Comparison of Different Cytokine Conditions Reveals Resveratrol as a New Molecule for Ex Vivo Cultivation of Cord Blood-Derived Hematopoietic Stem Cells

NIELS HEINZ,a,b BIRGitta EHNRSTR ¨OM,b AXEL SCHAMBACH,b ADRIAN SCHWARZER,b UTE MODLICH,a BERNHARD SCHIEDLMEIERb

Key Words. Resveratrol • Cord blood • Hematopoietic stem cell • Expansion • Serial transplantation

ABSTRACT

Human cord blood (CB)-derived hematopoietic stem cells (HSCs) are an interesting source for HSC transplantation. However, the number of collected CB-HSCs is often too low for one transplantation; therefore, ex vivo expansion of CB-HSCs is desirable. Current expansion protocols are based on the use of cytokine combinations, including insulin-like growth factor-binding protein 2 (IGFBP2) and angiopoietin-like proteins, or combinations with “small molecules” such as stemregenin-1. The aim of our project was to compare the potential of different CB-HSC expansion strategies side-by-side by phenotypical analysis in vitro and serial engraftment properties in NOD/SCID/IL2rg−/− (NSG) immunodeficient mice. We further identified resveratrol, a naturally occurring polyphenol, as a new, alternative small molecule combined with cytokines to facilitate serum-free ex vivo expansion of human CB-HSCs. The cultivation in resveratrol preserved the CB-HSC phenotype in vitro most efficiently and was ∼2 times more potent than commonly used cytokine conditions (including stem cell factor, thrombopoietin, Fms-related tyrosine kinase 3 ligand, interleukin-6) and the recently established serum-free culture, including IGFBP2 and angiopoietin-like 5. Serial transplantation studies further confirmed resveratrol to support robust multilineage engraftment in primary and secondary NSG recipients. Therefore, our work proposes resveratrol as a new small molecule for improved ex vivo culture and modification of human HSCs based on an efficient ex vivo propagation of the HSC fate.

SIGNIFICANCE

Human cord blood (CB)-derived hematopoietic stem cells (HSCs) are an important source for HSC transplantations but restricted in their usage because of their low numbers. In gene therapy, modifications of HSCs rely on their ex vivo modification without losing their stemness properties. Therefore, ex vivo cultivation and expansion of CB-HSCs is important for their effective application in HSC transplantation and gene therapy. Several promising protocols for serum-free cultivation of HSCs using different combinations of cytokines or so-called small molecules are described. A direct comparison was performed of three described serum-free cytokine conditions, demonstrating that the natural occurring polyphenol resveratrol is able to support ex vivo cultivation of CB-HSCs. The results show that resveratrol is an additional candidate for improving ex vivo cultures of HSCs for transplantation and gene therapeutic applications in the future.

INTRODUCTION

Hematopoietic stem cells (HSCs) constitute a rare cell population within the bone marrow with the potential to self-renew and to produce all mature blood lineages of an individual throughout its lifetime. Because of these characteristics, HSCs are clinically used for the treatment of hematological disorders by bone marrow hematopoietic stem cell (BM-HSC) transplantation, including gene therapeutic applications. Human cord blood (CB)-derived HSCs constitute a valuable alternative to BM-HSCs, because they are easily accessible and the CB-CD34+ selected cells can be cryopreserved for further usage. Compared with BM-HSCs, the transplantation of CB-derived CD34+ cells with up to 2 human leukocyte antigen mismatches revealed a lower risk of graft-versus-host-disease (GvHD), a major side effect in allogeneic transplantations [1–3]. This reduced risk of GvHD is linked to a smaller number of T cells and more naive status of the CB-derived cells [4]. Therefore, CB is of increasing interest as a source of isolating HSCs for clinical applications. However, the major
limitation in the clinical use of CB-HSCs is the small size of single CB donations. Owing to the low numbers of CB-CD34⁺ cells from one donor cord, the hematopoietic and immunological recovery in recipients is often delayed, causing higher infection rates and transplant-related mortality. This limits the application of CB mainly to the treatment of pediatric patients. To overcome this limitation, double CB transplantations were successfully tested but a higher risk of GvHD was reported [5]. Another approach is the development of protocols for ex vivo expansion of CB-HSCs. Success from clinical studies has demonstrated an improved hematopoietic recovery early after cotransplantation of ex vivo expanded CB-CD34⁺ cells; however, the long-term engraftment was established from cotransplanted, nonexpanded CB-HSCs of a second donor [6, 7].

The major hurdle for ex vivo culture is the progressive loss of self-renewal capacity during in vitro cultivation. Under homeostatic conditions in vivo, HSCs are maintained mostly in a quiescent state in the bone marrow [8] to avoid exhaustion and damage. For ex vivo culturing, HSCs are induced to enter the cell cycle. These changes lead to several responses of the HSCs, including changes in lipid raft clustering and loss of polarization, accumulation of reactive oxygen species, endoplasmic reticulum stress, and genotoxic stress, which together ultimately lead to the loss of HSC function [9–13].

