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Thrombopoietin, flt3-ligand and c-kit-ligand modulate *HOX* gene expression in expanding cord blood CD133$^+$ cells

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Abstract. Haemopoietic stem/progenitor cell (HSPC) development is regulated by extrinsic and intrinsic stimuli. Extrinsic modulators include growth factors and cell adhesion molecules, whereas intrinsic regulation is achieved with many transcription factor families, of which the *HOX* gene products are known to be important in haemopoiesis. Umbilical cord blood CD133$^+$ HSPC proliferation potential was tested in liquid culture with 'TPOFLK' (thrombopoietin, flt-3 ligand and c-kit ligand, promoting HSPC survival and self-renewal), in comparison to 'K36EG' (c-kit-ligand, interleukins-3 and -6, erythropoietin and granulocyte colony-stimulating factor, inducing haemopoietic differentiation). TPOFLK induced a higher CD133$^+$ HSPC proliferation (up to 60-fold more, at week 8) and maintained a higher frequency of the primitive colony-forming cells than K36EG. Quantitative polymerase chain reaction analysis revealed opposite expression patterns for specific *HOX* genes in expanding cord blood CD133$^+$ HSPC. After 8 weeks in liquid culture, TPOFLK increased the expression of *HOX B3*, *B4* and *A9* (associated with uncommitted HSPC) and reduced the expression of *HOX B8* and *A10* (expressed in committed myeloid cells) when compared to K36EG. These results suggest that TPOFLK induces CD133$^+$ HSPC proliferation, self-renewal and maintenance, up-regulation of *HOX B3*, *B4* and *A9* and down-regulation of *HOX B8* and *A10* gene expression.

INTRODUCTION

Haemopoiesis is characterized by a high cell turnover requiring the continuous self-renewal of a primitive stem cell pool as well as controlled differentiation and development of their progenitors into a diverse array of mature blood cells. Such a developmental system calls for complex complementary extrinsic (growth factors and cell adhesion molecules) and intrinsic (signal transduction and transcription factor) stimuli. Dysregulation of one or several of these pathways may lead to impaired haemopoiesis (Tenen *et al*. 1997).

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CD133 is a highly hydrophobic 865 amino acid (120 kDa) glycoprotein. It has five transmembrane domains with eight N-linked glycosylation sites. CD133 is the human orthologue of the murine prominin 1 and as such was recently described as being a member of the prominin family (Miraglia *et al*. 1997; Yin *et al*. 1997; Corbeil *et al*. 1998; Fargeas *et al*. 2003). CD133 expression is mainly restricted to a small haemopoietic stem/progenitor cell (HSPC) subset of $CD34^+$ HSPC as well as primitive endothelial cells and several non-haemopoietic tissues, including the foetal brain, pancreas and kidney (Miraglia *et al*. 1997; Corbeil *et al*. 1999; Uchida *et al*. 2000). Several studies by our group and others have shown that CD133 expression on HSPC precedes that of CD34 on developing HSPC (Bhatia 2001; McGuckin *et al*. 2003b). CD133 cell function is unknown but the presence of a leucine-zipper motif (inclined to dimerization) on the second extracellular loop as well as its selective membrane location to plasma membrane protrusions suggests that it could mediate targeted cell–cell communication (McGuckin *et al*. 1999; Corbeil *et al*. 2000).

Haemopoietic reconstitution of the blood system relies on the transplantation of sufficient numbers of uncommitted HSPC to recipients to ensure both short- and long-term bone engraftment (Gilmore *et al*. 2000). One of the main limiting factors of this procedure is often the number of HSPC available from the source, not least when using umbilical cord blood units (Gabutti *et al*. 1993; Emerson 1996; Gluckman 2000). Our group and others have previously demonstrated the advantage of using cord blood $CD133^+$ HSPC which encompass more uncommitted HSPC than $CD34^+$ counterparts (de Wynter *et al*. 1998; Forraz *et al*. 2002a; McGuckin *et al*. 2003a). *Ex vivo* expansion technology may circumvent cord blood HSPC limitations and extend their use to adult haemopoietic stem cell transplantation, because it aims to generate *in vitro* HSPC amplification with growth factors, whilst limiting proliferation-induced differentiation (Aglietta *et al*. 1998).

