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Therapeutic benefits in thalassemic mice transplanted with long term cultured bone marrow cells

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Abstract

Objective—Autologous bone marrow (BM) cells with a faulty gene corrected by gene targeting could provide a powerful therapeutic option for patients with genetic blood diseases. Achieving this goal is hindered by the low abundance of therapeutically useful BM cells and the difficulty of maintaining them in tissue culture long enough for completing gene targeting without them differentiating. Our objective was to devise a simple long-term culture system, using unfractioned BM cells, that maintains and expands therapeutically useful cells for ≥4 weeks.

Materials and Methods—From 2 to 60 million BM cells from wild-type (WT) mice, or from mice carrying a truncated erythropoietin receptor transgene (tEpoR-tg), were plated with or without irradiated fetal-liver derived AFT024 stromal cells in 25 cm^2 culture flasks. Four-week cultured cells were analyzed and transplanted into sublethally irradiated thalassemic mice (1 million cells / mouse).

Results—After 4 weeks, the cultures with AFT024 cells had extensive “cobblestone” areas. Optimum expansion of Sca-1 positive cells was 5.5-fold with 20×10^6 WT cells/flask and 27-fold with 2×10^6 tEpoR-tg cells. More than 85% of thalassemic mice transplanted with either type of cells had almost complete reversal of their thalassemic phenotype for at least 6 months, including blood smear dysmorphology, reticulocytosis, high ferritin plasma levels and hepatic/renal hemosiderosis.

Conclusion—When plated at high cell densities on irradiated fetal-liver derived stromal cells, BM cells from WT mice maintain their therapeutic potential for 4 weeks in culture, which is sufficient time for correction of a faulty gene by targeting.

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Keywords

Hematopoietic stem cells; long term bone marrow culture; bone marrow transplantation; thalassemia; gene targeting

Introduction

Hematopoietic stem cells (HSC) have been the subject of intensive study since their identification in the 1960s by Till and McCulloch [1]. HSC are capable of self-renewal and of differentiation to all blood cell types. Indeed, bone marrow repopulation has been demonstrated with a single mouse HSC [2,3]. However, maintaining and expanding HSC in culture for extended times without differentiation is difficult, although much progress has been achieved by the use of culture systems based on the long-term bone marrow cell culture (LTBMC) system described by Dexter *et al.* [4]. For example, we have shown that mouse bone marrow (BM) cells can be maintained in culture for greatly extended periods of time, and their proliferation enhanced if a truncated erythropoietin receptor (tEpoR) under the control of a β actin promoter is introduced into the genome of the donor mice [5]. Others have achieved comparable long-term cultures of murine BM cells by including a Hox B4 transgene [6], or by the use of a cocktail of growth factors [7]. In the current study we describe a simple procedure, using unfractionated BM cells plated at high cell density on a feeder layer of irradiated fetal-liver derived stromal cells (AFT024), that allows survival of therapeutically-competent cells for ≥ 4 weeks, which is sufficient time for achieving gene targeting. [Stromal-based culture was chosen, as opposed to a liquid-based culture [7], because electroporated cells survive better in stromal cultures than in liquid cultures, and because fewer technical difficulties are encountered in the subsequent tests for targeted gene correction using color gene markers [8], or PCR screening, or protein expression, etc.] We demonstrate that the phenotype of severely thalassemic mice becomes close to normal for at least 6 months after receiving BM cells from wild-type or tEpoR transgenic donors that have been cultured for ≥4 weeks under our conditions.

