***Ex vivo* expansion of cord blood progenitors impairs their short-term and long-term repopulating activity associated with transcriptional dysregulation of signalling networks**

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Abstract

Objectives: Cord blood (CB) has been established to be an alternative source of haematopoietic stem/progenitor cells (HPC) for transplantation. The number of HPC per CB unit is limited, which results in engraftment delay. *Ex vivo* expansion of HPC improvement must overcome this.

Materials and methods: Flow cytometry was used to extensively phenotype HPC pre- and post-expansion and CFDA-SE staining was used to track cell divisions. The NSG mouse model was employed in transplantation studies to determine long and short term repopulation in human cells. Gene array analysis was used to evaluate signalling pathways regulated following *ex vivo* expansion of HPC.

Results: expansion of CD34⁺ HPC impaired their regenerative function. In this xenograft transplantation model we showed that repopulating activity of CB cells declined following expansion. Expanded HPC had delayed engraftment at early and late stages post-transplant. High resolution division tracking revealed that the cultured HPC had reduced expansion and self-renewal probability and increased differentiation rate compared to non-expanded cells. Gene expression analysis exposed significant modulation of a complex network of

genes and pathways that normally maintain HPC proliferation and limit their differentiation.

Conclusions: The decline in short-term engraftment is consistent with the loss of rapid SCID repopulating ability r(SRA) by expanded CD34⁺CD38⁺ cells recently reported (1). Our data raise concerns for future clinical applications of expanded HPC alone in transplantation.

Introduction

Umbilical cord blood (CB) is an increasingly utilized source of stem cells for allogeneic stem cell transplantation (SCT), due to its ready availability for recipients who cannot be identified to other HLA matched marrow or mobilized peripheral blood (PB) donors. Recent growth in CB transplantation methods has been promoted by the availability of these cells in CB banks, as well as by strong clinical data supporting use of HLA mismatched transplants with low risk of graft versus host disease [see (2) for review]. Wide utilization of CB, however, is limited by the relatively low number of stem cells per unit with most CB units insufficient for transplantation into an adult; thus, significant effort has been made to develop technologies for *ex vivo* expansion of CB stem cells to enable transplantation for adults, who indeed, comprise the majority of patients in need of SCT (3,4). Until recently however, no definitive evidence existed that current expansion approaches would improve performance of CB transplantation. In 2002, Shpall *et al.* (5) reported the first safe transplantation of *in vitro* expanded CD34⁺ cells using stem cell factor (SCF),

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thrombopoietin (TPO) and granulocyte-colony stimulating factor (G-CSF) in their expansion cocktail. Expanded and un-manipulated CB fractions derived from one CB unit were administered together, demonstrating that the approach was principally feasible, although faster haematopoietic recovery was not observed. Many factors may promote *ex vivo* expansion of haematopoietic stem cells (HSC) and a number of laboratories has attempted to systematically optimize combination of cytokines and other factors to increase the extent of HSC expansion (6). A clinical scale technique for massive expansion of haematopoietic progenitors without impairing activities of primitive and very primitive stem cells has previously been published (7). Recently, Delaney *et al.* demonstrated in a small clinical trial, that patients transplanted with dual CB units – one unexpanded and another *ex vivo* expanded, engrafted after a median of 16 days following transplantation, compared to 26 days in patients with two non-manipulated CB units (8). An immobilized form of Notch ligand together with cytokines was used in this trial to stimulate expansion of HPC.

Here we show that *ex vivo* expansion of CB-derived CD34⁺ HPC impaired their regenerative function. Rate of short-term engraftment in PB, T-cell generation and multi-lineage reconstitution of BM in mouse models was lower despite transplantation of expanded numbers of HPC. High resolution division tracking revealed that CD34⁺CD38⁺ HPC, that we would expect to promote short-term engraftment in the expanded product, had reduced rates of expansion and self-renewal probability and increased differentiation rate, compared to non-expanded cells. Gene expression analysis showed complex modulation of multiple genes and signal cascades, consistent with reduced self-renewal and increased differentiation rate in *ex vivo* expanded CD34⁺ HPC. In addition, decline in short-term engraftment in mice transplanted with expanded cells was associated with reduction in numbers of CD34⁺CD38[−] cells with short- and long-term SCID repopulating ability (SRA). Our data raise concerns for future clinical applications of *ex vivo* expanded HPC alone in transplantation.

Materials and methods

Cell culture

Umbilical CB was provided by Sydney Cord Blood Bank and all experiments were approved by the Sydney Children's Hospital Human Research Ethics Committee. Mononuclear cells were purified from CB by Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO, USA) density-gradient centrifugation. CD34⁺ cells were purified by positive-selection using a magnetic cell sorting system (autoMACS Miltenyi Biotec, Auburn, CA, USA) and cultured in a pre-established suspension cocktail (9).

To span 10 days of continuous division tracking, CD34⁺ cells were re-isolated and stained with CFDA-SE on day 6. For the 1st week of division tracking, CD34⁺ cell fractions were cultured with cytokines for 5 days and analysed every 24 h. To perform the 2nd week of division tracking, suspension culture cells from the first 5 days of culture were re-stained with CFDA-SE, CD34⁺ cells were purified by MACS, followed by daily cytometric analysis for the next 5 days (days 6–10). Alternatively, co-cultured cells from the first week were re-isolated and tracked in suspension or co-culture for days 6–10.