To overcome these limitations, different approaches were developed to increase the amount of HSCs from a single CB donor by modifying serum-free ex vivo culture conditions. These modifications encompass different cytokine combinations such as the use of insulin-like growth factor-binding protein 2 (IGFBP2) and angiopoietin-like (Angptl) proteins or pleiotrophin and the addition of small molecule components such as stemregenin-1 or the recently identified UM171 into the culture medium [14–19]. The combinations of serum-free culture conditions with different Angptl proteins were shown to expand human and murine HSCs ex vivo, and stemregenin-1 expanded human HSCs. Although the understanding of the mechanism is still incomplete, several effects have been demonstrated. The stimulation of HSCs with IGFBP2 modifies their cell cycle and regulates antipapoptotic proteins [18, 20]. Angptl proteins were shown to maintain quiescence of HSCs and to support the repopulation capacity of HSCs [21]. The HSC-supporting activity of stemregenin-1 was linked to its antagonistic activity on the aryl hydrocarbon receptor [17], a receptor present on HSCs [22].

Resveratrol is a naturally occurring polyphenol found in a variety of plants and fruits, including peanuts, mulberries, and grapes. Since its discovery in the 1940s, it has been linked to a wide range of health benefits in humans. The systemic administration of resveratrol has had beneficial effects in the treatment of cancer, diabetes, and neurological disorders [23]. Although a complete understanding of the mode of action is still missing, several cellular and molecular effects have been described, displaying a broad range of activities of resveratrol on cell functions. Resveratrol was shown, for example, to bind several receptors involved in hematopoiesis, including aryl hydrocarbon receptor and integrin αvβ3, both of which have been linked to HSC activity [24–26]. Furthermore, application of resveratrol via the food was able to partially restore the hematopoietic defect in vivo in a Fancd2 knockout mouse model [27], and it was shown that in vivo treatment of mice with resveratrol resulted in an increase of HSC numbers [28]. Moreover, resveratrol has been shown to inhibit cellular oxidative stress, endoplasmic reticulum stress, and lipid raft perturbation [29, 30]. We therefore tested resveratrol for its ability to improve ex vivo cultures of CB-derived HSCs as an additional component to enhance CB expansion protocols. Furthermore, we directly compared the effect of resveratrol with that of two recently described cytokine conditions, including IGFBP2 and Angptl5 and stemregenin-1, in several independent experiments with different CD34-enriched CB donations.

### MATERIALS AND METHODS

#### Isolation of CD34⁺ Cells

Human umbilical CB samples were collected from anonymized donors after written informed consent by Hannover Medical School (Hannover, Germany) approved by the institutional ethic committee. CD34⁺ CB cells were isolated from donations 1–4 hours after birth by model-based analysis of chip-sequence separation with the human CD34 MicroBead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com) according to the manufacturer’s protocol. In brief, CB was diluted threefold with phosphate-buffered saline (PBS), 2 mM EDTA, and 2% fetal calf serum (FCS) (PBS/EF; all from PAA Laboratories, Pasching, Austria, http://www.paa-laboratories.com) and the mononuclear cell fraction (MNC) was isolated via Ficoll (Biochrom, Berlin, Germany, http://www.biochrom.com) density centrifugation using Leucosep tubes (Greiner Bio-One, Frickenhausen, Germany, http://www.greiner.com) and washed twice with PBS/EF. The MNCs were counted, stained, and separated via LS columns (Miltenyi Biotec) according to the manufacturer’s instructions. The isolated CD34⁺ cells were analyzed for purity via flow cytometry, counted, and directly cryopreserved in FCS/10% dimethyl sulfoxide in liquid nitrogen for further usage. All experiments were performed with independent pools of CB-derived CD34⁺ cells.

#### Cultivation and Transplantation of CB-Derived Cells

Independent batches of CD34⁺ were thawed and directly pooled into serum-free StemSpan medium (StemCell Technologies, Vancouver, BC, Canada, http://www.stemcell.com), including stem cell factor (SCF) (50 ng/ml). The cells were centrifuged and resuspended in StemSpan medium, including SCF (50 ng/ml), and the living cell numbers were determined via live/dead counting using Neubauer improved chambers and trypan blue. The cells were directly transferred to the respective cytokine conditions for parallel cultivation within the different media. The media with the respective cytokine and small molecule conditions were renewed every second day during the whole culture period. All experiments were done with independent pools (i.e., from multiple donors) of CD34-enriched cord blood cells.