Materials and Methods

Long-term BM culture

BM cells were harvested by flushing femora and tibiae with phosphate-buffered saline (PBS) containing 2% heat-inactivated fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA) (Flow buffer). Clumps of BM tissue were gently dispersed using an 18 G needle with 10mL of a syringe, then centrifuged at 1,200 rpm (~450g) for 5 min and resuspended with ACK lysis buffer (0.15 M NH4Cl, 10 mM KHCO3, 0.1 mM Na2-EDTA, pH 7.4) to lyse red blood cells (5mL / mouse) for 5 min at room temperature. The lysis reaction was stopped by adding an equal volume of Flow buffer and washed by centrifuging. The pellet was resuspended with LTBMC medium containing 20% horse serum (STEMCELL Technologies Inc., Vancouver, Canada), 10^{-6} M Hydrocortisone (Phizer Inc., NY), 2mM L-Glutamine and 100 U/ml penicillin/100 μg/ml streptomycin in Iscove's modified Dulvecco's medium (IMDM; GIBCO, Grand Island, NY), and counted the cell number. To test the effects of cell density on cell proliferation, 2, 6.3, and 20 M tEpoR-tg BM cells and 2, 6.3, 20 and 60 M wild-type BM cells were plated separately onto irradiated AFT024 feeder cells with LTBMC medium (5 mL / 25 cm^2 flask) (plus 2 units EPO/mL for the tEpoR-tg cultures) at 33 °C, 5% CO2 in a humid atmosphere for 4 weeks. Half of the LTBMC medium was exchanged twice per week.

The murine fetal liver stromal cell line, AFT024 (a kind gift from IR Lemischka, Princeton University, Princeton, NJ) was maintained in high-glucose Dulbecco's Modified Eagle's

Medium (DMEM; GIBCO) supplemented with 15% FBS, 2mM L-Glutamine and 100 U/ml penicillin/100 μg/ml streptomycin in a 75 cm^2 flask (Falcon Plastics, Oxnard, CA) coated with 0.3% porcine gelatin (Sigma-Aldrich, St. Louis, MO) and maintained at 37°C. The stromal cells were passaged by 0.05% trypsin/EDTA (Invitrogen, Carlsbad, CA) when approximately 80% confluent and plated 1:5 to new gelatin-coated flasks. To prepare stromal feeders for long-term BM cultures, the AFT024 cells were irradiated with a 3 Gy γ-ray (Gammacell 40, Best Theratronics Ltd, Ontario, Canada) and plated at a density of 1.1 million (M) cells per 25 cm^2 on gelatin coated flasks.

Transplantation assays

Four-week cultured BM cells were detached from flasks by 0.05% trypsin/EDTA for 5 min, and dispersed with the LTBMC medium and filtered at 40 μm. After centrifuging, the medium was changed to Flow buffer. The cells were transplanted into sublethally irradiated (7.5 Gy) C654T ß-globin mutated mice (1.0×10^6 cells / mouse) through the retro-orbital sinus under anesthesia. The transplanted mice were maintained in microisolator cages with autoclaved food and acidified sterile water supplemented with neomycin sulfate for the first 2 weeks after transplant.

Animals

Male tEpoR-tg mice and wild-type (WT) GFP-tg mice, both on a C57BL/6J background, were used as BM donors. The tEpoR-tg mice have a truncated erythropoietin receptor transgene, driven by a human ß-actin promoter, inserted into the *Hprt* locus on the X chromosome [5]. The WT GFP-tg mice have an hrGFP transgene (Stratagene, La Jolla, CA) with a nuclear localization signal, also driven by the human ß-actin promoter and also inserted into the *Hprt* locus; the hrGFP transgene is flanked by two copies of a prototypic vertebrate insulator from the chicken (β-globin locus [9]. The recipient mice were C654T ß-globin mutated females (ß-thal) on a C57BL/6J background that have a mutant human ß-globin gene driven by the natural human ß-globin promoter in place of the normal mouse adult ß-globin genes [10]. The animals were maintained in microisolator cages with irradiated standard mouse chow in the animal facility at the University of North Carolina (Chapel Hill, NC) in accordance with Institutional Animal Care and Use Committee standards. All experimental mice were approximately 4 to 5 months old.

