## Simvastatin improves hematopoietic stem cell engraftment by preventing irradiation-induced marrow adipogenesis and radio-protecting the niche cells

Pre-transplant myeloablation increases bone marrow (BM) adipogenesis and destroys the niche cells, adversely affecting the hematopoietic stem cell (HSC) engraftment. This becomes a serious issue when donor HSCs are few or if their functionality is compromised. Therapeutic targeting of the niche[1] to increase HSC engraftment became popular after it was demonstrated that an increase in osteoblast numbers leads to an increase in HSC number.[2,3] Statins, the drugs used to treat hypercholesterolemia, have several clinically useful pleiotropic effects, including inhibition of adipogenesis in marrow-derived mesenchymal cells *in vitro* and *in vivo* prevention of irradiation-induced tissue and cell damage.[4,5] Therefore, we hypothesized that simvastatin treatment of stem cell transplant (SCT) recipients might improve HSC engraftment by preventing irradiation-induced adipogenesis and by radio-protecting the niche cells. We found that simvastatin treatment of recipient mice positively affects engraftment and expansion of donor HSCs by inhibiting marrow adipogenesis and radio-protecting niche cells. Simvastatin treatment of non-irradiated mice boosts the HSC numbers by remodeling the niche. These data provide evidence that simvastatin can be an effective niche-targeting agent to improve HSC engraftment by treating both recipients as well as donors. As simvastatin is already widely used, clinical application of this approach might be relatively straightforward.

To validate our hypothesis, we treated CD45.2 recipients with simvastatin (25 mg/kg body weight/day) or vehicle for one week prior and three weeks post transplantation with CD45.1 donor cells (Figure 1A). Hemogram analyses showed that the simvastatin-treated recipients had significantly higher platelet, neutrophil and total white blood cell count in their peripheral blood (PB) compared to the controls (*Online Supplementary Figure S1A*). Hemoglobin levels were comparable in both sets (*Online Supplementary Figure S1A*). Simvastatin-treated recipients had significantly low serum cholesterol levels and a high HDL : total cholesterol ratio. But the HDL : LDL ratio remained unaffected (*Online Supplementary Figure S1B*), showing that simvastatin treatment reduces irradiation-induced hypercholesterolemia. The donor cell engraftment in both control and simvastatin-treated recipients was more than 80%. Lineage analysis of PB confirmed that simvastatin treatment does not affect lineage commitment of the donor cells (Figure 1B). Immuno-phenotypic analyses (*Online Supplementary Appendix and Online Supplementary Table S1*) of BM cells revealed significantly high number of donor HSCs (FLT3$^-$

