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Improved hematopoiesis in anemic Sl/Sld mice by splenectomy and therapeutic transplantation of a hematopoietic microenvironment

P Anklesaria, TJ FitzGerald, K Kase, A Ohara and JS Greenberger
Improved Hematopoiesis in Anemic $ SI/SI^d $ Mice by Splenectomy and Therapeutic Transplantation of a Hematopoietic Microenvironment

By Pervin Anklesaria, T.J. FitzGerald, Kenneth Kase, Akira Ohara, and Joel S. Greenberger

The ability of a clonal hematopoiesis-supportive bone-marrow stromal cell line GBlneo $^d$ to engraft and alter the microenvironment-induced anemia of $ SI/SI^d $ mice was studied. Prior to stromal cell transplantation, $ SI/SI^d $ mice received 1 Gy total body irradiation (TBI) and 13 Gy to the right hind limb. Two months after intravenous (IV) injection of $ 5 \times 10^8 $ GBlneo $^d$ cells, 54.4% $\pm$ 17.0% donor origin (G418$^+$) colony-forming cells were recovered from the right hind limb of $ SI/SI^d $ mice. Long-term bone marrow cultures (LTBMCs) established from GBlneo $^d$-transplanted mice produced 189.5 CFU-GEMM-forming progenitors/flask over 10 weeks compared with 52.7 $\pm$ 6.2 CFU-GEMM forming progenitors/flask from irradiated nontransplanted $ SI/SI^d $ mice. A partial correction of macrocytic anemia was detected 2 months after GBlneo $^d$ transplantation in splenectomized, irradiated $ SI/SI^d $ mice (Hgb 7.2 $\pm$ 0.4 g/dL; MCV 68.3 $\pm$ 7.0 fL) compared to splenectomized, irradiated, nontransplanted $ SI/SI^d $ mice (Hgb 6.5 $\pm$ 1.1 g/dL; MCV 76.8 $\pm$ 9.5 fL) or control $ SI/SI^d $ mice (Hgb 5.4 $\pm$ 0.5 g/dL; MCV 82.4 $\pm$ 1.3 fL). Mean RBC volume distribution analysis showed a 2.5-fold increase in percentage of peripheral blood RBCs with MCV $\leq$ 45 fL and confirmed reduction of the MCV in splenectomized-GBlneo $^d$-transplanted mice compared to control $ SI/SI^d $ mice. A hematopoiesis-suppressive clonal stromal cell line derived from LTBMCs of $ SI/SI^d $ mice (SI$^d$neo$^d$) engrafted as effectively (43.5% $\pm$ 1.2% G418$^+$ CFU-F/flask) as did GBlneo $^d$ cells (38.3% $\pm$ 0.16% G418$^+$ CFU-F/flask) to the irradiated right hind limbs of C57Bl/6 mice. LTBMCs established after 2 or 6 months from SI$^d$neo$^d$-transplanted mice showed decreased hematopoiesis (182 $\pm$ 12 [2 months] and 3494.3 $\pm$ 408.1 [6 months]) CFU-GEMM progenitors/flask over 10 weeks compared to those established from GBlneo $^d$-transplanted mice (5980 $\pm$ 530 [2 months] and 7728 $\pm$ 607.6 [6 months]) CFU-GEMM progenitors forming/flask. Thus, transplantation of clonal bone-marrow stromal cell lines in vivo can stably transfer their physiologic properties to normal or mutant mice.

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MATERIALS AND METHODS

Mice. Adult, 5- to 8-week-old, severely anemic WCByF1 $ SI/SI^d $ mice and C57Bl/6 mice were obtained from Jackson Laboratories, Bar Harbor, ME. B6'Cast$^-GPI$-$a^+$ mice, obtained from Jackson Laboratories, were bred at the University of Massachusetts Medical Center.

Bone-marrow stromal cell lines. Derivation and characterization of clonal-marrow stromal cell lines (GBl/$ SI/SI^d $, S$^d$3, +/+ and D2XRII) have been described previously.$^{3,4,6}$ The embryo fibroblast cell line Bl/6embC was provided by Dr S. Aaronson, NCI, Bethesda, MD. Neomycin (G418$^+$) resistant subclones of S$^d$3 and D2XRII and Bl/6embC were established by retroviral vector-mediated gene transfer.$^{7}$ The subclones are designated S$^d$3neo$, D2XNeC$, and Bl/6embneo$^+$, respectively.

