroma rather than directly acting on the incoming human CD34$^+$ PBC graft.

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REFERENCES

1. Kang EM, M de Witte, H Malech, RA Morgan, S Phang, C Carter, SF Leitman, R Childs, AJ Barrett, R Little and JF Tisdale. (2002). Nonmyeloablative conditioning followed by transplantation of genetically modified HLA-matched peripheral blood progenitor cells for hematologic malignancies in patients with acquired immunodeficiency syndrome. Blood 99:698–701.
2. Thomas ED, CD Buckner, M Banaji, RA Clift, A Fefer, N Flournoy, BW Goodell, RO Hickman, KG Lerner, PE Neiman, GE Sale, JE Sanders, J Singer, M Stevens, R Storb, and PL Weiden. (1977). One hundred patients with acute leukemia treated by chemotherapy, total body irradiation, and allogeneic marrow transplantation. Blood 49:511–533.
3. Thomas ED, A Fefer, CD Buckner and R Storb. (1977). Current status of bone marrow transplantation for aplastic anemia and acute leukemia. Blood 49:671–681.
4. Krivit W. (2004). Allogeneic stem cell transplantation for the treatment of lysosomal and peroxisomal metabolic diseases. Springer Semin Immunopathol 26:119–132.
5. Parkman R. (1986). The application of bone marrow transplantation to the treatment of genetic diseases. Science 232: 1373–1378.
6. Pai SY, D DeMartiis, C Forino, S Cavagnini, A Lanfranchi, S Giliani, D Moratto, C Mazza, F Porta, L Imberti, LD Notarangelo and E Mazzolari. (2006). Stem cell transplantation for the Wiskott-Aldrich syndrome: a single-center experience confirms efficacy of matched unrelated donor transplantation. Bone Marrow Transplant 38:671–679.
7. Horwitz ME, AJ Barrett, MR Brown, CS Carter, R Childs, JI Gallin, SM Holland, GF Linton, JA Miller, SF Leitman, EJ Read and HL Malech. (2001). Treatment of chronic granulomatous disease with nonmyeloablative conditioning and a T-cell-depleted hematopoietic allograft. N Engl J Med 344:881–888.
8. Myers LA, DD Patel, JM Puck and RH Buckley. (2002). Hematopoietic stem cell transplantation for severe combined immunodeficiency in the neonatal period leads to superior thymic output and improved survival. Blood 99:872–878.
9. Cavazzana-Calvo M, S Hacein-Bey, G de Saint Basile, F Gross, E Yvon, P Nusbaum, F Selz, C Hue, S Certain, JL Casanova, P Bousso, FL Deist and A Fischer. (2000). Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. Science 288:669–672.
10. Ginn SL, JA Curtin, B Kramer, CM Smyth, M Wong, A Kakakios, GB McCowage, D Watson, SI AlexanderI, M Latham, SC Cunningham, M Zheng, L Hobson, PB Rowe, A Fischer, M Cavazzana-Calvo, S Hacein-Bey-Abina and IE Alexander. (2005). Treatment of an infant with X-linked severe combined immunodeficiency (SCID-X1) by gene therapy in Australia. Med J Aust 182:458–463.
11. Hacein-Bey-Abina S, F Le Deist, F Carlier, C Bouneaud, C Hue, JP De Villartay, AJ Thrasher, N Wulffraat, R Sorensen, S Dupuis-Girod, A Fischer, EG Davies, W Kuis, L Leiva and M Cavazzana-Calvo. (2002). Sustained correction of X-linked severe combined immunodeficiency by ex vivo gene therapy. N Engl J Med 346:1185–1193.
12. Aiuti A, S Slavin, M Aker, F Ficara, S Deola, A Mortellaro, S Morecki, G Andolfi, A Tabucchi, F Carlucci, E Marinello, F Cattaneo, S Vai, P Servida, R Miniero, MG Roncarolo and C Bordignon. (2002). Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. Science 296:2410–2413.
13. Malech HL, PB Maples, N Whiting-Theobald, GF Linton, S Sekhsaria, SJ Vowells, F Li, JA Miller, E DeCarlo, SM Holland, SF Leitman, CS Carter, RE Butz, EJ Read, TA Fleisher, RD Schneiderman, DE Van Epps, SK Spratt, CA

Maack, JA Rokovich, LK Cohen and JI Gallin. (1997). Prolonged production of NADPH oxidase-corrected granulocytes after gene therapy of chronic granulomatous disease. Proc Natl Acad Sci USA 94:12133–12138.