The cultivation was performed with serum-free StemSpan medium with the human cytokines and small molecules as follows: control (ctrl), SCF, thrombopoietin (THPO), Fms-related tyrosine kinase 3 ligand (Flt3-L), and interleukin-6 (IL-6) (each 100 ng/ml; all from Peprotech, Hamburg, Germany, http://www.peprotech.com); Rvt, SCF, THPO, Flt3-L, IL-6 (each 100 ng/ml), and 10 μM resveratrol (Sigma-Aldrich, Taukirchen, Germany, http://www.sigma-aldrich.com); SR-1, SCF, THPO, Flt3-L, IL-6 (each 100 ng/ml), and 10 μM stemregenin-1 (Cellagen Technologies, San Diego, CA, http://www.cellagentechnologies.com); and STA13, SCF (50 ng/ml), THPO (10 ng/ml), Flt3-L (50 ng/ml), IGFBP2 (100 ng/ml; R&D Systems, Minneapolis, MN, http://www.rndsystems.com), Angptl5 (100 ng/ml; Abnova, Taipei, Taiwan, http://www.abnova.com; supplemental online Table 3).
CD34+ or their progenies were transplanted into sublethally (2.5 Gy) irradiated NOD/SCID/IL2rg−/− NSG recipient mice by i.v. injection. To analyze the recipient mice, both tibias and femurs per mouse were isolated, and the bone marrow was flushed from the bones, collected, and analyzed separately for each individual recipient. For flow cytometric analysis, approximately 2 × 10⁶ cells were used for surface marker staining. For secondary transplantation, one half of the isolated bone marrow from the individual primary recipients was pooled and transplanted in equal amounts (approximately 2–2.5 × 10² cells per mouse) into sublethally irradiated secondary recipient mice.

**Phenotypical Analysis**

The purity of CD34-enriched cells was analyzed by staining with anti-human CD34-allophycocyanin (APC; eBioscience, San Diego, CA, http://www.ebioscience.com). The cultivated cells were analyzed for stem cell markers with anti-human CD34-APC, anti-human CD133-PE (both eBioscience), and 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) for exclusion of dead cells. Human chimerism in the peripheral blood was analyzed via an APC-conjugated anti-human CD45 antibody (eBioscience) after red blood cell lysis. Approximately 10⁶ total bone marrow cells were collected for determining the human chimerism and multilineage engraftment. The cells were subsequently stained with anti-human CD45-BV570, anti-human CD34-phycocerythrin (PE), and anti-human CD19-peridinin chlorophyll protein complex. Dead cells were excluded with DAPI. Measurements were either done with a FACS-Calibur flow cytometer and analyzed with the CellQuestPro software or performed on a LSRII-SORP flow cytometer and analyzed with the FACS Diva software (all from BD Biosciences, Heidelberg, Germany, http://www.bdbiosciences.com), where appropriate.

**Statistical Analysis**

Statistical analyses were performed with GraphPad Prisms, version 5, software (GraphPad Software, Inc., La Jolla, CA, http://www.graphpad.com). For the primary data analysis, the one-way analysis of variance (ANOVA) as a global test was performed. If the global ANOVA test (95% confidence interval) showed a significant difference among the four groups, a pairwise and two-sided comparison of the selected groups was performed. Owing to the experimental characteristic of the present study, this was done with a nonadjusted p value (i.e., a 95% confidence interval).

**RESULTS**

**Resveratrol Expands CB-CD34+ Cell In Vitro**

As the first approach, we aimed to compare the growth behavior and phenotype of CB-CD34+ cells cultured in different media in vitro. For this in vitro screen, immunomagnetically enriched CD34+ cells were cultured in different serum-free media for 9 days, a similar culture time to that described by Zhang et al. (5–10 days) [14, 15]. The basic medium contained the cytokines SCF, THPO, FLT3L, and IL-6 (ctrl), which are known to induce proliferation of CB hematopoietic stem cells [31]. This medium is commonly used as a standard cytokine condition for ex vivo cultures of CB cells. For a detailed comparison of the in vitro effects of resveratrol on CB-HSC, we tested the new small molecule stemeugenin-1, discovered by Boitano et al. [17], which was added to the basic ctrl medium (SR-1). Additionally, we used the serum-free cytokine medium established by Zhang et al. [14, 15], including IGFBP2 and Angptl5, together with SCF, THPO, and FLT3L (STA13). Similarly to SR-1, we included resveratrol in the basic cytokine medium ctrl for our analysis (Rvt). The optimal dosage of resveratrol was determined at 10 μM based on an in vitro screen of Rvt with different concentrations of resveratrol (0 to 50 μM) and subsequent flow cytometry screening for the preservation of the CD34 phenotype (supplemental online Fig. 1). No differences were found in the total cell numbers after cultivation in the different cytokine combinations (Fig. 1A). The total fold expansion after 9 days (total cells relative to the initial cell number) was 24-fold ± 9 for ctrl, 26-fold ± 12 for STA13, 27-fold ± 10 for SR-1, and 27-fold ± 9 for Rvt. In order to determine the effect of the different cytokine combinations on the cell surface phenotype of HSCs, we analyzed the cells after cultivation for the expression of the known HSC markers CD34 and CD133 by flow cytometry, because these markers positively define the stem cell-containing population also after in vitro cultivation [32]. Although no significant differences in CD34 marker expression were observed between the groups, a trend was seen that cultivation with Rvt and SR-1 preserved CD34 surface expression (60% ± 16% and 64% ± 16%, respectively) compared with ctrl (49% ± 14%) and STA13 (50% ± 12%), respectively (Fig. 1B). In addition, the cultivation in medium containing Rvt or SR-1 led to a significantly higher percentage of CD34+/CD133+ expression (13% ± 2% for Rvt and 13% ± 2% for SR-1) compared with the two cytokine combinations ctrl and STA13 (8.9% ± 1.6% and 8.2% ± 2.3%, respectively; Fig. 1C). The analysis of the mean fluorescence intensity of CD133 surface expression in the total cell population or the CD34+/CD133+ population revealed no major differences in the cell surface expression of CD133 between the tested groups (supplemental online Fig. 2). A representative flow cytometry analysis is shown in Figure 1D. Correlating the total fold expansion rates and the percentage of CD34+/CD133+ cells in the respective cytokine media also supported a significant increase in phenotypically HSCs after in vitro culture of 3.5-fold ± 0.9 for Rvt and 2.8-fold ± 0.4 for SR-1 compared with ctrl (1.9-fold ± 0.5) and STA13 (1.6-fold ± 0.9; Fig. 1E). These data are summarized in supplemental online Table 1 and demonstrate the potential of Rvt to improve ex vivo culture condition of human CB-derived HSCs.