Flow cytometry

Phenotypic analysis of unexpanded and *ex vivo* expanded CB cells was performed using flow cytometry. Cells were blocked and stained with monoclonal antibodies against a variety of haematopoietic markers (Table 1) (Becton Dickinson; San Jose, CA, USA). 1×10^5 events were acquired on a FACS Canto, and data analysed with FACSDiva software (Becton Dickinson). Viable cells were gated based on forward versus side scatter (FSC/SSC).

High resolution division tracking of CD34⁺ cells stained with CFDA-SE (Invitrogen, Mulgrave, Victoria, Australia) was performed as previously described (10). Interpretation of time series multi-type division tracking data was conducted as previously described (10). Population statistics were defined to characterise precursor cell frequency, self-renewal, apoptosis and differentia-

Table 1. Phenotype of unexpanded and expanded CB CD34⁺ cells. This table shows the % expression, as determined by flow cytometry, of a number of hematopoietic surface markers on unexpanded CD34⁺ cells and these cells after short-term expansion in culture with hematopoietic cytokines ($n = 5$)

Sample	CD38 [−]	CXCR4	VLA4	CD14	CD61	CD56	CD4	CD25	CD19	CD117	CD90	CD133	CD123	CD13
Unexpanded	25.7	3.2	87.2	5.1	3.6	0.25	2.4	2.9	4.1	6.5	10.3	75.9	1.7	2.2
Expanded	7.4	1.2	89.5	20.4	3.8	0.8	1.2	3.1	1.2	3	70.9	39.2	12.2	7.6
P-value	0.000005	0.0159	0.7652	0.0062	0.8878	0.0389	0.0718	0.8915	0.005	0.0468	0.0026	0.0036	0.0735	0.0002

tion. Numbers of cells at the start of culture that gave rise to CD34⁺ and CD34⁻ cells at each time point, was estimated and used to indirectly estimate precursor cell frequency and number of cells that had died.

Bone marrow transplantation model

Approval from the UNSW Animal Care and Ethics Committee (ACEC) was obtained for animal experiments. NOD-SCID Il2rg^{-/-} (NSG) mice were obtained from the Jackson Laboratory and bred in the animal facility at the Children's Cancer Institute Australia for Medical Research. Mice 6–8 weeks of age were irradiated with a sub-lethal dose of 2.5 Gy from a cobalt-60 source, 12 h before being transplanted with CB cells by intravenous (IV) injection. Unexpanded or expanded CB CD34⁺ cells were administered to all mice at cell dose equivalent to 1×10^5 unexpanded cells. Engraftment kinetics were analysed by detection of human cells in PB collected from the tail vein, at a number of different time points. Human and murine cells were distinguished by the species-specific expression of CD45 by flow cytometry. Proportions of cells labelled with human CD45 (hCD45) only, were taken as the level of engraftment. Mice were sacrificed at 20 weeks after transplantation and BM was harvested and assessed for human cell engraftment using the same analysis parameters that were employed to evaluate PB engraftment. Cells from BM were also analysed by flow cytometry to assess the contribution of different human haematopoietic cell lineages. Antibodies specific to human CD34, CD33, CD41a, CD19 and CD3, 4 and 8 were used to examine multi-lineage reconstitution of primitive cells, myeloid cells, platelets, B cells and T cells respectively in BM.

RNA extraction and real time-PCR

Total RNA was prepared using the RNeasy Micro Kit (Qiagen, Doncaster, Victoria, Australia). PCR was performed using standard techniques (Applied Bio systems, Mulgrave, Victoria, Australia). Template cDNA was synthesized using SuperScriptIII First-Strand Synthesis System for reverse transcription-PCR (RT-PCR) (Invitrogen). PCR mixtures (25 µl per reaction) contained cDNA, 0.2 µmol/l each of forward and reverse primers, and 12.5 µl iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). Reactions were performed in triplicate in 96-well plates using the iCycler iQ Real-Time PCR Detection System version 4.006 (Bio-Rad), and were analysed using software from the manufacturer. Amounts of transcript were determined based on a standard curve specific for each gene, and were normalized to the amount of β 2-microglobulin transcript in the same sample.

Expression analysis

Gene expression analysis was performed using Illumina array technology; pooled CD34⁺ cells from 4 CB samples were used in these experiments. Sample paired analysis was performed for RNA isolated from non-manipulated CD34⁺ cells or from CD34⁺ cells that had been expanded for 6 days. CD34⁺ cells were re-isolated following a 6-day expansion period. Preparation of total RNA, cRNA, hybridization, raw data analysis and data statistics were performed using BeadStation 500× system protocols and Beadstudio software according to the manufacturer's instructions. Sentrix Human-6 Expression BeadChips containing 25 000 human genes were used in our experiments. Genes with ≥ 1 -fold ratio (log) change were filtered for further analysis and validation. Raw data and normalized hybridization data are available from the gene expression omnibus (GEO) database, under accession number GSE21073. GEO analysis using the DAVID 2.1 tool was conducted to identify functional classes specifically associated with differentially expressed genes (11).

Validation of differential expression was conducted using real-time PCR and flow cytometry.

Statistical analysis

Results are expressed as mean $\pm$ SD. Differences between groups were examined for statistical significance using Student's *t*-test.