Flow cytometry analysis

Fresh BM cells and 4-week cultured BM cells were obtained as described above. Peripheral blood samples were collected from retro-orbital sinus under anesthesia and were lysed in ACK lysis buffer and washed twice with the Flow buffer. One million cells were incubated with 5% Fc-block (CD16/CD332) supernatant for 5 min on ice before staining. Appropriate amounts (0.03-0.05 μg / 1 million cells) of the following antibodies were added: Sca-1–-APC (Biolegend, San Diego, CA); Lineage markers including 5 kinds of antibodies: CD45R/B200–phycoerythrin (PE); CD3ε–PE, TER119–PE, Ly-6C (Gr-1)–PE and CD11b–PE (All from BD Biosciences, San Diego, CA); c-Kit (CD117)–Biotin (eBioscience, San Diego, CA). The cells were incubated on ice in the dark for 15-20 min, and then washed twice with Flow buffer. As secondary antibody reaction, 0.25 μg of Streptavidin–PE-Cy5.5 (eBioscience) was added and incubated with Flow buffer on ice in the dark for 5 to 10 min. After washing twice with Flow buffer, cells were resuspended with 300μL of Flow buffer. Samples were analyzed using a CyAn ADP Flow Cytometer (Beckman Coulter Inc., Fullerton, CA) and Summit™ Software ver. 4.3 (DakoCytomation, Glostrup, Denmark). A minimum of 50,000 events was acquired for each sample.

Peripheral blood smear

Blood smears, made from 1.8μL of blood collected in heparinized microhematocrit tubes, were air dried and stained with Wright stain or new methylene blue for reticulocyte counting. The reticulocytes were observed using the x100 oil-immersion lens and were counted the percentage per 1000 red blood cells.

Statistics

Results of experimental points from different experiments are reported as the mean, standard error of the mean (SEM). Significance levels were determined by either paired or non-paired Student's *t*-test analysis as indicated. For multiple comparisons from different experimental conditions, the statistical significance was evaluated using post-hoc analysis with Scheffé's test. The nonparametric Mann-Whitney *U* test was used for analyzing data from blood smears (Fig. 3C).

Results

Long-term Dexter-type cultures of BM cells from tEpoR-tg and wild-type mice BM cells from mice carrying a tEpoR transgene (tEpoR-tg) survive for up to 140 days and increase in number in long-term bone marrow cell cultures (LTBMC) when supplemented with erythropoietin (EPO) [5]. Cobblestone areas become predominant in these long term cultures as they progress. Comparable cultures with wild-type BM cells, with or without EPO, have no detectable cobblestone areas after 80 days. For our present experiments, aimed at finding conditions enabling productive LTBMC with wild-type BM cells, we therefore used irradiated fetal liver derived AFT024 cells [11] as stromal feeder cells because of their ability to facilitate the growth of bone marrow cells. We also chose to avoid EPO supplementation in the WT BM cultures because of its tendency to induce terminal differentiation.

Effects of BM Cell Numbers

We first determined the effects of increasing the numbers of cells per culture flask. Our investigation of this variable was guided by the thought that higher cell densities might encourage the development of cell clusters able to provide a micro-environment for stem cell proliferation comparable to that in normal bone marrow. As summarized in Fig. 1A, varying numbers (2, 6.3, 20 and 60 million) of BM cells, harvested from mice carrying the tEpoR-tg or from WT mice, were plated on AFT024 feeder layers in 25 cm^2 culture flasks (plus 2 units EPO/mL for the tEpoR-tg cultures). Table 1 and Fig. 1B and 1C show clear and marked differences between the results when the BM cells are from mice having the tEpoR transgene compared with those from WT mice. When the numbers of input tEpoR-tg BM cells were increased from 2 million (M) to 20M per flask, the fold increase in total cell number after 4 weeks decreased from 2.8-fold to 0.34-fold while the fold increase in Sca-1 (+) cells fell from 28- to 2.4-fold. In contrast, with WT BM cells, when input numbers were increased from 2M to 20M, the fold increase in the total cell number remained essentially constant at ~ 0.5-fold while the fold increase in Sca1(+) cells increased from 1.3- to 5.5-fold. At 60M input of WT BM cells, the fold increase in total cell number decreased to 0.21-fold, while the fold increase in Sca1(+) cells decreased to 1.7-fold. Thus the optimum input number for tEpoR-tg BM cells in our system is 2M per flask. Yet it is 20M for WT BM cells. Despite these differences the end point cultures are very similar; both have extensive “cobblestone” areas (Fig. 1D). Some flow data for single experiments (Fig. 1E and 1F) show that the percentages of Sca-1 (+) cells (outlined in blue) and Sca-1 (+)/c-Kit (+)/Lineage (−) cells (SKL, outlined in black) increase during the culture period; see also Table 1. [Deletion Here] We conclude that culturing tEpoR-tg BM cells at higher densities decreases the fold