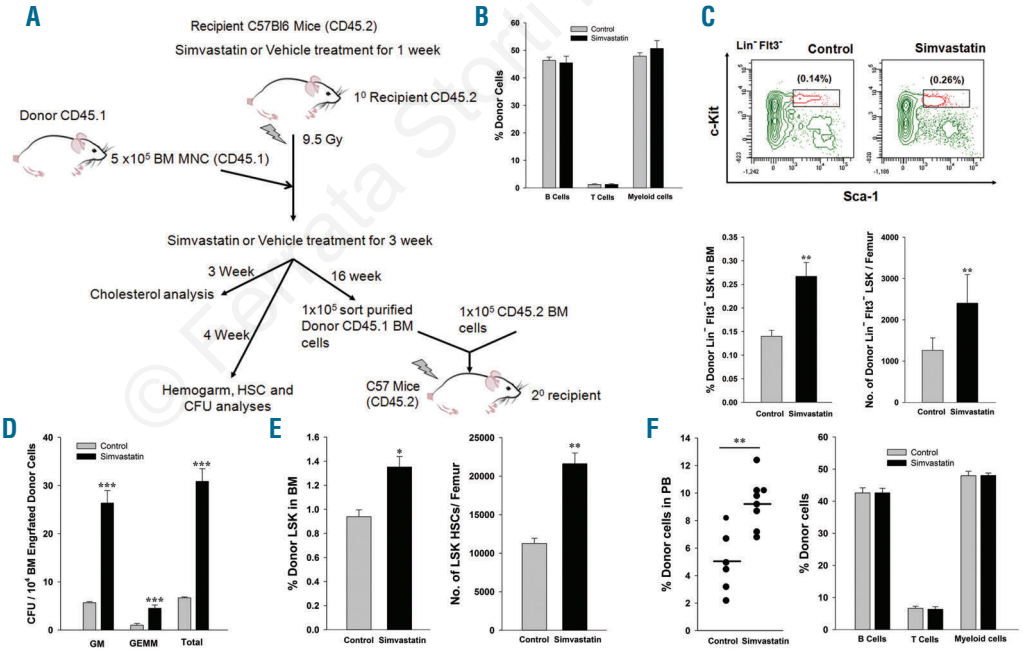


Figure 1. Simvastatin treatment augments donor cell engraftment. (A) Experimental scheme used to study the effect of simvastatin-treatment of recipients on HSC engraftment. (B) Measurement of lymphoid and myeloid lineage formation by donor cells in recipients' peripheral blood at four weeks post-transplant. Data represent mean ± SEM of 4 independent experiments. (C) Measurement of donor-derived HSCs in recipients' BM at four weeks post-transplant. The flow data show Sca1$^+$ c-Kit$^+$ cells analyzed in the Lin$^-$Flt3$^-$ population. The left bar diagram illustrates the % Lin$^-$ Flt3$^-$ LSK population. Right bar diagram shows the absolute numbers of Lin$^-$Flt3$^-$ LSK HSCs per femur. (N=6, Representative data for 4 independent experiments). (D) Colony formation unit (CFU) assay performed on the donor cells sorted from primary recipients' marrow. Colonies belonging to granulocyte-monocyte (GM) and granulocyte-erythrocyte-monocyte-megakaryocyte (GEMM) were scored. N=3; 3 plates/mouse, 3 mice/experiment. (E) Left panel shows the analysis of donor LSK in bone marrow (BM) of recipients at 16 weeks post transplant. Right panel shows the absolute numbers of LSK HSCs per femur. (N=5, Representative data for 3 independent experiments). (F) Competitive repopulation of the sorted donor BM cells collected from the primary recipients' marrow. Right panel shows multi-lineage repopulation analysis done at four weeks in the PB of secondary recipients. (N=8, Representative data for 3 independent experiments). All data are represented as mean ± SEM.*$P$<0.05, **$P$<0.01, ***$P$<0.001. Also see *Online Supplementary Figure S1*.

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LSK) (Figure 1C) in the simvastatin-treated recipients. Treatment of lineage-negative cells with simvastatin for seven days did not increase HSC numbers showing that simvastatin had no direct effect on HSCs (*Online Supplementary Figure S1C*).

Significantly higher numbers of colony forming units (CFU) present in the donor cells sorted from BM of simvastatin-treated recipients showed that simvastatin treatment promotes efficient regeneration of hematopoiesis (Figure 1D).

At 16 weeks, the number of donor LSK-HSC remained significantly high in the BM of simvastatin-treated recipients (Figure 1E). When the sort-purified engrafted donor cells were infused into secondary recipients to assess their long-term functionality, it was observed that the donor cells harvested from the simvastatin-treated primary recipients engrafted more efficiently in the secondary recipients (Figure 1F).

Collectively, these data demonstrate that simvastatin treatment of the SCT recipients significantly improved engraftment and expansion of the donor stem cells, and these expanded HSCs showed long-term functionality.

Marrow adipogenesis is the most detrimental effect of pre-transplant myeloablation. Naveiras *et al.* demonstrated that irradiation-induced marrow adipogenesis suppresses hematopoiesis and a pharmacological inhibition of PPAR-γ enhances donor cell engraftment.[6,7] Bone histology revealed strikingly reduced numbers of adipocytes in the marrow of simvastatin-treated recipients compared to controls (Figure 2A). A significantly reduced expression of *Ppar-γ* and an increased expression of *Runx-2* mRNA were observed in CD45$^-$ stromal cells sort-purified from simvastatin-treated recipients as compared to control recipients (Figure 2B). These data show that simvastatin prevents the irradiation-induced marrow adipogenesis by inhibiting the expression of PPAR-γ, a master regulator of adipogenesis.