Results

Characterisation of ex vivo expanded HPC

CD34⁺CD38⁺ cells from fresh CB were shown to retain rapid SRA (rSRA), while only CD34⁺CD38⁻ cells retained long-term SRA (LT-SRA) (1). In addition, *ex vivo* expansion was shown to reduce rSRA of CD34⁺CD38⁺ cells, so that both rSRA and LT-SRA was retained only in the CD34⁺CD38⁻ cell subset, following expansion (1). Around 60–70% freshly isolated CD34⁺ cells co-expressed CD38 on their cell surfaces and that percentage went up following expansion of CD34⁺ cells, with only a small proportion (as low as 2.2%) of HPC negative for CD38 (Fig. 1a). As well as rapid decline in the proportion of CD38⁻ cells, there was a concomitant fall in their numbers following expansion (Fig. 1c). *Ex vivo* expanded CD34⁺ cells also exhibited reduced proportion of phenotypically primitive CD34⁺CD133⁺ and CD34⁺CD19⁺ cells, and increased proportions of CD34⁺ cells co-expressing myelomonocytic markers CD13, CD14 and CD123 (Table 1). Accordingly the most primitive HPC were marginally

Figure 1. Multi-colour flow cytometry to determine phenotype of CD34⁺ cells before and after *ex vivo* expansion. (a) representative dot plot showing expression of CD38 and CD34 expression on CD34-purified population of unexpanded CB cells. Day 0 37% of CD34⁺ cells are CD38⁺. (b) using the same surface staining as in A, cells expanded for 6 days evaluated by flow cytometry. There is a clear shift up and to the left, indicating loss of CD34 antigen and increased CD38 positivity, with only 2% of cells CD34⁺CD38⁺. (c) Over 6 days in expansion culture, CD34⁺CD38⁺ cell numbers declined steadily leaving only a small subset in the graft.

expanded, or in some cases their numbers were reduced, following 5–6 day culture (data not shown).

Reduction in proportions of CD34⁺ HPC co-expressing CXCR4 (important for cell homing) was seen, following *ex vivo* expansion (Table 1). In contrast, expression of integrin VLA4 also shown to determine stem cell homing, was higher in *ex vivo* expanded CD34⁺ cells (Table 1).

Collectively these results indicate that *ex vivo* expansion of CD34⁺ cells lead to significant expansion of differentiating, but not of primitive, subsets.

HPC retaining CD34⁺ phenotype after expansion had less rSRA and LT-SRA than non-expanded CD34⁺ cells

Mice were transplanted with 1×10^5 non-expanded or expanded equivalents of 10^5 non-expanded CD34⁺ cells. Stem cell engraftment in NSG mice permits evaluation of rSRA in PB and later, LT-SRA in BM and spleen and T cell regeneration in PB (12). Short-term engraftment has previously been defined by the presence of human CD45⁺ haematopoietic cells within PB at 1–7 weeks post-transplant while long-term engraftment is characterized by capacity to establish multi-lineage haematopoietic development in bone marrow and spleen at 12–19 weeks post-transplant, and by presence of human T cells in PB and thymus (12).

Human CD45⁺ (hCD45) cells were detectable in 100% of mice transplanted with unexpanded HPC while only 60% of mice transplanted with expanded cells were hCD45⁺ (>0.5%) in PB by week 2, suggesting reduced numbers of cells with rSRA in the expanded graft (Fig. 2a). At later time points, hCD45⁺ cells were detectable in all mice, however, short-term engraftment in PB (week 3–7) was significantly lower in mice transplanted with the expanded graft (Fig. 2b). Consistent with previously reported data, hCD45⁺ cells identified in PB at this stage were mostly CD19⁺ (Fig. S1a), with

few CD33⁺ myeloid cells present (Fig. S1a). Human CD41a⁺ platelets could be identified in PB starting from week 5 (Fig. 2c), but with poorer kinetics of PB engraftment of this subset seen in the expanded group compared to unexpanded (Fig. 2c).

Similar to published data, T-cells were detected in PB at week 9 following transplantation, and again, poorer kinetics of PB engraftment with CD3⁺ T-cells were seen, from expanded HPC, compared to non-expanded cells (Fig. 2d). CD4⁺ and CD8⁺ T cells were identified in PB at week 20 demonstrating successful differentiation, and proportions of T-cell subsets following transplantation were not affected by expansion (data not shown). In addition, proportions of CD34⁺ progenitor cells were similar in both groups (Fig. S1b). Human CD45 engraftment in the BM was analysed 20 weeks post-transplant. There was a significantly lower proportion of hCD45 in BM of mice transplanted with expanded cells compared to the unexpanded group (Fig. 2e). Both groups provided successful lineage differentiation with no difference between percentages of CD19 and CD33 progenitors in the BM (Fig. 2f,g and Fig. S2a). The percentage of primitive CD34⁺ progenitor cells in BM was similar in both groups (Fig. 2h and Fig. S2b). Collectively, analysis of *ex vivo* expanded HPC in this model revealed that despite increased numbers of CD34⁺ HPC in the expanded graft, we did not observe improved engraftment, and in fact, this was inferior to its non-expanded counterpart, including both short-term and long-term reconstitution. It appears that delayed PB engraftment was due to inferior BM reconstitution in the *ex vivo* expanded group.