expansion of Sca1 (+) cells, whereas culturing WT cells at higher densities increases the fold expansion, with an optimum at 20M input cells per flask.

Effects of Feeder Cell Numbers

We next explored the influence of the numbers of AFT024 feeder cells on the survival of BM cells after long-term culture (Fig. 2A). We seeded various densities of the AFT024 feeder cells into flasks and plated 20M WT BM cells in each. We used 1.1 M cells per 25 cm^2 flask as a baseline (1×), as this is the approximate number of cells found in a confluent flask (Fig. 2B). Half of this is designated as 0.5× and so forth. The effect of AFT024 feeder density on the ability of BM cells to expand is illustrated in Fig. 2C. We found that the total number of BM cells per 20 M input cells increased progressively as the feeder cells in the flask were increased up to 2×. At higher densities the feeder layer rolled over, and most of the BM cells were lost. The numbers of Sca-1 (+) cells in the flasks are affected by the density of the feeder layer in the same manner as the total number (Fig. 2C, right panel). We therefore chose a standard density of 1.1 M AFT024 feeder cells per 25 cm^2 flask for all subsequent cultures in this study.

Therapeutic benefits in mutant βC654T mice transplanted with 4-week cultured BM cells

To determine the therapeutic potential of the cells obtained after 4 weeks in culture, the cultures were disaggregated, and the unfractionated dispersed cells were transplanted into βC654T thalassemic (ß-thal) mice [10] (1M cells / mouse) that had been irradiated with a sublethal dose of 7.5 Gy. This mouse has one copy of chromosome 7 with the normal mouse ß globin genes, and one copy of chromosome 7 in which both adult ß globin genes ($ß^{major}$ and $ß^{minor}$) have been replaced by a human ß-globin gene harboring a splicing mutation. Mice heterozygous for this mutant ß globin gene manifest classic signs of ß thalassemia intermedia, namely anisocytosis, poikilocytosis, and target cells in the peripheral blood smear. The transplanted mice were analyzed every two weeks to observe the erythrocyte morphology and the number of reticulocytes by blood smears. Improvement in the morphology was noted four to six weeks later, as shown in Fig. 3B. In order to perform semi-quantitative analyses of blood smears, we generated an index of five standard mixes of blood from WT with ß-thal mice (Fig. 3C). Cells cultured for 4 weeks in the optimal culture conditions for both tEpoR-tg and WT cells were transplanted into six or seven sublethally irradiated ß-thal mice (Fig. 3A). All transplanted mice lived to 6 months post-transplant, and significant improvements were seen in erythrocyte morphology, e.g. the index increased from 1 to 4-5 by 2 months post-transplant in all mice ($p < 0.01$, pre-transplant versus 1 month post-transplant). The percentage of reticulocytes also decreased favorably in all transplanted mice, to less than 1% from a starting value of approximately 3.7% (the average in ß-thal mice) (Fig. 3D). This improvement in reticulocyte percentage persisted for at least 6 months in all mice. We also have evidence that nucleated myeloid cells derived from the donor are present in the recipient (Supplementary Fig. E1).