Although the ideal growth factor combination optimized for true stem cell expansion remains to be identified, our group previously reported on the thrombopoietin, flt3-ligand and c-kit ligand (TPOFLK) promoting self-renewal whilst limiting differentiation (Forraz *et al*. 2002b, 2004; McGuckin *et al*. 2004). Further to extrinsic cytokine stimulation, we believe that HSPC *ex vivo* expansion may benefit from taking into account the intrinsic activation of transcription factors which can modulate HSPC fate both *in vivo* and *in vitro* (Zhu & Emerson 2002). For instance, *ex vivo* expansion of early uncommitted HSPC could be achieved with: activation of P glycoprotein pump genes (Bunting *et al*. 2000); the exogenous excess addition of all-*trans* retinoic acid; soluble Sonic Hedgehog protein (Purton *et al*. 2000); or the stimulation of Notch family of transcription factors (Varnum-Finney *et al*. 2000).

Amongst predominant intrinsic transcriptional pathways, the *HOX* gene family of transcription factors has been described by several studies as a key group of control genes for regulation (and dysregulation) of the haemopoietic system (Sauvageau *et al*. 1997; Antonchuk *et al*. 2002; Krosl *et al*. 2003). The Hox B4 protein was notably reported as a strong, positive regulator of HSPC self-renewal (Antonchuk *et al*. 2002; Kyba *et al*. 2002). *HOX B4* gene over-expression could specifically enhance the rate of HSPC expansion without impairing normal differentiation or functional repopulation potential and did not induce neoplastic transformation (Sauvageau *et al*. 1995; Thorsteinsdottir *et al*. 1999; Antonchuk *et al*. 2001, 2002). Blocking *HOX* gene function by the use of anti-sense oligonucleotides or gene knock-out, perturbs a number of distinct haemopoietic events. For instance, myeloid, erythroid and lymphoid haemopoiesis are all defective in *HOX A9* knock-out mice (Lawrence *et al*. 1997), and the anti-sense ablation of *HOX B5*, *HOX B6*, *HOX B7* (Takeshita *et al*. 1993), or *HOX A5* (Fuller *et al*. 1999) blocks erythroid differentiation. Mechanisms modulating the integration between extrinsic growth factor stimulation and intrinsic Hox transcription factors during HSPC *ex vivo* expansion remain to be

further characterized and may help elucidate HSPC 'proliferation–differentiation' versus 'maintenance–self-renewal' roles.

A small-array real-time *HOX* (SMART-*HOX*) quantitative polymerase chain reaction (Q-PCR) methodology enabling sensitive analysis of changes in *HOX* gene expression in human haemopoietic cells has previously been reported and validated (Thompson *et al*. 2003). This method has been utilized in this study to quantify comparatively the variations in:

1 *HOX B3*, *HOX B4* and *HOX A9*, which are up-regulated in early, uncommitted HSPC and are down-regulated during differentiation (Sauvageau *et al*. 1994, 1995; Shimamoto *et al*. 1998; Dorsam *et al*. 2003); and
2 *HOX B8* and *HOX A10* gene expression previously linked with committed myeloid cells (Krishnaraju *et al*. 1997; Bjornsson *et al*. 2001; Buske *et al*. 2001; Taghon *et al*. 2002).

Here, the study investigated a possible correlation between *ex vivo* expansion kinetics of cord blood CD133$^+$ cells when stimulated by two different growth factor combinations:

1 K36EG [c-kit ligand, interleukin (IL)-3, IL-6, erythropoietin and granulocyte colony-stimulating factor (G-CSF)] known to recruit cells into proliferation and differentiation (Aglietta *et al*. 1998; Forraz *et al*. 2002a; McGuckin *et al*. 2004); and
2 TPOFLK (thrombopoietin, flt-3 ligand and c-kit ligand) the synergism of which was reported to amplify early HSPC populations efficiently (Gilmore *et al*. 2000; McGuckin *et al*. 2004) and the expression of specific *HOX* genes (*HOX B3*, *HOX B4*, *HOX A9*, *HOX A10* and *HOX B8*) in expanded cord blood CD133$^+$ cells.