Ex vivo expanded CD34⁺ cells were characterized by reduced proliferation levels, probability of self-renewal and increased differentiation

To analyse potential mechanisms that determined reduced regenerative performance of HPC following

Figure 2. Unexpanded cells showing superior kinetics of engraftment compared to *ex vivo* expanded cells. CB CD34⁺ cells expanded for 6 days then transplanted into irradiated NSG mice. (a) 2 weeks after transplantation, human cells could be detected in the peripheral blood of the mice. There was significant increase in proportion of human CD45⁺ cells in mice transplanted with unexpanded cells compared to expanded. Furthermore, all mice in the unexpanded group had hCD45⁺ levels over the 0.5% threshold for PB engraftment, while only 60% of mice in the expanded group reached above this level. (b) mice that received unexpanded CD34⁺ graft continued to show significantly higher levels of human cell engraftment in the PB from week 2 post transplant through to week 7. From week 8, levels of human cells detected in PB were similar between group right through to termination of the experiment at week 20. (c) from week 5 post transplant human platelets were detected in the PB indicated by CD41 positivity. At all time points evaluated, the expanded group demonstrated significantly poorer kinetics of platelet engraftment compared to unexpanded. (d) human CD3⁺ T cells detected in PB of mice from 9 weeks post-transplant. While initially no difference was seen between unexpanded and expanded group, by week 12 there was significant difference with the expanded group again showing lower reconstitution; this was again seen at week 15. (e) Keeping with the trend from the PB data, there was significantly lower hCD45 engraftment seen in BM of mice that received an expanded graft compared to unexpanded, where the average was around 70% human reconstitution. Both unexpanded and expanded grafts promoted successful lineage differentiation as well as primitive cell proliferation in the BM, with no differences in proportion of CD19⁺ B-cell (f), CD33⁺ myeloid cells (g) or CD34⁺ progenitors (h) engrafted. *n* = 9 per group. Combined results of two experiments are presented. *P < 0.05 and **P < 0.01.

their *ex vivo* proliferation, high resolution division tracking was performed to compare rate of expansion, self renewal and differentiation of unexpanded and expanded CD34⁺ cells, as previously described (10). In these experiments we compared rate of expansion of fresh CD34⁺ cells with CD34⁺ cells cultured with cytokines for 6 days, then re-isolated using MACS technology.

Division tracking data was used to measure precursor cell frequency, mean generation number, and rate of phenotypic renewal (10). Total expanded HPC divided at a significantly slower pace, consistent with increased rate of cell differentiation and decreased self-renewal (Fig. 3a–d). CD34⁺ precursor expansion rate was negative in unexpanded samples indicating decline in CD34⁺

Figure 3. Cell expansion, differentiation and self renewal calculated using flow cytometry and division tracking. (a) cell expansion rate was higher in unexpanded samples when cultured in suspension or in co-culture with murine stroma and stroma augmented expansion rate in both samples. (b) probability of a cell undergoing self renewal was significantly decreased following *ex vivo* expansion regardless of culture conditions. Addition of stroma to cultures did not affect a cell's ability to self renew. (c) Not surprisingly, during *ex vivo* expansion proportion of cells expressing CD34 declines with concomitant increase in proportion of myeloid differentiation marker CD14. Presence of stroma in the culture moderated both of these observations. (e) expansion rate of CD34⁺ cells was negative in both unexpanded and expanded cultures indicating decline in precursor frequency, and this effect was significantly greater following expansion. Co-culture slightly modulated the effect in the unexpanded sample, however, there was dramatic decline in precursor expansion of expanded cells on stroma. (f) decline in CD34⁺ expansion accompanied by reciprocal increase in expansion of CD34⁻ precursors. Rate of CD34⁻ expansion in expanded samples was significantly higher than unexpanded and addition of stroma made no difference to this. * $P < 0.05$ and ** $P < 0.01$.

cell precursors, and this decline was further accelerated in the expanded group (Fig. 3e). This negative CD34⁺ precursor expansion rate was accompanied with a reciprocal increase in CD34⁻ precursor expansion rate confirming that speed of CD34⁺ cell differentiation increased in cultures previously exposed to proliferative stimuli (Fig. 3f). Compared to unexpanded CD34⁺ cells, there was a shorter lag time before first mitosis at the start of culture, in expanded samples (Table 2), which was not surprising considering that the majority of unexpanded cells were in the G₀/early G₁ phase of cycling when cultures were established (13). An increased cell cycle time was seen in expanded cells consistent with reduced overall expansion and mean generation numbers (Table 2). Cell cycle time for expanded CD34⁻ cells only marginally increased compared to non-expanded

ones and was significantly shorter than for CD34⁺ cells (Table 2). The rate of apoptosis was not significantly different in expanded cells however a remarkable increase in differentiation was seen following expansion compared to unexpanded cells (Table 3). Thus lower renewal rate of expanded CD34⁺ cells can be attributed to increased cell differentiation.