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to the right hind limb (RHL) or both hind limbs (BHL) delivered by a linear accelerator as described. We used 1 Gy TBI and 10 Gy to the RHL due to the relative sensitivity of S/I/S*4 mice to TBI.2 Irradiated mice were transplanted with 5 x 10^10 stromal cells of each line (GBlneo', S'/neo', B/I/6 emboNeoC or D2Xneo') by IV injection (single schedule). For sequential boost irradiation-transplantation studies with S/I/S*4 mice, 1 Gy TBI and 10 Gy to the RHL was delivered by linear accelerator on day 0. A single-cell suspension of the GBlneo' cell line was injected IV 48 hours later. Two months after the first irradiation schedule, the same group of mice received 1 Gy TBI and 10 Gy to the left hind limb (LHL). Another injection of the GBlneo' cell line was administered 48 hours later (multiple transplant schedule). Control irradiated nontransplanted mice received 2 Gy TBI and 10 Gy to both hind limbs (BHL).

Splenectomy and irradiation of S/I/S* mice. S/I/S* mice (5 to 6 weeks old) were anesthetized using an inhalation anesthetic methoxyflurane (metofane, Pitman Moore, Inc, Washington Crossing, NJ). A small incision was made in the flank region, and the spleen was gently removed and cauterized as described.14 The incision was sutured with a 5.0 chromic gut and the skin flaps held together with wound clips using Mikron auto clip (Clay Adams). Two weeks after splenectomy, mice were irradiated (1 Gy TBI and 10 Gy BHL) and transplanted (single schedule) with the GBlneo' marrow stromal cell line as described above. Transplanted and control animals were followed for clinical improvement of their sampled blood Hgb concentration, hematocrit, and mean corpuscular volume (MCV), as described below.

Measurement of stromal cell engraftment. The CFU-F assay was carried out as described. Briefly, control-irradiated–nontransplanted or irradiated-transplanted mice were sacrificed at time intervals indicated, and the harvested bone-marrow cells from each hind limb were counted and plated at different cell concentrations in 60-mm dishes (Falcon, Lincoln Park, NJ) in the absence or presence of 200 µg/mL G418 (Gibco). CFU-F colonies (>25 cells) were counted at days 7 and 14.

Measurement of hematopoietic recovery in vivo and in vitro. Peripheral blood from transplanted and control-irradiated–nontransplanted mice was analyzed monthly. Blood from the tail vein (80 µL) was collected in heparinized microhematocrit capillary tubes (Fisher Scientific, Springfield, NJ) and mixed with 20 mL of diluent (NaCl [6.38 g/L], Boric acid [1.0 g/L], sodium tetraborate [0.2 g/L], and EDTA-2K [0.2 g/L]). RBCs, WBCs, platelets, Hgb concentration, hematocrit, and MCV were measured using an automated TOA-II Sysmex (Coulter, Hialeah, FL). Peripheral blood smears were made and stained with Wright's-Giemsa stain.

The functional integrity of transplanted stromal cells was evaluated by establishing LTBMCs from each hind limb as described.15 Hematopoietic recovery was quantitated by measuring cumulative nonadherent CFU-GE/MM–forming progenitor cells removed weekly for over 10 weeks in culture.16 In other experiments, different ratios of the two stromal cell lines were added to same flasks to test for dominance of biologic properties.

**Statistical evaluation.** The differences between the means was determined by standard Student's t test and chi-square analysis.

**RESULTS**

Recovery of bone-marrow stromal cells in vitro after TBI and isolated hind-limb boost irradiation in vivo. The effects of TBI (1 to 2 Gy) and high-dose irradiation to the RHL (13 Gy) on regeneration of normal C57BI/6 and S/I/S* mouse-marrow stromal cells in vivo were studied first. Two days following 1 Gy TBI and 13 Gy to the RHL, the femoral CFU-F from normal C57BI/6 mice decreased to 53% of the level detected in control-nonirradiated mice (Fig 1). By 60 days after irradiation, a further decrease (26% of control) in the colony-forming potential of marrow stromal cells from irradiated C57BI/6 mice was observed (P < .05). Improvement in the colony-forming ability of stromal cells from irradiated mice at 180 days after irradiation was not significant (Fig 1).