To elucidate the underlying mechanisms of enhanced HSC expansion by Rvt, we performed gene expression analysis of CD34+/CD133+ CB cells after ex vivo expansion (supplemental online Fig. 3; supplemental online data). Compared with ctrl, gene set enrichment analysis [33, 34] of ex vivo expanded CB cells further demonstrated enrichment of cell cycle-associated gene sets during Rvt cultivation by preserving the HSC-characteristic signature (supplemental online Fig. 4). In contrast, cell cycle- and HSC-associated gene sets were not enriched in CD34+/CD133+ cells during cultivation in the cytokine conditions STA13 or SR-1.

**Resveratrol Leads to Improved Engraftment of Ex Vivo Cultivated CB-HSCs into Primary and Secondary NSG Mice**

Next, we addressed the question whether Rvt also improved the ability of in vitro cultivated CB-HSC derived cells to engraft in NSG mice. Initially, we compared the effects of 9 days of cultivation and expansion in Rvt with the ctrl medium and STA13 on engraftment. Four sublethally irradiated NSG mice were transplanted with the progeny of initially 20,000 CD34+ cultivated cells per mouse. As a reference point, 4 mice were transplanted with 20,000 uncultured CD34+ CB cells per mouse from the same pool of CB cells also
After 9 months, the mice were sacrificed, and the peripheral blood and bone marrow were assessed for the contribution of human hematopoiesis. Noteworthy, the peripheral blood of the mice receiving freshly thawed cells (day 0 [d0]) displayed the highest human chimerism of all groups (55% ± 11%). As expected, the basic cytokine cocktail did not support CB-HSCs well during 9 days of in vitro culture (ctrl 14% ± 6%). In contrast, ex vivo expansion in STAI3 and Rvt improved the contribution of human blood cells to the peripheral blood (32% ± 24% and 19% ± 17%, respectively) after transplantation. In addition, in the bone marrow of the recipient mice, the highest human chimerism was achieved by the transplantation of noncultured fresh cells (62% ± 11%). In the bone marrow compartment, cells transplanted after cultivation in ctrl medium displayed the expected loss of repopulation property (25% ± 25%), which could be rescued at least partially by applying Rvt (57% ± 26%) or STAI3 (52% ± 25%) to the in vitro culture. Compared with the supportive HSC culture condition STAI3, Rvt showed a similar level of human engraftment in primary NSG mice.

To verify that the human engraftment in NSG mice was based on long-term HSCs and to support our findings of the positive effect of Rvt on the CB-HSC culture, we performed serial transplantations of the bone marrow of primary recipients into secondary recipient NSG mice. Four secondary NSG mice were transplanted with similar numbers (range 2–2.5 × 10^7) of all total BM cells pooled from all primary recipients of one group. The human chimerism of the pooled BM was analyzed before transplantation (Fig. 2E) and was highest in the Rvt and STAI3 transplanted groups (~66%–67%). In addition, it was lower in the pool that was transplanted with ctrl cultured cells (22%). After 9 months, the secondary recipient mice were sacrificed, and the contribution of human hematopoietic cells in the peripheral blood and bone marrow of the secondary
recipients was determined (Fig. 2C, 2D). The peripheral blood and bone marrow chimerism in mice that had received Rvt cultured cells was 1.4% ± 0.3% (blood) and 13% ± 15% (BM). In contrast, the cultivation of CB-HSCs in the basic cytokine cocktail led to a nearly complete loss of repopulation in secondary NSG mice (0.04% ± 0.04% [blood] and 0.9% ± 1.3% [BM]). These data clearly demonstrate the beneficial effect of the Rvt culture conditions to preserve HSC potential in vitro. The culture of HSCs in STAI3 conditions also failed to efficiently engraft secondary recipients in this experiment, because the peripheral blood chimerism was 0.6% ± 0.7% and the bone marrow chimerism only 0.03% ± 0.05%, although the primary recipients had a chimerism similarly as high as that in the Rvt mice.