Co-culture with murine BM stromal MS5 cells used to mimic interaction of human HPC in the BM niche shortened the cell cycle time of unexpanded CD34⁺ cells from 21 to 16 h (Table 2) and this was even more obvious in expanded cultures with stromal cells, from 37 to 22 h (Table 2). However, cell cycle time for unexpanded CD34⁺ cells in co-culture with stroma still remained significantly shorter than for expanded CD34⁺ cells (16 h versus 22 h). Reduced cell cycle time in MS5

Table 2. Effect of expansion and co-culture with stroma on the lag time and cell cycle time of CD34⁺ and CD34[−] cells. The time before the first cell division following initiation of culture (lag time) and cell cycle time were derived from division tracking data using previously defined formulas [10]

Sample	Lag time (h)		Cell cycle time (h)	
	CD34 ⁺	CD34 [−]	CD34 ⁺	CD34 [−]
Unexpanded				
Suspension	31.6 (8.2)	49.3 (4.7)	21.3 (2.2)	17.2 (3.6)
Co-culture	36.4 (1.6)	48 (0.6)	16.3 (0.6)	18.1 (6.3)
Expanded				
Suspension	14.8 (3.3)	23.5 (3.2)	37.2 (3.8)	19.5 (1.5)
Co-culture	25.1 (4.4)	20.6 (5.3)	21.8 (1.6)	18.3 (1.4)

Table 3. Effect of expansion and co-culture with stroma on apoptosis and differentiation of CD34⁺ cells. Percentages of apoptotic cells and cells undergoing differentiation were derived from division tracking data using previously defined formulas [10]

Sample	Apoptosis (%)	Differentiation (%)
Unexpanded		
Suspension	1.89	3.74
Co-culture	3.67	1.88
Expanded		
Suspension	2.42	13.79
Co-culture	4.00	14.74

co-cultures correlated with increased mean generation number, however it still remained lower in expanded cells compared to unexpanded. Unexpanded and expanded CD34⁺ cells co-cultured with stroma did not exhibit increased renewal after 5 days culture compared to suspension culture, and renewal probability was lower in expanded CD34⁺ cells co-cultured with stroma compared to unexpanded CD34⁺ cells. Collectively, these results demonstrate that although co-culture with stroma promoted proliferation of *ex vivo* expanded CD34⁺ HPC and delayed their differentiation, self-renewal probability remained much lower compared to unexpanded CD34⁺ cells, which may explain their inferior regenerative function observed in this mouse transplantation model.

Ex vivo expansion modulates critical regulatory pathways in CD34⁺ HPC

To gain insight into the molecular mechanisms that underlie functional defects in *ex vivo* expanded HPC, we examined gene expression in these cells, on a genome-wide scale, and compared it to gene expression in unexpanded CD34⁺ HPC. Pooled CD34⁺ cells from four CB samples, were used in these experiments. Approxi-

mately 40% of the genes (10 000 out of 45 000 probes) presented on the array were expressed in all three conditions. Cytokine-induced expansion of CD34⁺ cells was associated with modulated expression of 4720 genes (40% of the expressed genes) – 2518 genes were up-regulated and 2202 down-regulated, suggesting global transcriptional modulation of gene expression in *ex vivo* expanded CD34⁺ HPC.

Cell cycle-related genes

Expanded CD34⁺ HPC exhibited a gene expression pattern consistent with their actively cycling nature – increased transcription of genes involved in DNA replication, repair, and cell division, relative to unexpanded CD34⁺ HPC (Table S1). Up-regulation of genes associated with G₁/S-phase cell cycle events, mitosis, spindle formation, DNA replication and chromatin modification was seen including genes encoding cyclin A1, A2, B1, B2 and F, cell division cycle molecules (cdc) 2, 6, 7, 20, 23, 25A, B, C and 45L, cell division cycle associated molecules (cdca) 1–5 and 8, centromere proteins (cenp) A, E, F, J, M and O, centrosomal proteins (cep) 55, 72 and 152, E2F transcription factors (E2F) 2, 6 and 7, translation initiating factors EIF 2B5, 2S1, 2S2, 4Ebp 1 and 5b. All of these genes were differentially expressed in *ex vivo* expanded and unexpanded CD34⁺ HPC. In addition, altered expression of genes belonging to the family of Mini-Chromosome Maintenance (MCM) complex genes known to regulate chromatin activity during cell cycling, was seen in expanded CD34⁺ cells. *Ex vivo* expansion of CD34⁺ cells induced expression of DNA-damage response gene *Chk1* that preserves genome integrity in proliferating cells (14). Modulation of multiple members of histone family clusters was registered in *ex vivo* expanded CD34⁺ cells, consistent with previously shown histone gene modulation in cytokine-stimulated HPC (15). Down-regulation of genes encoding cyclin dependent kinase inhibitors (cdkn) 1A (p21) and 1C (p57) both belonging to the Cip1/Kip family and known to be major determinants of stem cell quiescence (16,17), was seen in *ex vivo* expanded CD34⁺ cells. We have demonstrated that p57 modulation is associated with quiescence induced by inhibition of GSK3 β shown to prevent stem cell exhaustion during *ex vivo* culture of HPC (9,18). One of the critical regulators of quiescence in the BM niche is the TGF β pathway (19). This pathway, and more specifically SMAD7, also regulates p57 in the niche and following expansion we saw a decline in expression of two members of the TGF β family, TGFB1 and 3 and also SMAD7. In addition, down-regulation of other quiescence-related genes BMP8B and GATA2 as well as

several members of the TNF α family of genes was seen in *ex vivo* expanded CD34⁺ cells (20,21–23).

Ex vivo expanded CD34⁺ cells expressed higher levels of BCL-2 and BCL-2L12 and lower levels of MCL1 compared to their unexpanded counterparts. BCL-2 was shown to play an important role in protection of *ex vivo* cultured HPC from apoptosis, while functional dependence on MCL1 for self-renewal has recently been demonstrated to be a defining characteristic of human stem cells (24). Thus the pattern of MCL1/BCL-2 expression in *ex vivo* expanded CD34⁺ cells, correlated with induction of differentiation and loss of stem cell function.