Lymphoid differentiation from 4-week cultured BM cells *in vivo*

All the WT mice that we used as donors carry a ubiquitously expressed GFP transgene [9], see Materials and Methods, as a marker enabling the recognition of donor cells in thalassemic mice receiving a transplantation. To make this distinction, we performed flow analysis on peripheral blood cells obtained from transplanted ß-thal mice 5 to 6 months post-transplant, and the cells were measured for GFP (+) cells as well as for the B-cell marker CD19, and the T-cell marker CD3 epsilon (CD3ε), which recognizes CD3ε chains in the T-cell CD3 receptor complex. The percentages of CD19 (+) cells recovered were 17% from transplanted cells ($CD19^+GFP^+$, outlined in blue) and 45% from host cells ($CD19^+GFP^-$, see Fig. 3E). As for T cells, 7% were derived from transplanted cells ($CD3ε^+GFP^+$, outlined in blue) and 16% were derived from the host ($CD3ε^+GFP^-$)(Fig. 3F). The culture conditions

used in this study do not support long term survival or proliferation of lymphoid cells (data not shown). Accordingly, this result suggests that the transplanted cells may include common lymphoid progenitors (CLPs) that give rise to T and B cells. Additionally, 4% of the Sca-1 (+) cells in peripheral blood were derived from the transplanted cultured cells ($Sca\text{-}1^{+}GFP^{+}$, outlined in blue) and 8% were from host cells ($Sca\text{-}1^{+}GFP^{-}$, see Fig. 3G). The presence of mixed chimerism in the lymphoid series is probably a function of the sublethal dose of radiation employed.

Post-Transplant Improvement in Organ Function

ß-thal mice manifest phenotypes similar to those seen in human ß thalassemia intermedia, including iron deposition, splenomegaly and ventricular hypertrophy. Some of these effects were reversed in ß-thal mice transplanted with 4-week cultured BM cells from tEpoR-tg or WT mice . Thus, no iron deposition was observed in all 7 mice receiving tEpoR-tg cells and 6 mice receiving WT cells (very low iron deposition was observed in one mouse kidney) (Supplementary Fig. E2A). And at 6 months post transplant using the 4-week cultured BM cells from the tEpoR-tg mice, the ß-thal phenotype was found to be reversed in the following specific areas; (1) the heart weight was reduced ($p < 0.01$) (left histogram in Supplementary Fig. E3A). (2) Left ventricular end-diastolic internal dimension (LVID) was reduced in transplanted mice ($p < 0.05$) to levels > indistinguishable from WT (right histogram in Supplementary Fig. E3A). (3) spleen size, which is more than 3 times larger in ß-thal mice than in normal mice, was reduced ($p < 0.05$) to a size indistinguishable from control (Supplementary Fig. E3B). (4) Plasma ferritin levels were improved ($p < 0.01$) from nearly three times the control mouse level to approximately the same level as the control mice (left histogram in Supplementary Fig. E3C). (5) Hematocrit, hemoglobin and leukocyte value also improved (Supplementary Fig. E3C). These results demonstrate that transplantation of 4-week cultured BM cells can reverse the pathologic phenotype of ß thalassemia intermedia in this murine model.

Discussion

In the experiments presented, we have demonstrated that BM cells with normal murine (β-globin genes from mice with or without a tEpoR transgene, when transplanted after four weeks in culture at high cell densities in the presence of irradiated AFT024 stromal cells, can provide therapeutic benefits in thalassemic mice. After transplantation, the morphology of erythrocytes in the recipient mice improved very substantially and the reticulocyte number was normalized. The transplanted cells were also shown to have differentiated to lymphocytes. The therapeutic benefits in thalassemic mice resulting from transplanting our 4-week cultured BM cells persisted for at least 6 months, demonstrating that the cultures include long term repopulating cells. Because we did not carry out serial transplantation tests, we cannot conclude that the 4-week cultures include “true” hematopoietic stem cells. But we can conclude that our simple culture system enabled us to reach our objective of maintaining therapeutically useful wild-type BM cells for 4 weeks in culture, which is sufficient time for carrying out corrective gene targeting using suitably designed DNA constructs, such as those described by us previously [8].