Transplantation of Different Cell Doses to Minimize Variations in Chimerism

The chimerism within each group displayed a high variation. This was not only observed in the primary recipients, but could also be detected in the secondary recipient mice. We therefore performed a second experiment with the same settings with another pool of CB-derived CD34+ cells to validate the potential use of Rvt for enhanced in vitro culture of CB-HSCs and the reproducibility of the CB expansion. In this experimental setup, we also included a group of mice that were transplanted with cells cultured with SR-1. Although this experiment used the same amount of highly enriched CB-derived CD34+ cells as the initial experiment, the mean chimerism value in the peripheral blood (Fig. 3A) was only 23% ± 13% in the d0 group. Because the mice already transplanted with the d0 cells displayed a reduction of chimerism, the ex vivo cultivated cells were also engrafted with reduced chimerism, as measured in the peripheral blood (Fig. 3A). This observation was also reflected in the bone marrow (Fig. 3B), in which the human chimerism was 19% ± 10% for d0, 14% ± 14% for ctrl, 5.6% ± 7.5% for STA13, 13% ± 14% for Rvt, and 18% ± 17% for SR-1.

To reduce the variation in the engraftment within each group, in the next experiment we used 100,000 CD34+ starting cells per mouse (i.e., fivefold more initial input cells under the same experimental settings as described above). As observed previously, the corresponding d0 group receiving cells without in vitro culture displayed the highest chimerism value in the peripheral blood.
More importantly, compared with the ctrl medium, the Rvt and STAI3 conditions improved the human contribution in the peripheral blood; however, this was not entirely reflected within the bone marrow (Fig. 3D).

Combined Analysis of Serial Transplantation Experiments Revealed Supportive Effects of Resveratrol on CB Expansion

In order to compare all the experiments, we calculated the relative human engraftment by normalizing each value within each experiment to the value of the mouse with the highest chimerism in the ctrl group of the same experiment (Fig. 4A, the peripheral blood; Fig. 4B, the BM). The peripheral blood chimerism of the primary recipients was improved after cultivation in the conditions Rvt and STAI3 compared with the cultivation under ctrl conditions. By applying the same normalization for the analysis of the secondary recipients, we also observed that the uncultivated cells resulted in a robust engraftment level of human cells in the peripheral blood (Fig. 4C) and bone marrow (Fig. 4D). As expected, cultivation of CD34+ cells with the ctrl medium condition led to a strong reduction in the number of SCID repopulating cells. However, the implementation of the Rvt and STAI3 cytokine conditions enhanced the engraftment levels, not only for the BM compartment, but also for the level of human cells in the circulation after transplantation. Taken together, 56% of mice secondarily transplanted with cells cultivated in ctrl medium showed human engraftment (5 of 9 recipient mice; defined as ≥0.1% human CD45+ cells in the BM compartment). In contrast, 67% of secondary mice engrafted with Rvt and STAI3 (6 of 9 recipient mice) cultivated cells (supplemental online Table 2).

To further address the question of whether the engraftment of the cultivated cells also led to a multilineage repopulation of the hematopoietic system, we analyzed the presence of CD19 (B-cells) and CD33 (myeloid)-positive cells within the human donor-derived compartment. These data demonstrate a robust multilineage engraftment shown for all cultivation conditions in primary (Fig. 5A) and secondary (Fig. 5B) recipient mice. In particular, resveratrol was able to provide sustained human engraftment in primary and secondary recipients, thereby leading to human-derived multilineage engraftment.

**DISCUSSION**

We tested resveratrol for its ability to improve in vitro cultivation of CB-derived HSCs. Resveratrol is a naturally occurring polyphenol whose activity is linked to several signaling cascades involved in cell fate determination. Resveratrol has been shown to induce apoptosis in leukemic cells, rescue hematopoietic defects in mice, increase the HSC numbers of mice in vivo, and inhibit...
differentiation of murine LSK (lineage-negative, c-Kit+, Sca-1+) cells in vitro [28, 35–38]. Furthermore, we tested and compared two recently described cytokine conditions, one including IGFBP2/Angptl5 and the other using the small molecule stemregenin-1, which had been described to enhance in vitro expansion of human CB-HSCs. In control cultures, we used a standard cytokine condition as the control, which is not only widely used, but has also been shown to lead to stem cell differentiation [14, 15, 17, 31]. This side-by-side comparison was intended to identify the potential of resveratrol to enhance in vitro cultures and to analyze the reproducibility of published culture conditions.

In our study, a higher preservation of the more stringent CD34+/CD133+ stem cell phenotype after cultivation with Rvt and SR-1 was detected. In contrast, no major differences in the preservation of the stem cell marker CD34+ could be observed. Based on the total fold expansion of the HSC-containing

Figure 4. Combined data for primary and secondary recipients after normalization. For a better comparison of the in vivo data from the different experiments, the chimerism values of all experiments were normalized and integrated into a single graph as relative engraftment levels. For normalization within each independent experiment, the value of the mouse with the highest chimerism of d0 group was artificially set to 1. All other values were then normalized relative to that value. (A): Peripheral blood chimerism of primary mice. (B): Bone marrow chimerism of primary mice. (C): Peripheral blood of secondary mice. (D): Bone marrow chimerism of secondary mice. Abbreviations: BM, bone marrow; ctrl, control medium containing SCF, THPO, Flt3-L, IL-6; Flt3-L, Fms-related tyrosine kinase 3 ligand; d0, day 0; h, human; IL, interleukin; neg, negative; PB, peripheral blood; rel., relative; Rvt, medium containing SCF, THPO, Flt3-L, IL-6 plus resveratrol; SCF, stem cell factor; STA13, medium containing SCF, THPO, Flt3-L, insulin-like growth factor-binding protein 2, angiopoietin-like protein; THPO, thrombopoietin.