Efficient engraftment of donor HSCs critically depends on optimal niche function. Osteoblasts, sinusoidal endothelial cells, Nestin positive (Nestin$^{+ve}$) MSCs and EPCs play a critical role in donor cell engraftment.[8,9] Since myeloablation destroys these niche cells,[10] we analyzed these cells in BM of simvastatin-treated recipients. A distinctly higher density of micro-capillaries and trabeculae seen in the BM of the simvastatin-treated recipients (*Online*

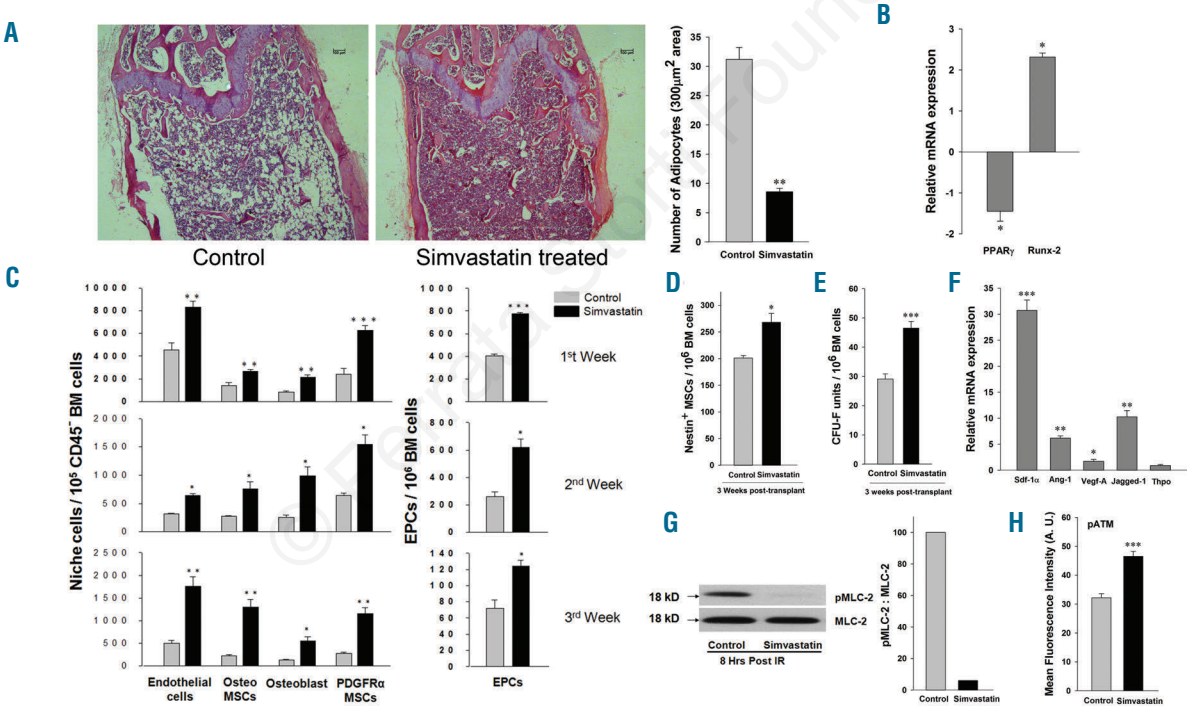


Figure 2. Simvastatin inhibits irradiation-induced adipogenesis and radio-protects bone marrow (BM) niche cells. (A) Histological characterization of paraffin-embedded bone sections from control and simvastatin-treated recipients using hematoxylin and eosin staining (H&E) done at four weeks post-transplant. Bar represents 100 μm. (n=4, repeated 3 times). Number of adipocytes formed in the bone marrow is graphically represented in the right panel. (N=3). (B) Quantification of *Ppar-γ*- and *Runx*-2-specific mRNAs in CD45$^-$ stromal cells sort-purified from control and simvastatin-treated recipients is shown. (n=4 per experiment, repeated three times). (C) Quantification of recipients' niche cells done at weekly intervals post-transplant. 6-8 mice/group/experiment, n=3. (D) Measurement of Nestin$^+$ MSCs in the recipients' marrow at 3 weeks post transplant. 6-8 mice/group/experiment n=3. (E) Colony forming unit-fibroblast (CFU-F) assay performed on BM cells collected from control and simvastatin-treated recipients at 3 weeks post-transplant. n=3. 6 plates/set/experiment. (F) Q-RT-PCR done on CD45$^-$ niche cells sorted from control and simvastatin-treated recipients at 3 weeks post-transplant. Experiment was repeated 3 times. 4 mice/set/experiment. (G) Western blot analysis of irradiated M210B4 stromal cells, with or without simvastatin-treatment, to examine the level of irradiation-induced phosphorylation of MLC-2, an immediate down-stream target of Rho kinase. Right panel depicts densitometric analysis of the bands. (H) Mean fluorescence intensity (MFI) measurements of immuno-stained control irradiated and simvastatin-treated irradiated M210B4 stromal cells using anti-p-ATM-Ser1981 antibody. Ataxia telangiectasia mutated (ATM), a serine-threonine kinase, is both activated and recruited to DNA double-strand breaks (DSBs), with its phosphorylation at the Ser-1981 site being involved in DDR.[11] MFI was measured using Image J software. A.U.: Arbitrary Units. Nuclei in 10 independent non-overlapping fields were scored. Data are represented as mean ± SEM. Also see *Online Supplementary Figure S2*. *$P$<0.05, **$P$<0.01,***$P$<0.001.

*Supplementary Figure S2A*), suggested that simvastatin preserves niche integrity. Flow analyses of niche cells revealed a significantly higher numbers of endothelial cells, osteoblasts, osteoblastic precursor MSCs, PDGFRα⁺ MSCs (Figure 2C and *Online Supplementary Figure S2B*), and endothelial precursor cells (EPCs) (Figure 2C and Online Supplementary Figure S2C) in the BM of simvastatin-treated recipients. The number of Nestin⁺-MSCs (Figure 2D and *Online Supplementary Figure S2B*) and the number of CFU-fibroblast (CFU-F) were also significantly high in the sim-