MATERIALS AND METHODS

CD133$^+$ cell immunomagnetic cell separation

Human umbilical cord blood specimens were collected from full-term, third-stage labour deliveries after elective Caesarean sections from informed and haematologically normal volunteers in accordance with the South Thames Local Research Ethics Committee. Cord blood mononuclear cells (MNC) were separated through a density gradient method using research grade Ficoll–Paque solution (*d*: 1.077 g/cm^3, Pharmacia Biotech, Uppsala, Sweden). CD133$^{+/-}$ cells were obtained from MNC after immunomagnetic separation using the CD133 mini-magnetic activated cell sorting (MACS) selection kit (Miltenyi Biotec, Bergish Gladbach, Germany) following the manufacturer's instructions as previously reported (Forraz *et al*. 2002a; Whiting *et al*. 2003; McGuckin *et al*. 2004).

Immunophenotyping and flow cytometry

Cells were incubated in human gammaglobulins (20 min, 4 °C, 2% in phosphate-buffered saline, Sigma Aldrich, Poole, UK) to block non-specific Fc receptors. Cells were subsequently labelled (30 min, 4 °C) with monoclonal mouse anti-human phycoerythrin-conjugated CD133 (Miltenyi Biotec) and peridinin chlorophyll protein-conjugated CD34 antibodies (BD-Pharmingen, San Diego, CA, USA). Following appropriate washing procedures cells were finally fixed in paraformaldehyde (1%, BDH, Poole, UK). Fluorescent events were acquired on a Becton Dickinson FACScan flow cytometer with CELLQUEST software prior to analysis with WINMDI software.

Cord blood LinNeg expansion in TPOFLK-stimulated liquid cultures

Cord blood CD133$^+$ cells were grown in duplicate in 9-cm^2 tissue culture slide flasks (Nunc, Hereford, UK) at a concentration of 2.7×10^4 cells/ml of Iscove's-modified Dulbecco's medium (IMDM, Life Technologies, Paisley, UK) supplemented with 10% foetal calf serum

(PAA Laboratories, Yeoville, UK), gentamicin (50 μg/ml; Life Technologies) and either the TPOFLK cytokines mix: thrombopoietin (10 ng/ml), flt3-ligand (50 ng/ml) and c-kit-ligand (20 ng/ml) or the K36EG cytokines mix: c-kit-ligand (20 ng/ml), IL-3 (50 ng/ml), IL-6 (20 ng/m), erythropoietin (6 U/ml), G-CSF (10 ng/ml).

$CD133^+$ cells were cultured at 37 °C, 5% CO_2 in a humidified atmosphere and counted weekly. Aliquots were removed for clonogenic assays. All cytokines were purchased from R & D Systems Ltd, Abingdon, UK.

Clonogenic assays in methyl cellulose

Cell aliquots were seeded in 200 μl IMDM, 10% foetal calf serum and 800 μl Methocult solution (H4230, Stem Cell Technologies, London, UK), supplemented with erythropoietin (6 U/mL), c-kit-ligand (20 ng/ml), IL-3 (50 ng/ml), IL-6 (20 ng/ml), G-CSF (10 ng/ml), and grown for 14 days at 37 °C, 5% CO_2 in a humidified atmosphere, prior to scoring for colony-forming cells (CFC). All cytokines were purchased from R & D Systems Ltd.

RNA isolation, cDNA preparation and Q-PCR

Total RNA and cDNA were prepared as previously reported (Thompson *et al*. 2003) using standard reagents: Trizol, murine Maloney leukaemia virus native reverse transcriptase, random primers (all from Invitrogen, Paisley, UK). As a result of the small numbers of cells, glycogen (Invitrogen) was used as an inert carrier molecule for RNA following the manufacturer's instructions. Real-time Q-PCR was carried out using *Taq*Man probe-based chemistry (Applied Biosystems, Foster City, USA). The 5′-reporters for the *HOX* genes and endogenous controls (18SrRNA) were 6-carboxyfluorescein (FAM) and VIC™ (Applied Biosystems), respectively. Relative fold expression based on the $\Delta\Delta C_T$ method (reviewed by Ginzinger 2002) was corrected for RNA equivalents using 18S rRNA C_T (threshold cycle) values.

Statistical analysis

When applicable, results were analysed statistically and expressed as mean ± SEM from experiments performed in at least duplicate. Statistical significance was calculated by the Student's *t*-test.

RESULTS

$CD133^+$ cell purification

Cord blood MNC immunolabelled against CD133 or CD34 antigens contained a significantly lower frequency of $CD133^+$ cells [0.39 ± 0.04% (mean ± SEM); range 0.19–0.70] than $CD34^+$ cells (0.59 ± 0.05%; range 0.21–1.36; $P = 0.002$, $n = 31$).