14. Ott MG, M Schmidt, K Schwarzwaelder, S Stein, U Siler, U Koehl, H Glimm, K Kuhlcke, A Schilz, H Kunkel, S Naundorf, A Brinkmann, A Deichmann, M Fischer, C Ball, I Pilz, C Dunbar, Y Du, NA Jenkins, NG Copeland, U Luthi, M Hassan, AJ Thrasher, D Hoelzer, C von Kalle, R Seger and M Grez. (2006). Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1–EVI1, PRDM16 or SETBP1. Nature Med 12:401–409.
15. Peled A, I Petit, O Kollet, M Magid, T Ponomaryov, T Byk, A Nagler, H Ben-Hur, A Many, L Shultz, O Lider, R Alon, D Zipori and T Lapidot. (1999). Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. Science 283:845–848.
16. Lapidot T, A Dar and O Kollet. (2005). How do stem cells find their way home? Blood 106:1901–1910.
17. Liles WC, HE Broxmeyer, E Rodger, B Wood, K Hubel, S Cooper, G Hangoc, GJ Bridger, GW Henson, G Calandra and DC Dale. (2003). Mobilization of hematopoietic progenitor cells in healthy volunteers by AMD3100, a CXCR4 antagonist. Blood 102:2728–2730.
18. Kahn J, T Byk, L Jansson-Sjostrand, I Petit, S Shivtiel, A Nagler, I Hardan, V Deutsch, Z Gazit, D Gazit, S Karlsson and T Lapidot. (2004). Overexpression of CXCR4 on human CD34$^+$ progenitors increases their proliferation, migration, and NOD/SCID repopulation. Blood 103:2942–2949.
19. Brenner S, N Whiting-Theobald, T Kawai, GF Linton, AG Rudikoff, U Choi, MF Ryser, PM Murphy, JM Sechler and HL Malech. (2004). CXCR4-transgene expression significantly improves marrow engraftment of cultured hematopoietic stem cells. Stem Cells 22:1128–1133.
20. Kawai T, U Choi, L Cardwell, SS DeRavin, N Naumann, NL Whiting-Theobald, GF Linton, J Moon, PM Murphy and HL Malech. (2007). WHIM syndrome myelokathexis reproduced in the NOD/SCID mouse xenotransplant model engrafted with healthy human stem cells transduced with C-terminus truncated CXCR4. Blood 109:78–84.
21. Tian C, J Bagley, D Forman and J Iacomini. (2006). Inhibition of CD26 peptidase activity significantly improves engraftment of retrovirally transduced hematopoietic progenitors. Gene Ther 13:652–658.
22. Kollet O, A Spiegel, A Peled, I Petit, T Byk, R Hershkoviz, E Guetta, G Barkai, A Nagler and T Lapidot. (2001). Rapid and efficient homing of human CD34(+)CD38 (−/low)CXCR4(+) stem and progenitor cells to the bone marrow and spleen of NOD/SCID and NOD/SCID/B2m(null) mice. Blood 97:3283–3291.
23. Kim HK, M De La Luz Sierra, C Williams, AV Gulino and G Tosato. (2006). G-CSF down-regulation of CXCR4 expression identified as a mechanism for mobilization of myeloid cells. Blood 108:812–820.
24. Christopherson KW, 2nd, G Hangoc and HE Broxmeyer. (2002). Cell surface peptidase CD26/dipeptidylpeptidase IV regulates CXCL12/stromal cell-derived factor-1 alpha-mediated chemotaxis of human cord blood CD34$^+$ progenitor cells. J Immunol 169:7000–7008.
25. Herrera C, C Morimoto, J Blanco, J Mallol, F Arenzana, C Lluis and R Franco. (2001). Comodulation of CXCR4 and CD26 in human lymphocytes. J Biol Chem 276: 19532–19539.
26. Christopherson KW, 2nd, G Hangoc, CR Mantel and HE Broxmeyer. (2004). Modulation of hematopoietic stem cell homing and engraftment by CD26. Science 305:1000–1003.
27. Buhling F, D Kunz, D Reinhold, AJ Ulmer, M Ernst, HD Flad and S Ansorge. (1994). Expression and functional role of dipeptidyl peptidase IV (CD26) on human natural killer cells. Nature Immun 13:270–279.
28. Buhling F, U Junker, D Reinhold, K Neubert, L Jager and S Ansorge. (1995). Functional role of CD26 on human B lymphocytes. Immunol Lett 45:47–51.
29. Fleischer B. (1994). CD26: a surface protease involved in T-cell activation. Immunol Today 15:180–184.
30. Fleischer B. (1987). A novel pathway of human T cell activation via a 103 kD T cell activation antigen. J Immunol 138:1346–1350.
31. Chen T, D Smyth and CA Abbott. (2004). CD26. J Biol Regul Homeost Agents 18:47–54.
32. Brenner S, NL Whiting-Theobald, GF Linton, KL Holmes, M Anderson-Cohen, PF Kelly, EF VaninF, AM Pilon, DM Bodine, ME Horwitz and HL Malech. (2003). Concentrated RD114-pseudotyped MFGS-gp91phox vector achieves high levels of functional correction of the chronic granulomatous disease oxidase defect in NOD/SCID/beta− microglobulin−/− repopulating mobilized human peripheral blood CD34$^+$ cells. Blood 102:2789–2797.
33. Choi U, SS DeRavin, K Yamashita, N Whiting-Theobald, GF Linton, NA Loktionova, AE Pegg and HL Malech. (2004). Nuclear-localizing O6-benzylguanine-resistant GFP-MGMT fusion protein as a novel in vivo selection marker. Exp Hematol 32:709–719.
34. Cosset FL, Y Takeuchi, JL Battini, RA Weiss and MK Collins. (1995). High-titer packaging cells producing recombinant retroviruses resistant to human serum. J Virol 69:7430–7436.
35. Goerner M, PA Horn, L Peterson, P Kurre, R Storb, JE Rasko and HP Kiem. (2001). Sustained multilineage gene persistence and expression in dogs transplanted with CD34(+) marrow cells transduced by RD114-pseudotype oncoretrovirus vectors. Blood 98:2065–2070.
36. Tailor CS, A Nouri, Y Zhao, Y Takeuchi and D Kabat. (1999). A sodium-dependent neutral-amino-acid transporter mediates infections of feline and baboon endogenous retroviruses and simian type D retroviruses. J Virol 73:4470–4474.
37. Malech HL, U Choi and A Brenner. (2004). Progress toward effective gene therapy for chronic granulomatous disease. Jpn J Infect Dis 57:S27–S28.
38. Kawai T, U Choi, NL Whiting-Theobald, GF Linton, S Brenner, JM Sechler, PM Murphy and HL Malech. (2005). Enhanced function with decreased internalization of carboxy-terminus truncated CXCR4 responsible for WHIM syndrome. Exp Hematol 33:460–468.
39. Tanaka T, F Kitamura, Y Nagasaka, K Kuida, H Suwa and M Miyasaka. (1993). Selective long-term elimination of natural killer cells in vivo by an anti-interleukin 2 receptor