The number of colony-forming CFU-F progenitors/hind limb obtained from nonirradiated S/I/S* mice was 53% lower than that of control nonirradiated C57BI/6 mice. At two
days after irradiation, the number of CFU-F colonies obtained from irradiated SI/SI\(^4\) mice was not significantly lower than that of control-nonirradiated SI/SI\(^4\) mice. However, by 60 days after irradiation, there was a 41% decrease in the colony-forming ability of stromal cells from irradiated SI/SI\(^4\) mice (P < .05, Fig 1). These data indicated that the schedule of TBI and boost irradiation would reduce the number of colony-forming host stromal cells and should provide space to support the seeding of engrafted stromal cells at this site.

Support of hematopoietic progenitor cells by stromal cell cultures derived from different ratios of SI\(^{1146}\) and GB

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Ratio Plated*</th>
<th>Cumulative CFU-GEMM Progenitors/Flask †</th>
</tr>
</thead>
<tbody>
<tr>
<td>SI(^{1146})</td>
<td>1</td>
<td>21.5 ± 2.5</td>
</tr>
<tr>
<td>GB</td>
<td>1887 ± 2 18</td>
<td></td>
</tr>
<tr>
<td>SI(^{1146}) : GB</td>
<td>1:1</td>
<td>33 ± 1.8</td>
</tr>
<tr>
<td>SI(^{1146}) : GB</td>
<td>1:10</td>
<td>584.5 ± 8.5</td>
</tr>
<tr>
<td>SI(^{1146}) : GB</td>
<td>1:50</td>
<td>555 ± 43.2</td>
</tr>
<tr>
<td>SI(^{1146}) : GB</td>
<td>1:100</td>
<td>450 ± 79.6</td>
</tr>
</tbody>
</table>

* Stromal cells plated at different ratios in 25-cm\(^2\) flasks in duplicate to form confluent cultures with no further cell division possible, thus maintaining a stable ratio. Day 36 nonadherent hematopoietic cells (3 \times 10\(^6\) cells) from C57BL/6 LTBMCS were then engrafted to each flask. At weekly intervals, nonadherent cells produced/flask were harvested and assayed for CFU-GEMM--forming progenitors.
† Results are expressed as mean ± SD day 34 cumulative CFU-GEMM--forming progenitors/flask from two separate experiments.
C57BI/6 CFU-F colonies were still detected in GBlneo transplanted schedule transplants also showed no significant hematopoietic recovery of the peripheral blood counts at 60 days (Table 3, group IVb). In contrast, at 6 months, 22.5% G418® (Table 3, group 2). In contrast, at 6 months, 22.5% G418® mice were similar to those from nonirradiated (1 Gy TBI) 60 days showed no significant recovery in vivo at 120 days (Table 2, group IVa). S/I/SI® mice receiving sequential boost irradiation of both hind limbs and multiple schedule transplants also showed no significant hematopoietic recovery in vivo at 120 days (Table 2, group IVb).

To determine whether the failure to achieve detectable hematopoietic recovery in the GBlneo® transplanted mice was attributable to the inability of these cells to overcome the suppressive microenvironment of the S/I/SI® anemia, we next repeated the transplant studies in splenectomized S/I/SI® mice. A group of S/I/SI® mice were splenectomized 15 days before TBI and hind-limb irradiation (10 Gy BHL) and were then transplanted with the GBlneo® cell line. Splenectomized, irradiated, GBlneo®-transplanted mice had significantly increased WBC counts and Hgb concentration (9.5 ± 1.4 x 10^7/μL and 7.2 ± 0.4 g/dL, respectively [Table 3, group V]) at 2 months, compared with nonirradiated S/I/SI® mice (WBC counts: 4.6 ± 0.6 x 10^7/μL; Hgb concentration: 5.4 ± 0.5 g/dL. Table 3, group III P < .05). The splenectomized GBlneo®-transplanted mice at 2 months had reduced MCV (68.3 ± 7.0 FL) compared with that from control mice (82.4 ± 1.3 FL; P < .05, Table 3, group II). RBC volume distribution analysis demonstrated clear differences in RBC volumes comparing S/I/SI® mice to normal C57BI/6 mice, with only a small overlap between the two populations (Fig 2). The median MCV for normal RBCs from C57BI/6 mice received 1 Gy TBI and hind-limb irradiation (10 Gy BHL) and were then transplanted with GBlneo® mice. A group of S/I/SI® mice were prepared the mice with 2 Gy TBI and 20 Gy to both hind limbs (BHL, single schedule) or by sequential irradiation transplantation (multiple schedule), as described in Materials and Methods. In both experiments none of the control-irradiated–nontransplanted mice survived. LTBMCs established from the left hind limb of transplanted mice (1 Gy) supported multipotential progenitor cell production less efficiently (46.5 CFU-GEMM forming progenitors/flask) compared to both hind limbs (BHL, single schedule) or by sequential irradiation transplantation (multiple schedule).