Figure 5. Each cytokine condition displayed multilineage engraftment in primary and secondary recipient NOD/SCID/IL2rg−/− (NSG) mice. Multilineage engraftment of primary (A) and secondary (B) NSG mice was assessed based on staining of ∼10^6 total bone marrow cells for the presence of human CD19 (lymphoid; white bars) and human CD33 (myeloid; black bars) surface marking on bone marrow cells within the human CD45-positive cell compartment. Combined analysis of all primary and secondary animals transplanted during the present study. Abbreviations: BM, bone marrow; ctrl, control medium containing SCF, THPO, Flt3-L, IL-6; Flt3-L, Fms-related tyrosine kinase 3 ligand; d0, day 0; h, human; IL, interleukin; Rvt, medium containing SCF, THPO, Flt3-L, IL-6 plus resveratrol; SCF, stem cell factor; STA13, medium containing SCF, THPO, Flt3-L, insulin-like growth factor-binding protein 2, angiopoietin-like protein; THPO, thrombopoietin.
compartment, Rvt showed the highest in vitro expansion of CD34+/CD133+ double-positive stem cells compared with the standard condition and also to the established stem cell expansion conditions STA3 and SR-1. Our study, therefore, demonstrated that resveratrol not only inhibited the in vitro differentiation of murine LSK cells, as previously reported [37], but, more importantly, also of human CB-derived stem cells, thereby enabling a robust amplification of CD34+/CD133+ stem cells in vitro.

We further confirmed the beneficial effects of Rvt by serial transplantation, the most stringent assay for the analysis of the HSC potential [39]. We showed that Rvt preserved HSC function most efficiently. Furthermore, Rvt cultivated cells led to multilineage engraftment in primary and secondary recipients, similar to the other tested and already established conditions, SR-1 and STA3, demonstrating the feasibility of resveratrol in improving HSC cultures.

The high variability of the chimerism values within the groups, although transplanted with equal amounts of cells originating from the same pool of cells, was problematic. Our attempt to increase the input cell numbers by fivefold did not affect the variances observed for the different groups. One possible explanation might be the very low numbers of CB HSCs [40] in the ex vivo starting cultures and their potentially highly variable proliferation and self-renewal capacities, described as “stem cell heterogeneity” [41, 42]. Based on studies by John Dick’s laboratory and more recent analysis using barcoded vectors by Connie Eaves’ group, estimates show that only 0.02% of CD34+ cells are long-term repopulating HSCs [40, 43]. Furthermore, we observed substantial variations between the different experiments performed in our study. These differences were also obvious in the different nonmanipulated starting populations (d0 values). This has also been observed in a previous study [44]. These observations might be caused by intrinsic differences in the HSC subtypes, such as were shown for murine HSCs [45], which respond differentially to specific external stimuli. This effect might be even more pronounced in the case of CB donations from different individuals (i.e., from a genetically heterogeneous background).

Several molecular functions have been described for resveratrol, including reducing oxidative stress, influencing the cell cycle, and binding of receptors present on HSCs [24, 30, 46, 47], which ultimately influence stem cell behavior and are therefore of interest for deciphering the role of resveratrol in HSC culture. Gene expression analysis performed in our study linked the HSC potential [39]. We showed that Rvt preserved HSC function more efficiently. Furthermore, Rvt cultivated cells led to multilineage expansion by Rvt to an enhanced cell cycle status on the one side by preserving the HSC phenotype on the other side. However, the mechanistic action of resveratrol and a precise quantification of HSC numbers in the context of HSC ex vivo cultures should be addressed in future studies, which will help to understand the biological prerequisite for optimal ex vivo culture conditions.

Optimization of culture conditions are also of importance for gene therapeutic applications. Efficient targeted genome editing and homologous recombination relies on cell cycle transition (i.e., ex vivo culturing and modifying HSCs without compromising repopulation potential). Recently, the group of Luigi Naldini demonstrated the practical significance of optimizing culture conditions to enhance gene transfer and gene editing in human HSCs [48].

**CONCLUSION**

We conclude that resveratrol is a promising addition to optimize ex vivo culture protocols for human HSCs. The use of resveratrol enabled robust in vitro expansion of human HSCs comparable to other established conditions, including stemregenin-1 and AngptL5, which in our study also improved the outcome of ex vivo cultures.

**ACKNOWLEDGMENTS**

We thank Rena-Mareike Struss for help with animal work; Thomas Neumann for help with microarray work; Rolf Baumann, Hans Grundke, Jörg Frühauf, and Bernd Polivka (Radiotherapy, Hannover Medical School) for irradiation of animals; Ralf Hass and Marion Haidukiewicz for human CB samples; C.C. Zhang for helpful discussions; Christopher Baum for helpful support and suggestions; Kay-Martin Hanschmann (Department of Biostatistics, Paul-Ehrlich-Institute) for statistical advice; and Constance Taylor for critical reading of the manuscript. This study was supported by grants from the Federal Ministry of Education and Research (BMBF) within the supporting program “Cell-Based Regenerative Medicine” (CB-HERMES: Grant 01 GN 0930) and the Deutsche Forschungsgemeinschaft-funded REBIRTH Cluster of Excellence and SF738. N.H. received a “Hochschulinterne Leistungsförderung” grant from Hannover Medical School.