beta chain monoclonal antibody in mice. J Exp Med. 178:1103–1107.
40. Tanaka T, M Tsudo, H Karasuyama, F Kitamura, T Kono, M Hatakeyama, T Taniguchi and M Miyasaka. (1991). A novel monoclonal antibody against murine IL-2 receptor beta-chain. Characterization of receptor expression in normal lymphoid cells and EL-4 cells. J Immunol 147: 2222–2228.
41. McKenzie JL, OI Gan, M Doedens and JE Dick. (2005). Human short-term repopulating stem cells are efficiently detected following intrafemoral transplantation into NOD/SCID recipients depleted of $CD122^+$ cells. Blood 106:1259–1261.
42. Papayannopoulou T. (2004). Current mechanistic scenarios in hematopoietic stem/progenitor cell mobilization. Blood 103:1580–1585.
43. Peranteau WH, M Endo, OO Adibe, A Merchant, PW Zoltick and AW Flake. (2006). CD26 inhibition enhances allogeneic donor-cell homing and engraftment after in utero hematopoietic-cell transplantation. Blood 108: 4268–4274.
44. Graf L, M Iwata and B Torok-Storb. (2002). Gene expression profiling of the functionally distinct human bone marrow stromal cell lines HS-5 and HS-27a. Blood 100: 1509–1511.

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