However, throughout this study, the mean purity of cord-blood-derived $CD133^+$ immunomagnetically selected cells was 94.40 ± 1.02% (range 84.51–97.15, $n = 10$) and $CD133^+$ cells mean recovery from MNC was 74.80 ± 3.76% (range 60.36–86.64, $n = 10$). Figure 1 represents CD133 and CD34 antigen percentage distribution within MNC, $CD133^-$ and $CD133^+$ harvested cells, respectively.

TPOFLK induced high proliferation of CB $CD133^+$ cells in 8-week expansion liquid culture assay

TPOFLK and K36EG cytokine mixes were compared for their ability to expand cord blood $CD133^+$ cells over 8 weeks *in vitro*. In six distinct experiments, all performed in duplicate,

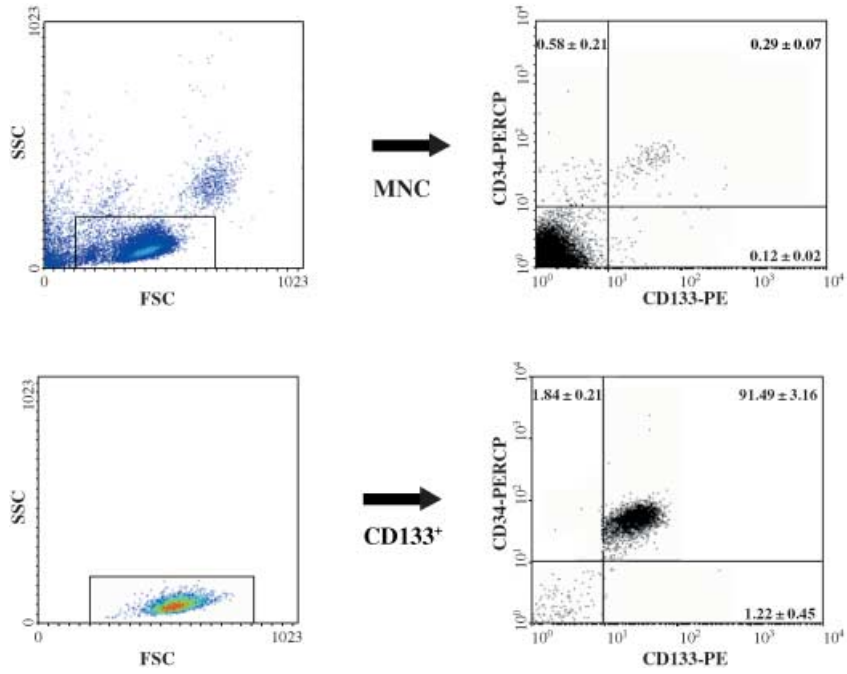

Figure 1. CD133 and CD34 antigen percentage distribution on cord blood MNC and CD133⁺ cells. The left panels show forward scattered (FSC) versus side scattered (SSC) flow cytometric density plots representing the size and internal complexity/granularity of the three cell populations. The rectangles highlight strategic regions enabling electronic gating of cells with 'blast' morphology (medium size, large nucleus, little cytoplasm). This further allowed CD133 and CD34 co-distribution analysis for each cell population. CD133 and CD34 were labelled with mouse anti-human specific monoclonal antibodies labelled with phycoerythrin and peridinin chlorophyll protein fluorochromes, respectively. Flow cytometric plots are from a representative example, whereas data are expressed as mean percentage (± SEM) distribution of (clockwise) $CD133^-$ $CD34^+$ $CD133^+$ $CD34^+$ and $CD133^+$ $CD34^-$ cell subsets of 10 distinct experiments.

TPOFLK-stimulated liquid cultures induced a higher cumulative total viable cell fold increase than the K36EG cytokine mix. After 8 weeks, the optimal cumulative cell fold increase was $4.68 \times 10^8 \pm 1.51 \times 10^8$ in TPOFLK and $7.96 \times 10^6 \pm 1.71 \times 10^6$ in K36EG-supplemented cultures ($n = 6$) (Fig. 2). Interestingly, this difference in expansion was statistically significant from week 3 to week 8 ($P < 0.05$) of liquid culture. TPOFLK thus appeared to be a very potent stimulus for sustained cord blood $CD133^+$ cell long-term proliferation.