We used a novel high density culture system for our experiments with the aim of replicating the crowded marrow micro-environment as much as possible in *ex vivo* culture. We used unfractionated BM cells rather than purified stem cells with the aim of providing as many adjunct stromal cells as possible to support the stem cells. Even so, in order to achieve longer survival times of the BM cells, we found it necessary to include irradiated AFT024 stromal cells in the cultures. A number of studies describing co-culture with AFT024 stromal cells have been published [11,12]. A potential problem is associated with the use of the immortal liver-derived AFT024 cells, namely the ability of bone-marrow derived cells to

fuse with other cells [13]. If a donor BM cell fused with an AFT024 stromal cell (an immortalized cell line), it is possible that the fused cell could proliferate in an uncontrolled, potentially malignant manner. However, we have not seen cells of this type in our cultures, or any abnormal cell growth in the mice receiving our cultured cells. Nevertheless, in order to provide a safer therapeutic option, our future studies will include attempts to obtain a source of stromal cells other than AFT024 . For example, one might envision performing gene correction procedures to reverse genetic defects in newborns using unfractionated nucleated cells from umbilical cord blood cultured at high cell density on a HUVEC type feeder layer prepared from umbilical cord endothelial cells [14] or from amniotic mesenchymal cells [15].

Some comment is required on the considerable difference in optimal plating density that we have observed between bone marrow cells from WT mice and those from mice carrying the tEpoR-tg. As indicated above, we suggest that the WT cells require plating at a high density in order for the development of hematopoietically beneficial niches and/or sources of hematopoietic factors that can stimulate the survival and expansion of therapeutically useful cells. However, at this (high) cell-density addition of EPO causes the feeder cell layer to curl up. For this reason, and because EPO tends to promote differentiation of WT BM cells, we did not include it in the WT cultures. In contrast, therapeutically useful cells in mice carrying the tEpoR-tg respond to EPO by dividing. Consequently, we add it to their medium, and they readily increase in number even at low cell densities. At high cell densities the tEpoR-tg cultures, like the WT cultures, are adversely affected by EPO.

In summary, as one of many steps towards developing a practical gene targeted approach for the treatment of inherited hematologic and immunologic diseases, we have demonstrated that unfractionated BM cells, plated at high cell densities on irradiated fetal-liver derived stromal cells, maintain their therapeutic potential for 4 weeks in culture, a window of time sufficient for correcting a faulty gene by targeting.

Acknowledgments

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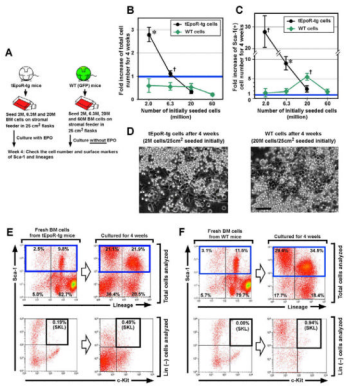

Figure 1. Analyzing 4-week cultured BM cells from tEpoR-tg and wild-type mice

(A) Experimental overview. Bone Marrow (BM) cells were harvested from tEpoR-tg and wild-type (WT) mice and plated at different cell densities on stromal feeders. Erythropoietin (EPO) was added to the tEpoR-tg cell flask. The cultured cells were analyzed at 4 weeks. **(B) and (C)** Fold increase of total cell number (*B*) and Sca-1 (+) cell number (*C*) at 4 weeks relative to the initial cell number. Varying numbers (2, 6.3, 20 and 60 million) of BM cells were seeded initially and counted after 4 weeks. Blue highlighted line indicates one fold increase. A student's *t*-test was used to assess statistical significance between tEpoR-tg and WT cells. (*$p < 0.01$; $^{\dagger}p < 0.05$). **(D)** Cobblestone area from tEpoR-tg (left) and WT (right) cells at week 4. The initial cell numbers are shown. Bar represents 50 μm. **(E) and (F)** Flow cytometry analyses of cells cultured 4-weeks (right two images) compared with the fresh BM (left two images) from tEpoR-tg (*E*) and WT (*F*) mice. Analyses of Sca-1/Lineage markers are shown in the upper two figures, and Sca-1/c-Kit/Lineage negative (SKL) are shown in the lower two figures.