<table>
<thead>
<tr>
<th>Group*</th>
<th>Peripheral Blood Analysis</th>
<th>WBC x 10^7/μL</th>
<th>RBC x 10^7/μL</th>
<th>PLT x 10^7/μL</th>
<th>Hgb g/dL</th>
<th>HCT %</th>
<th>MCV (FL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Normal C57BI/6</td>
<td>7.5 ± 1.7</td>
<td>8.1 ± 0.2</td>
<td>514 ± 145</td>
<td>12.1 ± 0.4</td>
<td>36.4 ± 6.6</td>
<td>36.6 ± 4.4</td>
<td></td>
</tr>
<tr>
<td>II. Nonirradiated S/I/SI®</td>
<td>4.6 ± 0.67</td>
<td>2.1 ± 0.2</td>
<td>234.9 ± 70.2</td>
<td>5.4 ± 0.5</td>
<td>17.5 ± 1.2</td>
<td>82.4 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>III. Irradiated-nontransplanted S/I/SI®</td>
<td>4.5 ± 0.7</td>
<td>2.9 ± 0.1</td>
<td>142 ± 5</td>
<td>6.6 ± 0.1</td>
<td>20.8 ± 0.15</td>
<td>71 ± 2</td>
<td></td>
</tr>
<tr>
<td>IV. S/I/SI® transplanted with GBlneo®</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) At 60 days (RHL)</td>
<td>4.3 ± 0.5</td>
<td>2.57 ± 0.37</td>
<td>411 ± 200</td>
<td>6.0 ± 1.1</td>
<td>18.1 ± 3.4</td>
<td>70 ± 2.9</td>
<td></td>
</tr>
<tr>
<td>b) At 120 days (BHL)†</td>
<td>3.26 ± 0.5</td>
<td>1.9 ± 0.57</td>
<td>153.3 ± 53</td>
<td>4.9 ± 1.6</td>
<td>17.0 ± 6.0</td>
<td>86.6 ± 7.5</td>
<td></td>
</tr>
<tr>
<td>V. S/I/SI® splenectomized and transplanted with GBlneo® (BHL)</td>
<td>9.5 ± 1.4‡</td>
<td>3.15 ± 0.2</td>
<td>152.6 ± 43.4</td>
<td>7.2 ± 0.4‡</td>
<td>19.1 ± 3.8</td>
<td>68.3 ± 7.0‡</td>
<td></td>
</tr>
<tr>
<td>VI. S/I/SI® splenectomized irradiated nontransplanted</td>
<td>7.6 ± 1.9</td>
<td>2.24 ± 0.6</td>
<td>201.3 ± 118.0</td>
<td>5.5 ± 1.1</td>
<td>16.6 ± 3.5</td>
<td>76 ± 8.5</td>
<td></td>
</tr>
</tbody>
</table>

*Results are expressed as mean ± SD for at least three to five mice per group.
†In group (b) mice received sequential irradiation-transplantation (multiple schedule), and all control-irradiated–nontransplanted mice died within 15 days after second irradiation.
‡P < .05 as compared to control-nontransplanted S/I/SI® mice.