TPOFLK expansion of cord blood $CD133^+$ cells maintained more primitive CFC than K36EG

After isolation, $CD133^+$ cells contained significantly increased levels of CFC with 547 ± 82 CFC per 10^4 $CD133^+$ cells compared to the more heterogeneous MNC (5 ± 2 CFC per 10^4 MNC, $n = 6$, $P < 0.01$). Comparative analysis of CFC frequency from expanded $CD133^+$ cells revealed that TPOFLK-supplemented liquid culture systems maintained more CFC than K36EG supplemented cultures at weeks 4, 6 and 8 ($P < 0.05$) (Fig. 3). The optimal window for CFC output appeared to be between week 2 and week 6 where TPOFLK-expanded $CD133^+$ cells produced between 23 ± 5 CFC (weeks 2 and 4) and 25 ± 6 CFC (week 6) per 10^4 seeded cells. CFC production in TPOFLK-stimulated liquid cultures significantly decreased at week 8 (6 ± 1 CFC; $P < 0.05$). TPOFLK cytokine mix therefore induced higher $CD133^+$ cell proliferation whilst expanding more primitive CFC than K36EG.

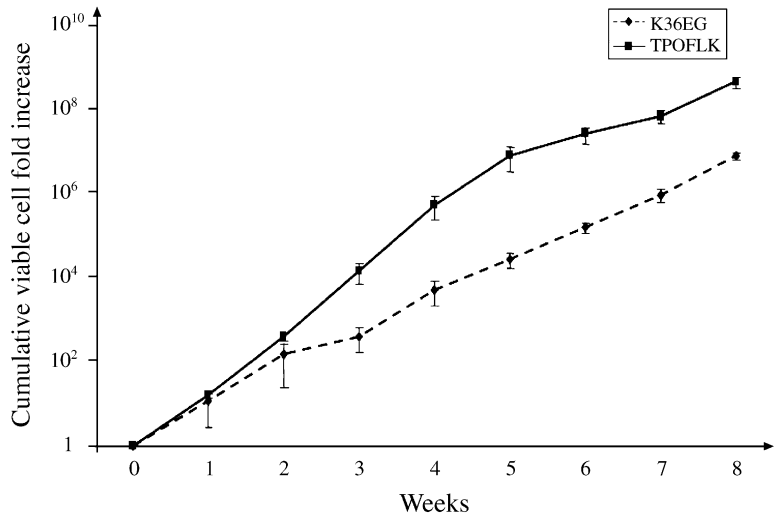

Figure 2. Comparison of cord blood CD133$^+$ cells. Eight-week expansion in liquid culture stimulated either by TPOFLK or K36EG. TPOFLK-stimulated liquid cultures induced a higher cumulative total viable haemopoietic cell fold increase from CD133$^+$ cells than K36EG cytokine mix ($P < 0.05$ from week 3 to week 8). Results are expressed as mean ± SEM of six distinct experiments performed in duplicates.

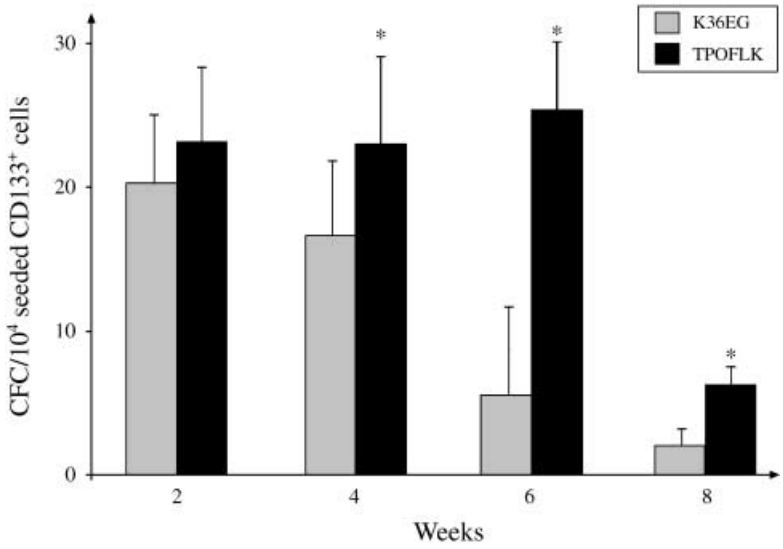

Figure 3. Comparison of 8-week cord blood CD133$^+$ cell TPOFLK- or K36EG-stimulated expansion in liquid culture clonogenic assay. Methylcellulose-based clonogenic assays were performed every 2 weeks from aliquots of expanding CD133$^+$ cells under TPOLFK or K36EG stimuli. This permitted frequency calculation of colony-forming-cells (CFC) per 10^4 CD133$^+$ expanding cells. TPOFLK maintained a higher CFC frequency over 8 weeks than K36EG. This was particularly significant at weeks 4, 6 and 8 (*$P < 0.01$). Results are expressed as mean ± SEM of six distinct experiments performed in duplicates.