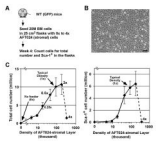

Figure 2. Effects of Feeder Cell Numbers

(A) Various densities (0 to 4-fold (×) versus typical confluence (1 ×)) of AFT024 stromal cells were tested with 20 million (M) BM cells from WT (GFP) mice in 25 cm^2 flasks. **(B)** Typical density (1 ×) of AFT024 feeder cells was used in this study for all other experiments. Scale Bar: 100μm. **(C)** The total number of cells (in the left panel) attached to the 25 cm^2 flask was counted at Week 4. The densities of 0.5× to 2× of the feeder layer maintained higher numbers of cells. In flasks containing higher numbers of feeder cells (4× density, 4.4M cells/25 cm^2 flask), the feeder layer often detached from the bottom of the flask and rolled over with the bone marrow cells, causing a decrease in the final cell number (denoted by a dashed line). The number of Sca-1 (+) cells was shown in the same manner as the total number in the right panel.

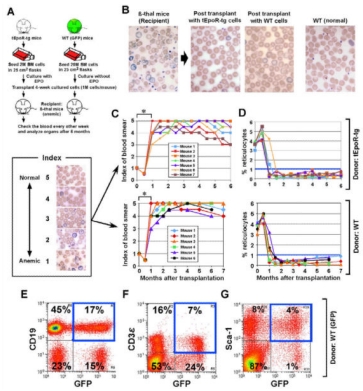

Figure 3. Therapeutic benefits in β-thal mice transplanted with 4-week cultured BM cells from tEpoR-tg and WT mice

(A) Experimental overview. BM cells were harvested by the same manner as shown Fig. 1A. The respective optimal culture conditions, as described in Fig. 1, were used for both tEpoR-tg and WT cells. One million unfractionated cells were transplanted to an irradiated β-thal mice after 4-weeks in culture. **(B)** Peripheral blood smears pre- and post-transplantation. Smears from pre-transplant (blood smear from a β-thal mouse) (left), 4 to 6 weeks post transplant (middle two pictures) and WT (non-transplanted) control mice (right). **(C)** Improvement of erythrocyte morphology 6 to 7 months post-transplant. Transplant-recipient mice were tracked with tEpoR-tg (upper) and WT (lower) cells, respectively. A Mann-Whitney *U* test was used to assess statistical significance between Month 0 and 1 within the same donor mice. (* $p < 0.01$; tEpoR-tg, n = 7; WT, n = 6;) **(D)** Improvement of reticulocyte amount in the peripheral blood 6 to 7 months post transplant. The same mice analyzed in Fig. 1C were analyzed here. The normal reticulocyte percentage is less-than 1%, and emphasized by a blue horizontal line. **(E), (F) and (G)** Flow analysis of peripheral blood from the recipient mice transplanted with WT (GFP) cells. CD19 for B cells (*E*), CD3ε for T cells (*F*) and Sca-1 (*G*) were analyzed 5 to 6 months post-transplant by GFP-positivity. The blue outline emphasizes both GFP and the marker (+) cells.

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Table 1

Cell numbers using tEpoR-tg and WT mice by 4-week culture.