**AUTHOR CONTRIBUTIONS**

N.H. and B.S.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; B.E.: collection and assembly of data, data analysis and interpretation; A. Schwarzer: data analysis and interpretation; A. Schambach: data analysis and assembly of data; U.M.: data analysis and interpretation, manuscript writing, final approval of manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

B.S. has uncompensated stock options. The other authors indicated no potential conflicts of interest.

**REFERENCES**

1. Rocha V, Wagner JE Jr., Sobocinski KA et al. Graft-versus-host disease in children who have received a cord-blood or bone marrow transplant from an HLA-identical sibling. Eurocord and International Bone Marrow Transplant Registry Working Committee on Alternative Donor and Stem Cell Sources. N Engl J Med 2000;342:1846–1854.

2. Rocha V, Gluckman E. Clinical use of umbilical cord blood hematopoietic stem cells. Biol Blood Marrow Transplant 2006;12(suppl 1):34–41.

3. Rubinstein P, Carrier C, Scaradavou A et al. Outcomes among S62 recipients of placental-bone blood transplants from unrelated donors. N Engl J Med 1998;339:1565–1577.

4. Garderet L, Dulphy N, Douay C et al. Polyclonal and naive but completely formed repertoire: Characteristics of a polyclonal and naive but completely formed repertoire. Blood 1998;91:340–346.

5. MacMillan ML, Weisdorf DJ, Brunstein CG et al. Acute graft-versus-host disease after unrelated donor umbilical cord blood transplantation: Analysis of risk factors. Blood 2009;113:2410–2415.