Stem cell function

Expanded CD34⁺ HPC exhibited significant modulation of signalling pathways regulating stem cell function (Table S2). Down-regulation of two major effectors of Notch signalling – HES1 and HES4, as well as two Notch ligands – DLL1 and JAG2 – were seen in *ex vivo* expanded CD34⁺ HPC. Suppressed Notch signalling during *ex vivo* expansion of HPC may explain the success of adding exogenous Notch ligand in the Delaney study (8). Down-regulation of genes belonging to the *Hox* family of developmentally regulated genes including *HoxA2*, *A4* and *A5*, *Hox B2*, *B3* and *B5* was observed in *ex vivo* expanded CD34⁺ HPC. *HoxB4* was not regulated in these samples and while the role of this family member has been well established (25), the role of the other *Hox* genes modulated during expansion here has not been defined before. Expression of *MEIS1*, *MLL5*, *MLLT6* and 7 genes regulating *Hox* signalling (26), was also down-regulated in CD34⁺ cells following expansion. In addition, significant up-regulation of *DNMT1* and *DNMT3B* genes, acting to maintain sustained methylation of *Hox* genes, was also seen in *ex vivo* expanded HPC (27), suggesting that dysregulation of *Hox* gene expression may result from down-regulation of *MLL* allowing *DNMT1*-mediated methylation of the *Hox* gene promoter.

Wnt signalling is another important stem cell regulator (28). Wnt ligands are secreted by stromal cells in the BM niche and regulate Wnt signalling in stem cells (29,30). Our gene expression analysis revealed that both unexpanded and expanded CD34⁺ cells expressed 3 Wnt ligands – non-canonical Wnt16 and 5B, and canonical Wnt2B, all at very low levels. Expansion did not modulate expression of Wnt ligands; however, it did result in up-regulation of FZD9, the receptor for canonical Wnt2B, and FZD2, another component of canonical Wnt signalling, suggesting a role for canonical Wnt signalling in *ex vivo* proliferation of CD34⁺ cells. β -catenin is expressed at very low levels in fresh CD34⁺ cells, and only a small proportion of cells exhibit β -catenin

staining in the nucleus (18). β -catenin accumulates in *ex vivo* expanded CD34⁺ cells then rapidly declines as soon as cells start to differentiate (31). Active Wnt/ β -catenin signalling is important for CD34⁺ cell proliferation and suppression of Wnt signalling by quercetin inhibited this (Fig. S2). Thus down-regulation of β -catenin signalling during *ex vivo* culture correlated with reduction in proliferation rate and self-renewal of CD34⁺ HPC. The *NKD2* gene, which encodes Wnt antagonist naked-2, was up-regulated in CD34⁺ cells following expansion and furthermore, expression of JUN, a downstream target of β -catenin, was down-regulated in expanded CD34⁺ cells. Loss of JUN was shown to destabilise a complex network of genes limiting myeloid differentiation and impaired cell responsiveness to Notch signalling due to transcriptional deregulation of the *HES1* gene (32). Consistently, *HES1* down-regulation was also observed in expanded CD34⁺ cells. In addition, expression of the gene encoding LEF, a β -catenin transcription partner, was down-regulated in CD34⁺ cells, which may account for the defective β -catenin transcription activity and down-regulation of JUN. However, this acts in concert with other important regulators such as Notch, and their unbalanced regulation may affect stem cell expansion and differentiation during *ex vivo* culture (31).

Down-regulation of 8 members of Kruppel-like factors (KLF) important transcription factors regulating stem cell function (33), was also observed in expanded CD34⁺ cells, as well as 2 genes encoding prostaglandin E receptors – PTGER2 and 4. Prostaglandin E has recently been shown to promote short-term engraftment (34), consequently decline in expression of these receptors may account for the reduced response to prostaglandin E in *ex vivo* expanded HPC and to affect short-term engraftment.

Differentiation

Ex vivo expansion of CD34⁺ HPC up-regulated expression of genes that promote and down-regulate expression of genes limiting stem cell differentiation (Table S3). Elevated expression of CCAAT/enhancer binding protein CEBP α , as well as two other transcription factors, ETS-related molecules ETV4 and 5, all regulating differentiation, was seen in expanded CD34⁺ cells. Expression of GATA-1 shown to regulate myeloid differentiation, was noted in expanded CD34⁺ cells (35). Significantly reduced expression of transcription factors EGR1, 2 and 3, recently shown to control stem cell differentiation and self-renewal was also seen in expanded CD34⁺ HPC (36).

Modulation of differentiation markers CD14 and CD38 was not shown in the array, although flow cytometry revealed increased proportions CD14⁺ and CD38⁺

surface staining in expanded CD34⁺ HPC, suggesting post-transcriptional modulation of these proteins at this stage of *ex vivo* culture. In addition, up-regulation of five genes encoding haemoglobin (HB), an erythroid cell constituent, was seen in the expanded CD34⁺ group. CD19 and other lymphoid determinants CD1D, CD37, CD3D, CD6, CD69, CD7, CD79A, CD82, 83, and CD99, were lower in *ex vivo* expanded CD34⁺ cells, consistent with the early loss of lymphoid differentiation, demonstrated in the BM transplantation model. These results reveal a complex network of genes modulated during early stages of differentiation of *ex vivo* expanded CD34⁺ cells: differentiating CD34⁺ HPC lose B-cell gene expression and up-regulate erythroid gene expression while expression of myelomonocytic characteristics appears to be up-regulated at transcriptional level at later stages, together with loss of CD34 expression.