The physiologic function of GBlneo® cells in different groups of transplanted S/I/SI® mice was next evaluated by in vitro culture of marrow. The first group consisted of mice receiving 1 Gy TBI and 13 Gy to the RHL. Marrow cultures established from irradiated-nontransplanted S/I/SI® mice showed increased cumulative progenitor cell production per flask (RHL, 10 Gy; 52 ± 10 progenitors/flask) compared to those from nonirradiated control mice (16 ± 4 progenitors/flask; P < .01; Fig 3). The cumulative number of nonadherent CFU-GEMM–forming progenitors produced in LTBMCs from RHL (10 Gy) of GBlneo® transplanted S/I/SI® mice was 189.5 progenitors/flask. This was higher than that produced in LTBMCs from control-nonirradiated or control-irradiated–nontransplanted mice. In contrast, LTBMCs established from the left hind limb of transplanted mice (1 Gy) supported multipotential progenitor cell production less efficiently (46.5 CFU-GEMM forming progenitors/flask; Fig 3). In the next two groups tested, we attempted to replace a dominant fraction of the marrow microenvironment of S/I/SI® mice by preparing the mice with either 2 Gy TBI and 20 Gy to both hind limbs (BHL, single schedule) or by sequential irradiation transplantation (multiple schedule), as described in Materials and Methods. In both experiments none of the control-irradiated–nontransplanted mice survived. LTBMCs established from GBlneo®-transplanted mice (2 Gy TBI and 20 Gy BHL) at 4 months showed increased cumulative CFU-GEMM–forming progenitors/flask in both right (298.8 ± 32.7) and left (415.8 ± 36.5) hind limb cultures compared to nonirradiated S/I/SI® mice (P < .05; Fig 4). In separate experiments, 2 months after mice received the second irradiation transplantation, the cumulative number of CFU-GEMM–forming progenitors obtained per flask was higher in LTBMCs established from engrafted RHL (136.1 ± 32) and LHL (78.6 ± 15.4) of GBlneo®-transplanted mice compared with those from control-nonirradiated S/I/SI® mice (P < .05; Fig 4).
Thus the transplanted GBlneo' cells functioned both in vivo in splenectomized SI/SI' mice to support hematopoiesis and in vitro after explant to LTBMC.

Transplantation of the SI'neo' stromal cell line to irradiated C57BL/6 mice. Normal C57BL/6 mice that received TBI and RHL irradiation (1 Gy TBI and 10 Gy RHL) were transplanted with the SI'neo' stromal cell line. At 2 and 6 months after transplantation, adherent stromal cells explanted from femurs and tibias of transplanted mice were selected in G418, and the percentage of donor-origin stromal cells recovered was 43.5% ± 1.2% and 8% ± 4.5% respectively (Table 2, group 3). Thus the SI'neo' cell line engrafted in vivo to Bl/6 mice as efficiently as the Gbline' cell line (Table 2, group 1). Marrow cultures established from mice transplanted with the Gbline' stromal cell line had significantly higher CFU-GEMM–forming progenitors per flask (2 months 5980 ± 530; 6 months 7728 ± 607, P < .05) compared with those from either control-irradiated–nontransplanted or SI neo -transplanted mice (Fig 5). In contrast, the cumulative number of hematopoietic progenitors supported by LTBMCs established at 2 and 6 months after transplantation with SI'neo' transplanted mice was 182 ± 12 and 3494 ± 408 CFU-GEMM progenitors/flask, respectively, and was no higher than that detected with marrow cultures.

Fig 2. RBC volume distribution analysis. Peripheral blood from normal C57BL/6 ( ), SI/SI' ( ), splenectomized Gbline'-transplanted SI/SI' ( ), and splenectomized-irradiated–nontransplanted SI/SI' ( ) mice at 4 months was diluted 1:10,000 and run at 1 1/2 amp and ½ 1/current setting of a Coulter Counter. MCV (fL) was estimated using standard latex particles (d = 3.14μmol/L). Results are expressed as mean ± SD percentage of total cells counted. At least five mice were analyzed per group. *P<.06 compared with control SI/SI' mice.