			Numbers of input BM cells (million) / 25-cm2 flask			
			2	**6.3**	**20**	**60**
tEpoR-tg mice	Day 0	Input Sca-1(+) cells * (million)	0.10 ± 0.02	0.32 ± 0.06	1.0 ± 0.2	N/A
		Input SKL cells † (thousand)	1.8 ± 0.1	5.5 ± 0.4	17.5 ± 1.3	N/A
	Week 4	Total cell number (million)	5.0 ± 0.9	7.1 ± 0.8	6.8 ± 1.8	N/A
		Cell number of Sca-1(+) (million)	2.8 ± 0.8	2.8 ± 0.6	2.6 ± 0.9	N/A
		Cell number of SKL (thousand)	32 ± 11‡	34 ± 16	21 ± 8	N/A
	Day 0 vs. Week 4	Fold increase in total cell number §	2.8 ± 0.3 ¶	1.1 ± 0.1 #	0.34 ± 0.09	N/A
		Fold increase in Sca-1(+) cell number **	28 ± 7 #	8.9 ± 1.7 ¶	2.4 ± 0.8	N/A
		Fold increase in SKL cell number	19 ± 8 #	7.0 ± 3.5	1.4 ± 0.6	N/A
WT mice	Day 0	Input Sca-1(+) cells * (million)	0.11 ± 0.02	0.33 ± 0.06	1.1 ± 0.2	3.2 ± 0.5
		Input SKL cells † (thousand)	1.6 ± 0.2	5.2 ± 0.5	16.5 ± 1.7	49.5 ± 5.1
	Week 4	Total cell number (million)	1.2 ± 0.6	3.5 ± 1.0	11 ± 2.6	14 ± 3.0
		Cell number of Sca-1(+) (million)	0.15 ± 0.12	0.15 ± 0.12	5.7 ± 1.1 ††	4.7 ± 1.5
		Cell number of SKL (thousand)	0.26 ± 0.12	0.73 ± 0.35	69 ± 40	93 ± 42
	Day 0 vs. Week 4	Fold increase in total cell number	0.59 ± 0.30	0.55 ± 0.16	0.54 ± 0.13	0.21 ± 0.04
		Fold increase in Sca-1(+) cell number ‡‡	1.3 ± 1.1	1.7 ± 0.7	5.5 ± 0.8 #	1.7 ± 0.6
		Fold increase in SKL cells number	0.18 ± 0.10	0.17 ± 0.10	3.9 ± 1.9	2.2 ± 1.2

The number shows mean ± SEM (n=4). N/A = not available. Post-hoc analysis with Scheffé's test was used to make multiple comparisons within the same row, unless otherwise specified.

*Values were calculated by multiplying total numbers of input BM cell (the upper row) by the percentages of Sca-1(+) cells by flow analyses. Sca-1 (+) cells contain both c-Kit (+) and c-Kit (−) cells.

†Values were calculated by multiplying total numbers of input BM cell (the upper row) by the percentages of SKL cells by flow analyses. SKL cells are Sca-1(+)/c-Kit(+)/Lineage(−).

‡$p < 0.05$ (Student's *t*-test between "Input SKL cells, Day 0, tEpoR-tg mice" and "Cell number of SKL, Week 4, tEpoR-tg mice" with the same number of cells seeded initially.)

§Values were calculated by "Total cell number" of tEpoR-tg mice at the Week 4 ÷ "Numbers of input BM cells". P<0.01 between 2 and 6.3, 2 and 20 millions/flask compared in the same row.

‡‡Values were calculated by "Cell number of Sca-1(+)" at the Week 4 ÷ "Input Sca-1(+) cells" at Day 0. P<0.05 between 2 and 20 millions/flask compared in the same row.

¶$p < 0.01$. (Student's *t*-test between tEpoR-tg and WT mice with the same number of cells seeded initially) as also indicated in Fig. 1B and 1C.

[#] $p < 0.05$. (Student's *t*-test between tEpoR-tg and WT mice with the same number of cells seeded initially)

[**] Values were calculated by “Cell number of Sca-1(+)” at the Week 4 ÷ “Input Sca-1(+) cells” at Day 0. P<0.05 between 2 and 6.3 millions/flask; P<0.01 between 2 and 20 millions/flask compared in the same row.

[††] $p < 0.01$ (Student's *t*-test between “Input Sca-1(+) cells, Day 0, WT mice” and “Cell number of Sca-1(+), Week 4, WT mice” with the same number of cells seeded initially)