Immunophenotype

Involvement of the immune system in transplantation is complicated and many factors are involved (Table S4). Just recently, two papers have been published presenting new findings concerning immune properties of expanded HSC and the niche, during transplantation. Zheng *et al.* have reported that expanded murine HSC express T-cell inhibitory molecule PD-L1, which plays a role in T-cell modulation, as well as maintenance of peripheral tolerance (37–39). Although most data were from murine models, the study did find that PD-L1 was up-regulated in human CD34⁺ cells although no *in vivo* data were presented for this (39). The second study presents data showing that quiescent HSC persist in an immune privileged

niche following transplantation (40). This niche is located at endosteal surfaces where HSC are surrounded in close proximity to a population of regulatory T cells (Treg) that express anti-inflammatory molecules, transforming growth factor- β [TGF- β and interleukin-10 (IL-10)].

Here, we have shown that *ex vivo* expansion acts to modulate the immunophenotype of the expanded product. Reduced expression of several members of the inflammatory CCL chemokine family (41) interferon target genes, TNF ligands and receptors, IL8 and IL1 β was seen in CD34⁺ HPC, following expansion. Reduced expression of multiple pro-inflammatory cytokines and chemokines in expanded grafts may be beneficial in the clinical setting, to reduce inflammation in the BM induced by the patient's pre-conditioning. However, on the contrary, reduced expression of pro-inflammatory cytokines and chemokines may negatively affect migration and homing of transplanted progenitor cells to the BM.

All expanded precursor cells exhibited down-regulation of genes encoding several HLA-class 1 and -class 2 molecules. Flow cytometric analysis revealed that only a small proportion of unexpanded CD34⁺ cells expressed HLA-ABC staining while a significant proportion of CD34⁺ cells were positive for HLA-DR, DP and DQ (Fig. 4a,b). *Ex vivo* expanded grafts expressed more HLA-ABC and less HLA-DR, DP and DQ, suggesting that this may be better tolerated by the recipient immune system (Fig. 4c,d) which may be stimulated by foreign class II molecules. Reducing allostimulatory capacity of expanded grafts may help to reduce graft failure, particularly in the case of non-myeloablative conditioning protocols. In this context, and taken together with the new data recently published, heavily transfused and

Figure 4. Levels of HLA Class I and Class II expression modulated by *ex vivo* expansion. (a) unexpanded CD34⁺ cells expressed low levels of HLA-class I molecules while almost 70% of unexpanded CD34⁺ cells were positive for HLA-class II antigens (b). Following expansion, this expression pattern changed with almost 40% of cells being double positive for CD34 and Class I (c). Interestingly, amount of Class II expression declined by almost 9-fold (d). This finding may be important as Class II antigens are important mediators of graft versus host disease.

allo-immunized patients with BM failure syndromes might benefit from allogeneic SCT with an *ex vivo* expanded graft.

Homing and adhesion

Dramatic down-regulation of CXCR4, the specific receptor for chemokine SDF1, was seen in expanded CD34⁺ cells, and was validated by flow cytometry (Table 1) (Table S5). Two molecules that regulate CXCR4, RGS1 and 2, were also modulated during expansion, and together this may account for inferior homing of *ex vivo* expanded progenitor cells. Down-regulation ITGA9, integrin $\alpha 9\beta 1$, an important niche component that interacts with osteopontin (42), was also observed in expanded CD34⁺ cells. A large number of cytoskeletal and extracellular matrix-associated genes such as for MMP3, 9, 11, 15, 23A, 28 and 7, as well as specific members of the collagen family, actins and actin-associated proteins, filamins and tubulins and related proteins, were modulated in expanded HPC, suggesting cytoskeleton remodelling. Interestingly, expanded CD34⁺ HPC seemed to have similar characteristics to cells under stress, as a large number of regulated genes were involved in the ubiquitin-proteasome complex, such as those for USP8, 25 and 38 and UBE2C and 2T, and detoxifier systems, such as ABCE1, and CYP1B1, 27A1 and 2S1 (website).

All together these results demonstrate modulation of gene expression in CD34⁺ cells following *ex vivo* expansion, that may account for reduction in stem cell engraftment.

Discussion

The ability to increase numbers of human HPC in grafts through *ex vivo* expansion could provide improved treatment options for clinical transplantation. It is assumed that *ex vivo* expanded, differentiating progenitor cells must efficiently promote short-term haematopoietic recovery, critical for survival and rapid recovery of patients receiving allogeneic transplants. Here we show that cytokine supplemented culture of CB-derived CD34⁺ cells induced rapid proliferation resulting in expansion of CD34⁺ HPC, including committed CD34⁺CD38⁺ cells, but not primitive CD34⁺CD38⁻ progenitor cells. Early decline in expression of B-cell determinant CD19 and accumulation of HPC expressing myeloid cell determinants CD13 and CD14, CD123, as well as CD38 and CD90, was observed in CD34⁺ HPC following expansion. *Ex vivo* expansion of CD34⁺ HPC was also accompanied by generation and further expansion of CD34⁻ differentiat-

ing progenitor cells expressing CD38, CD14 and CD13. Although there is no evidence that these progenitor cells may contribute to short-term or long-term engraftment in pre-clinical and clinical settings, they may have an impact as accessory cells in modulation of engraftment (13).