Fig 3. Hematopoiesis in LTBMCs established from hind limbs of irradiated and Gbline'-transplanted SI/SI' mice. At 2 months after transplant, LTBMCs were established from LHL and LHL of three to five mice per group of control-irradiated SI/SI' mice (-- ), irradiated-nontransplanted RHL-10 Gy (--- ), and LHL-1 Gy (--- ). Gbline' transplanted RHL-10 Gy (--- ), and LHL-1 Gy (--- ). Results are expressed as mean ± SD of cumulative CFUGEMM progenitors/flasks.
established from control-irradiated–nontransplanted mice (219 ± 9 [2 months] and 3225 ± 363 [6 months] CFU-GEMM–forming progenitors/flask; Fig 5). Thus both the GBlneo' and SI'neo' cells transferred their in vitro biologic properties to the in vivo marrow microenvironment of C57Bl/6 mice.

**DISCUSSION**

The present studies demonstrate that the GBlneo' marrow stromal cell line engrafts in vivo and improves hematopoietic recovery in splenectomized SI/S14 mice. Another stromal cell line, SI'neo', does not support hematopoiesis in vitro but engrafts in vivo and transfers its biologic properties to irradiated C57Bl/6 mice. Thus distinct physiologic properties of two clonal stromal cell lines that are expressed in vitro are maintained following transplantation in vivo.

The engrafted GBlneo' cells in splenectomized irradiated SI/S14 mice induced an increase in the HgB concentration as well as WBC counts in the peripheral blood of these mice as compared to that of control or splenectomized mice. The present data confirm and extend a previous report that demonstrated a partial stimulation of erythropoiesis in splenectomized SI/S14 mice implanted with intact spleens from +/+ mice.14 A decrease in the average MCV of peripheral
blood RBCs was also observed in splenectomized GBneo' transplanted mice. Furthermore, LTBMCs established at 2 and 6 months from GBneo'-transplanted Si/Si" mice showed increased longevity compared to marrow cultures from control Si/Si" mice. This may be attributable to a growth advantage in vitro of GBneo' stromal cells that further increase the favorable ratio of supportive stromal cells in vitro. LTBMCs established from control-irradiated Si/Si" mice supported hematopoeisis more efficiently than those from nonirradiated-control Si/Si" mice. Thus in Si/Si" mice, high-dose irradiation may help create a "niche" in the marrow cavity to support transplanted stromal cells and may also eliminate endogenous stromal cells that suppress hematopoeisis.

It is not yet known what characteristics are important for stable engraftment of stromal cell lines in vivo. A clonal murine embryonic fibroblast (Bl/6embneo' c) and bone-marrow stromal cell line (GBneo') that support hematopoeisis in vitro can engraft stably in vivo for up to 12 months to irradiated marrow sinususes of normal mice (unpublished observations). However, GBneo' cells were not detected by the CFU-F assay in the bone marrow of mutant Si/Si" mice at 6 or 12 months. One possible explanation for these data may be that the number of donor-origin cells was below the level of sensitivity of the assay used (G418') for detecting donor-origin cells. Another possibility could be that either migration or regeneration of endogenous Si/Si" stromal cells in irradiated marrow sinususes may have eliminated or reduced the numbers GBneo' cells. Results obtained in vitro using stromal cultures made up of different ratios of Si"neo' and GBneo' cells indicated that one tenth the number of Si"neo' cells could suppress support of hematopoiesis by GBneo' cells. While the Si"neo' stromal cell line did not support hematopoiesis in vitro, it did engraft in vivo and stably transferred its suppressive microenvironment. The stromal cell line D2Xneo', which does support hematopoiesis in vitro, did not engraft in vivo (unpublished observations). Thus the parameter of in vivo engraftment of a stromal cell line does not necessarily correlate with hematopoietic support in vitro and appears to be an independent biologic marker.

The present results indicate improved hematopoiesis in anemic Si/Si" mice after splenectomy and engraftment of a clonal bone-marrow stromal cell line. These data support the theory that the splenic microenvironment may be involved in the pathogenesis of some types of chronic anemia.19-22 Our attempts to replace a dominant fraction of the hematopoietic microenvironment involved sequential high-dose irradiation of two marrow niches and removal of the abnormal spleen microenvironment by splenectomy. While the parameters of stromal-cell transplantation need to be optimized, the present results provide evidence that therapeutic stromal cell transplant may be a viable alternative in diseases associated with a defective marrow microenvironment.20-27

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