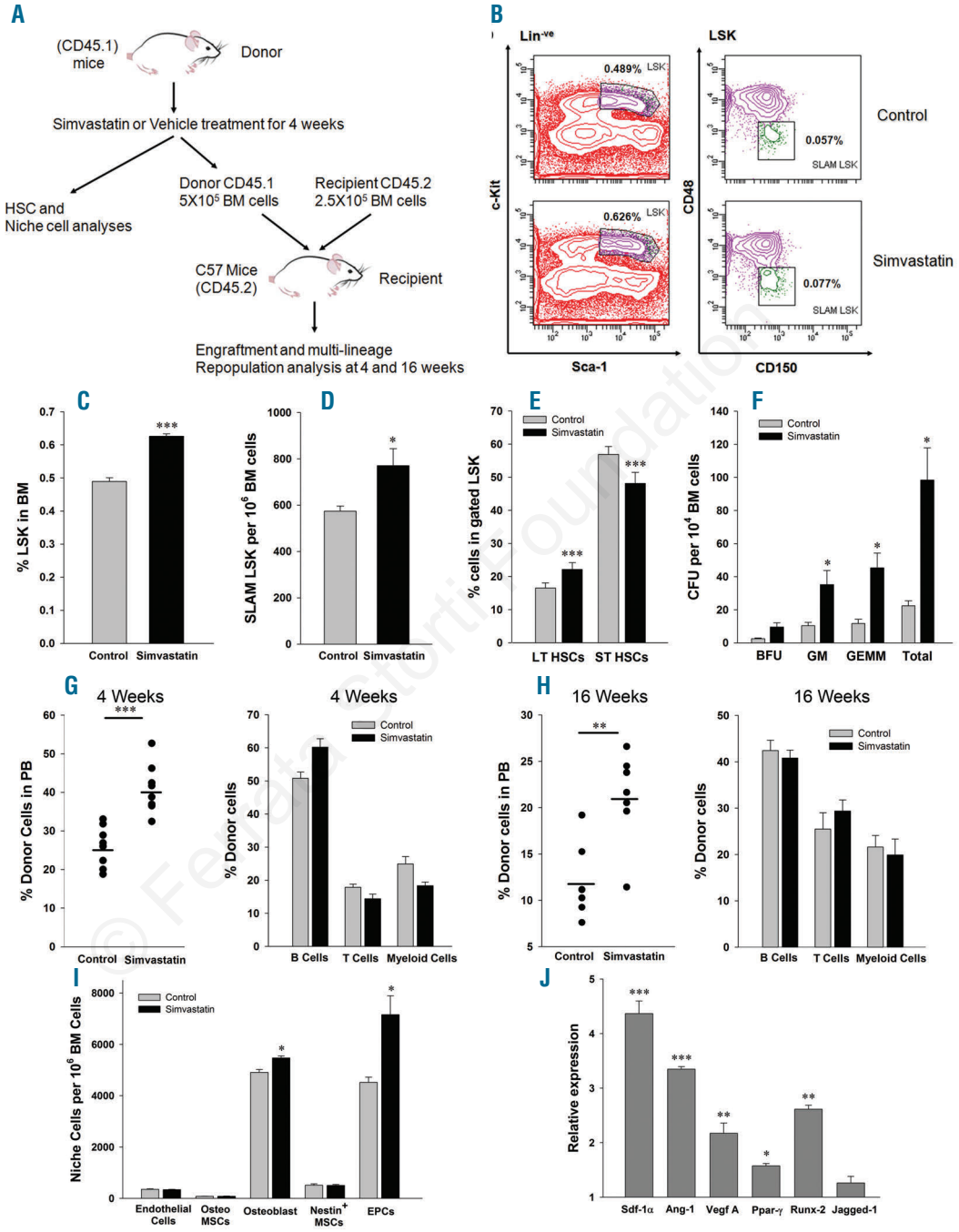


**Figure 3.** Simvastatin boosts HSC number in non-irradiated donors. (A) Experimental scheme for simvastatin-treatment is illustrated. (B) Representative flow panel depicting analyses of SLAM LSK HSCs in the marrow of control and simvastatin-treated donors. Quantification of LSK HSCs (C), SLAM LSK HSCs (D) and LT- and ST-HSCs (E) in the marrow of control and simvastatin-treated donors is depicted. Data are representative of 4 independent experiments done. (F) CFU assays done on the marrow cells of control and simvastatin-treated donors. Colonies belonging to burst-forming unit erythroid (BFU-E), granulocyte-monocyte (GM) and granulocyte-erythrocyte-monocyte-megakaryocyte (GEMM) type were visually scored using a phase contrast microscope. n=4, 6 plates/set/experiment. Engraftment of marrow cells collected from control and simvastatin-treated donors in irradiated recipients at four weeks (G) and 16 weeks (H) post transplant is illustrated. Panels on right side show percentage of myeloid and lymphoid cells present in the engrafted donor cell population.[8-10] mice/set/experiment, n=3. (I) Immunophenotypic analyses of various niche components in the marrow of control and simvastatin-treated donors. (J) Q-RT-PCR done on CD45⁻ stromal cells sorted from the marrow of control and simvastatin-treated donors. n=3. Data are represented as mean ± SEM. 5-6 mice/set/experiment were used. **P*<0.05, ***P*<0.01, ****P*<0.001. Also see *Online Supplementary Figure S3*.

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vastatin-treated recipients (Figure 2E). The recipients' niche cells support the donor HSC-proliferation by secreting various cytokines in response to myeloablative stress. Quantitative RT-PCR carried out on the sorted CD45$^-$ stromal cells revealed a much higher expression of CXCL-12 (*Sdf-1α*), *Ang-1-, Jagged-1*-and *Vegf-A*-specific mRNA in the cells of simvastatin-treated recipients (Figure 2F and *Online Supplementary Table S2*). As most effects of statins are attributed to the inhibition of regulatory proteins, including Rho Kinases, a potential use of statins as radio-protecting agents has been advocated.[5] Western blot analysis of the lysates prepared from simvastatin-treated and untreated, irradiated M210B4 stromal cells showed that simvastatin inhibited the phosphorylation of myosin light chain-2 (MLC-2), an immediate down-stream target of Rho kinase (Figure 2G). We also found a stronger expression of p-ATM-Ser 1981[11] in the simvastatin-treated irradiated cells compared to irradiated controls (Figures 2H and *Online Supplementary Figure S2D*). These data show that simvastatin protects the niche cells against irradiation *via* inhibition of Rho kinase and efficient activation of DNA damage repair (DDR) mechanisms. It may be interesting to see whether simvastatin treatment given within 24-48 h post irradiation mitigates the irradiation-induced damage. Simvastatin treatment results in an increased pool of niche cells in the BM microenvironment. This eliminates the extra cost associated with exogenous infusion of *ex vivo* expanded niche cells like MSCs or EPCs for enhancement of HSC engraftment.[12] Systemic administration of EGF and IGF-1 has been shown to improve post-transplant recovery;[13,14] however, these cytokines may induce unwarranted proliferation of residual neoplastic cells that may have escaped myeloablation. Simvastatin treatment of recipients will result in the protection of resident niche cells, and these protected niche cells would secrete the HSC-supportive cytokines in the proximity of the HSCs resulting in a much higher local concentration, thus saving the high cost of production and side-effects associated with systemic infusion of these cytokines.