A novel xenotransplantation model, the NSG mouse model, has been used here to study side by side engraftment kinetics of unexpanded and expanded CB CD34⁺ cells. This murine system is superior to the traditional NOD/SCID model as it enables evaluation of short term reconstitution as well as of long term, providing a complete picture of the engraftment process in much the same way that clinical transplants are evaluated. Furthermore, NSG mice support a broad range of human haematopoietic development, including human platelet and T-cell development, whereas studies that employ other NOD/SCID strains evaluate human lymphoid engraftment based solely on B cells. Using the NSG model we show, not only that LT-SRA of CB CD34⁺ cells is impaired following *ex vivo* expansion, but also rSRA of the graft declines following expansion. Reduced engraftment correlates with reduced numbers of CD34⁺CD38⁻ cells in the expanded graft. These cells were shown to be responsible for both rSRA and LT-SRA in the *ex vivo* expanded graft (1). It is relevant that the absolute number of CD34⁺CD38⁺ cells of the expanded product in our conditions was significantly higher than of fresh CB. CD34⁺CD38⁺ cells from fresh CB retain rSRA while the same subset in *ex vivo* expanded CB is unable to support rSRA (1). Thus delayed engraftment mediated by expanded CD34⁺ cells was associated with impaired function of CD34⁺CD38⁺ cells as well as reduced numbers of CD34⁺CD38⁻ cells.

High resolution division tracking revealed that *ex vivo* expanded HPC had reduced expansion rate and self renewal probability, and increased differentiation rate, which may account for impaired regenerative potential of the expanded progenitor cells. Gene expression analysis identified modulated gene expression that may account for the lower self-renewal probability and increased differentiation rate of expanded CD34⁺ cells. A major shift from 'quiescent' and 'stem cell' to 'cell division' and 'differentiation' expression pattern was observed in expanded CD34⁺ HPC. In this way *ex vivo* expansion modulated a complex network of genes and pathways that limit stem cell proliferation and differentiation during haematopoietic development; stem cell maintenance genes become silenced while differentiation specific genes are up-regulated. Genes regulating stem cell quiescence play a critical role in the maintenance of the primitive status of HPC *in vivo* (43,44), so reduced expression of these regulatory molecules determined in

our gene expression analysis, correlated with inferior engraftment, suggesting a role for these genes in *ex vivo* maintenance of primitive HPC, during culture.

Several methods to improve clinical engraftment, mostly focusing on short-term reconstitution, have been recently reported. Using immobilised Notch ligand to activate Notch signalling during *ex vivo* expansion has produced improved short-term engraftment in a small phase 1 clinical trial (8). Co-culture with MSC has also been shown to improve short-term engraftment in a further phase 1 clinical trial (45) and administration of prostaglandin E2 was seen to improve short-term engraftment in a pre-clinical BM transplantation model (46). We are currently investigating the effect of small molecule inhibitors of GSK3 β previously show to improve long-term engraftment (9).

Author contributions

TH Conception and design, collection and/or assembly of data, data analysis and interpretation and manuscript writing. FY collection and/or assembly of data and data analysis and interpretation. K-HK: collection and/or assembly of data and data analysis and interpretation. RN data analysis and interpretation. ES data analysis and interpretation. TAO financial support, data and data analysis and interpretation and final approval of manuscript. AD Conception and design, financial support, data and data analysis and interpretation manuscript writing and final approval of manuscript.

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Disclosure of potential conflicts of interest

The authors indicate no potential conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Peripheral blood engraftment dominated by B-cell progenitors. (a) at week 7 human lineage-specific markers were also detected in peripheral blood of transplanted mice. Half all cells expressed the B-cell marker CD19 while only 2% had differentiated into CD33⁺ myeloid cells. There was no difference between proportions of cells expressing these lineage markers in unexpanded and expanded groups. (b) proportion of hCD45⁺CD34⁺ cells in PB of mice at week 20 was similar in unexpanded and expanded groups.

Fig. S2 Expansion had no effect on proportion or extent of lineage commitment in BM of all mice at 20 weeks post-transplant. (a) CD19 and CD33 percentages were similar in BM from mice receiving both unexpanded and expanded grafts. Dot plots presented show CD19 versus CD33 within the hCD45⁺ gate. (b) There was no difference in proportion of hCD45⁺CD34⁺ cells in BM of unexpanded and expanded groups. These dot plots show hCD45 versus CD34 within the whole population.

Fig. S3 Expansion had no effect on proportion or extent of lineage commitment in BM of all mice at 20 weeks post-transplant. (a) Following short term treatment with

Wnt inhibitor Quercetin, suppressed CD34⁺ cell proliferation in a dose-dependent manner. Using CFSE analysis we see that following 5 days culture, 51% of cells in control cultures had undergone less than five divisions whereas after treatment with 10 mM Quercetin, this percentage increased to 87%, meaning that the majority of cells were not actively proliferating when Wnt is inhibited. (b) After treatment with Wnt inhibitor Quercetin, fold expansion of CD34⁺ cells in culture dramatically decreased in a dose-dependent manner, * $P < 0.05$ and ** $P < 0.01$.

Table S1 Modulation of genes regulating cell cycle

Table S2 Modulation of 'stem cell' genes

Table S3 Modulation of genes regulating differentiation

Table S4 Modulation of genes regulating immunophenotype

Table S5 Modulation of genes regulating homing and adhesion

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