6. Delaney C, Heimfeld S, Brashem-Stein C et al. Notch-mediated expansion of human cord blood progenitor cells capable of rapid myeloid reconstitution. Nat Med 2010;16:232–236.
7 de Lima M, McNiece I, Robinson SN et al. Cord-blood engraftment with ex vivo mesenchymal-cell coculture. N Engl J Med 2012;367:2305–2315.
8 Wilson A, Laurenti E, Oser G et al. Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. Cell 2008;135:1118–1129.
9 Mihrarada K, Sigurdsson V, Karlsson S. Dppa5 improves hematopoietic stem cell activity by reducing endoplasmic reticulum stress. Cell Reports 2014;7:1381–1392.
10 Chen Y, Ma X, Zhang M et al. Gadd45a regulates hematopoietic stem cell stress responses in mice. Blood 2014;123:851–862.
11 van Galen P, Kreso A, Mbone N et al. The unfolded protein response governs integrity of the haematopoietic stem-cell pool during stress. Nature 2014;510:268–272.
12 Ludin A, Gur-Cohen S, Golani K et al. Reactive oxygen species regulate hematopoietic stem cell self-renewal, migration and development, as well as their bone marrow microenvironment. Antioxid Redox Signal 2014;21:1605–1619.
13 Vannini N, Roch A, Naveiras O et al. Identification of in vitro HSC fate regulators by differential lidl raft clustering. Cell Cycle 2012;11:1535–1543.
14 Zhang CC, Kaba M, Ge G et al. Angiopoietin-like proteins stimulate ex vivo expansion of hematopoietic stem cells. Nat Med 2006;12:240–245.
15 Zhang CC, Kaba M, Iizuka S et al. Angiopoietin-like 5 and IGFBP2 stimulate ex vivo expansion of human cord blood hematopoietic stem cells as assayed by NOD/SCID transplantation. Blood 2008;111:3419–3423.
16 Himburg HA, Muramoto GG, Daher P et al. Pleiotrophin regulates the expansion and regeneration of hematopoietic stem cells. Nat Med 2010;16:475–482.
17 Boitano AE, Wang J, Romeo R et al. Aryl hydrocarbon receptor antagonists promote the expansion of human hematopoietic stem cells. Science 2010;329:1345–1348.
18 Huynh H, Iizuka S, Kaba M et al. Insulin-like growth factor-binding protein 2 secreted by a tumorigenic cell line supports ex vivo expansion of mouse hematopoietic stem cells. Stem Cells 2008;26:1628–1635.
19 Fares I, Chagraoui J, Gareau Y et al. Cord blood expansion: Pyrimidoindole derivatives are agonists of human hematopoietic stem cell self-renewal. Science 2014;345:1509–1512.
20 Huynh H, Zheng J, Umikawa M et al. IGF binding protein 2 supports the survival and cycling of hematopoietic stem cells. Blood 2011;118:3236–3243.
21 Zheng J, Huynh H, Umikawa M et al. Angiopoietin-like protein 3 supports the activity of hematopoietic stem cells in the bone marrow niche. Blood 2011;117:470–479.
22 Singh KP, Casado FL, Opanashuk LA et al. The aryl hydrocarbon receptor has a normal function in the regulation of hematopoietic and other stem/progenitor cell populations. Biochem Pharmacol 2009;77:577–587.
23 Baur JA, Sinclair DA. Therapeutic potential of resveratrol: The in vivo evidence. Nat Rev Drug Discov 2006;5:495–506.
24 Lin HY, Lansing L, Merillon JM et al. Integrin alphaVbeta3 contains a receptor site for resveratrol. FASEB J 2006;20:1742–1744.
25 Umemoto T, Yamato M, Ishihara J et al. Integrin αvβ3 regulates thrombopoietin-mediated maintenance of hematopoietic stem cells. Blood 2012;119:83–94.
26 Casper RF, Quesne M, Rogers JM et al. Resveratrol has antagonist activity on the aryl hydrocarbon receptor: Implications for prevention of dioxin toxicity. Mol Pharmacol 1999;56:784–790.
27 Zhang QS, Marquez-Loza L, Eaton L et al. Fancd2/- mice have hematopoietic defects that can be partially corrected by resveratrol. Blood 2010;116:5140–5148.
28 Rimmelé P, Lofek-Czubek S, Ghaffari S. Resveratrol increases the bone marrow hematopoietic stem cell and progenitor cell capacity. Am J Hematol 2014;89:E235–E238.
29 Yun JM, Chien A, Jialal I et al. Resveratrol up-regulates SIRT1 and inhibits cellular oxidative stress in the diabetic milieu: Mechanistic insights. J Nutr Biochem 2012;23:699–705.
30 Touzet O, Philips A. Resveratrol protects against protease inhibitor-induced reactive oxygen species production, reticulum stress and lipid raft perturbation. AIDS 2010;24:1437–1447.
31 Murray LJ, Young JC, Osborne LJ et al. Thrombopoietin, ft3, and kit ligands together suppress apoptosis of human mobilized CD34+ cells and recruit primitive CD34+ Thy-1+ cells into rapid division. Exp Hematol 1999;27:1019–1028.
32 Drake AC, Khoury M, Leskov I et al. Human CD34+ CD133+ hematopoietic stem cells cultured with growth factors including Angptl5 efficiently engraft adult NOD-SCID IL2rγc-/- (NSG) mice. PLoS One 2011;6:e18382.
33 Mootha VK, Lindgren CM, Eriksson KF et al. PGC1-alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nat Genet 2003;34:267–273.
34 Subramanian A, Tamayo P, Motha VK et al. Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci USA 2005;102:15545–15550.
35 Gatouillat G, Balasse E, Joseph-Pietras D et al. Resveratrol induces cell-cycle disruption and apoptosis in chemoresistant B16 melanoma. J Cell Biochem 2010;110:893–902.
36 Bai Y, Mao QQ, Qin J et al. Resveratrol induces apoptosis and cell cycle arrest of human T24 bladder cancer cells in vitro and inhibits tumor growth in vivo. Cancer Sci 2010;101:488–493.
37 Matsui K, Eozo S, Oritani K et al. NAD-dependent histone deacetylase, SIRT1, plays essential roles in the maintenance of hematopoietic stem cells. Biochem Biophys Res Commun 2012;418:811–817.
38 Gautam SC, Xu X, Dumaguin M et al. Resveratrol selectively inhibits leukemia cells: A prospective agent for ex vivo bone marrow purging. Bone Marrow Transplant 2000;25:639–645.
39 Purton LE, Scadden DT. Limiting factors in murine hematopoietic stem cell assays. Cell Stem Cell 2007;1:263–270.
40 Bhatia M, Wang JCY, Kapp U et al. Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. Proc Natl Acad Sci USA 1997;94:5320–5325.
41 Muller-Sieburg CE, Sieburg HB, Bernitz JM et al. Stem cell heterogeneity: Implications for aging and regenerative medicine. Blood 2012;119:3900–3907.
42 McKenzie JL, Gan OI, Doedens M et al. Individual stem cells with highly variable proliferative and self-renewal properties comprise the human hematopoietic stem cell compartment. Nat Immunol 2006;7:1225–1233.
43 Cheung AM, Nguyen LV, Carles A et al. Analysis of the clonal growth and differentiation dynamics of primitive barcoded human cord blood cells in NSG mice. Blood 2013;122:3129–3137.
44 Blank U, Ehrnström B, Heinz N et al. Angptl4 maintains in vivo repopulation capacity of CD34+ human cord blood cells. Eur J Haematol 2012;89:198–205.
45 Challen GA, Boles NC, Chambers SM et al. Distinct hematopoietic stem cell subtypes are differentially regulated by TGF-β1. Cell Stem Cell 2010;6:265–278.
46 Li YG, Zhu W, Tao JP et al. Resveratrol protects cardiomyocytes from oxidative stress through SIRT1 and mitochondrial biogenesis signaling pathways. Biochem Biophys Res Commun 2013;438:270–276.
47 Hisleh T, Halicka D, Lu X et al. Effects of resveratrol on the G(0)-G(1) transition and cell cycle progression of mitochondrially stimulated human lymphocytes. Biochem Biophys Res Commun 2002;297:1311–1317.
48 Genovese P, Schirol G, Escobar G et al. Targeted genome editing in human repopulating haematopoietic stem cells. Nature 2014;510:235–240.