A strategy that expands the resident stem cell pool in the donor BM can help to achieve an improved hematopoietic recovery post transplant. Therefore, we examined whether simvastatin positively regulates steady-state hematopoiesis as well, by treating donor mice with simvastatin for four weeks (Figure 3A). Quantification of HSC subsets showed that simvastatin significantly boosted the number of LSK-HSCs, SLAM-LSK-HSCs and LSK-CD34$^-$ (LT-HSCs) in the BM of simvastatin-treated donors (Figure 3B-E), without affecting marrow cellularity or hemogram (*Online Supplementary Figure S3A-C*). Comparable numbers of various progenitors present in the BM of control and simvastatin-treated mice showed that the increased HSC numbers were not related to a block in differentiation (*Online Supplementary Figure S3D and E*). Cell-cycle analysis of BM cells did not reveal any difference in the cell-cycle status of HSCs, suggesting that HSC expansion in the simvastatin-treated donors were not due to their excessive proliferation (*Online Supplementary Figure S3F*). CFU assays revealed that marrow cells of simvastatin-treated donors contained a significantly higher number of functional progenitors (Figures 3F). Competitive transplants were then performed to assess their engraftment ability. Short-term (4 weeks) (Figure 3G and *Online Supplementary Figure S3G*) and long-term (16 weeks) (Figure 3H) analyses of donor cells in the recipients' PB showed that BM cells from simvastatin-treated donors established a significantly higher level of chimerism at both time points and gave rise to a multi-lineage hematopoiesis without introducing any lineage bias (Figure 3G and H, right panels). Collectively, these results show that simvastatin treatment of non-irradiated mice expands the pool of functionally superior HSCs without inducing any undue myeloproliferation. Immuno-phenotypic analyses of BM cells revealed that simvastatin-treated donors harbored a significantly higher number of osteoblasts and EPCs in their BM (Figure 3I). Other niche cells (Figure 3I) and CFU-F (*Online Supplementary Figure S3H*) were not affected by the simvastatin treatment. Commensurate with these data, CD45$^-$ stromal cells sort-purified from simvastatin-treated donors showed significantly increased expression of *Sdf-1α*, *Vegf-A*, *Ang-1* and *Runx-2* mRNA (Figure 3J). The increase in *Jagged-1*-specifc mRNA was only marginal. A small increase in *Ppar-γ*-specific *mRNA* by simvastatin suggested that it did not interfere with natural mechanisms involved in the BM adipogenesis (Figure 3J). Collectively, these data demonstrate that, under steady-state conditions, simvastatin treatment expands the HSC pool through modulation of the BM niche. Co-infusion of EPCs with HSCs enhances donor cell engraftment.[12] Therefore, in addition to treating transplant recipients, treatment of donors with simvastatin may further enhance engraftment levels due to the presence of higher numbers of HSCs and EPCs in the graft.

In conclusion, our data show that simvastatin qualifies as a niche-targeting agent for use in clinical SCT. Using a clinically well-established drug like simvastatin with niche-protective effects is advantageous, since time-consuming phase I/II trials are not required, unlike newly discovered drugs. Since the pharmacokinetics of simvastatin is known, its efficacy in improving the outcome of SCT, especially in allogeneic settings, can be examined in large-scale clinical trials.

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