TPOFLK and K36EG differentially modulated *HOX* gene expression in expanding cord blood CD133$^+$ cells

Real-time Q-PCR allowed comparative analysis of *HOX B3*, *HOX B4*, *HOX B8*, *HOX A9* and *HOX A10* gene expression in expanded CD133$^+$ cells in triplicate. Using this method, we compared, from baseline (freshly isolated cord blood CD133$^+$ cells), the fold difference for expression of the above genes in expanded CD133$^+$ cells in either TPOFLK- or K36EG-stimulated liquid cultures.

Such comparison was analysed between week 4 and week 8, during which a significant difference in CD133$^+$ cell proliferation between TPOFLK- and K36EG-treated liquid culture systems was observed ($P < 0.05$). Furthermore, as a result of the limited number of CD133$^+$ cells available per cord blood unit a compromise had to be reached in the usage of expanding cells for functional assays and gene expression profiling. TPOFLK and K36EG caused different effects on the expression of *HOX B3*, *HOX B4*, *HOX A9* (associated with proliferating HSPC), and *HOX B8* and *HOX A10* (associated with committed myeloid progenitors) at week 4 and week 8 of expansion (Fig. 4). In both TPOFLK- and K36EG stimulated liquid cultures, expanding CD133$^+$ cells, *HOX B3*, *HOX B4*, *HOX B8* and *HOX A10* gene expression levels were similar after 4 weeks. *HOX A9* gene expression at week 4 was slightly higher in K36EG-expanded CD133$^+$ cells when compared to TPOFLK-expanded CD133$^+$ cells. From week 4 in TPOFLK-stimulated CD133$^+$ cells, *HOX B3*, *HOX B4* and *HOX A9* gene expression was down-regulated until week 6 and then progressively up-regulated to levels between 10 and 100 times higher than in K36EG-expanded CD133$^+$ cells. Further to this, K36EG induced greater up-regulation of *HOX B8* and *HOX A10* gene expression in these genes at week 8 of culture. Taken together, these data suggest a differential effect on *HOX* gene expression in CD133$^+$ cells expanded with TPOFLK compared to K36EG.

DISCUSSION

Activation of transcriptional intrinsic pathways to complement growth factor-stimulated systems is the focus of growing scientific interest and is important for the development of novel *ex vivo* HSPC expansion protocols. We and others have shown that CD133 is a good alternative marker for CD34 for HSPC selection when establishing functional assay standards. A majority of CD133$^+$ cells co-express CD34 but encompass earlier and less committed HSPC than their CD34$^+$ counterparts (de Wynter *et al*. 1998; Bhatia 2001; McGuckin *et al*. 2003a).

Previous reports from our group notably demonstrated that CD133$^+$ HSPC were able to produce pan-lineage haemopoietic differentiation when grown in K36EG cytokine mix (Forraz *et al*. 2002a; McGuckin *et al*. 2004). TPOFLK was reported to enhance HSPC self-renewal and to optimize cell survival by inhibiting apoptosis (Luens *et al*. 1998; Kohler *et al*. 1999; McGuckin *et al*. 2004).

Our 8-week CD133$^+$ cell expansion assay results are in accordance with these data. Even after 1 week in culture, TPOFLK induced more cord blood CD133$^+$ cell proliferation than K36EG, although such a difference was not initially significant. However, TPOFLK supplementation resulted in a 60-fold more CD133$^+$ cumulative cell fold increase than the K36EG mix after 8 weeks. TPOFLK did not only produce more HSPC proliferation but cells retained a greater proliferative potential (CFC output) than K36EG, suggesting maintenance at an earlier stage of development. TPOFLK action proved not only to induce high HSPC proliferation whilst maintaining self-renewal, but TPOFLK extrinsic functional signalling correlated with *HOX* transcription factors intrinsic transcriptional regulation.

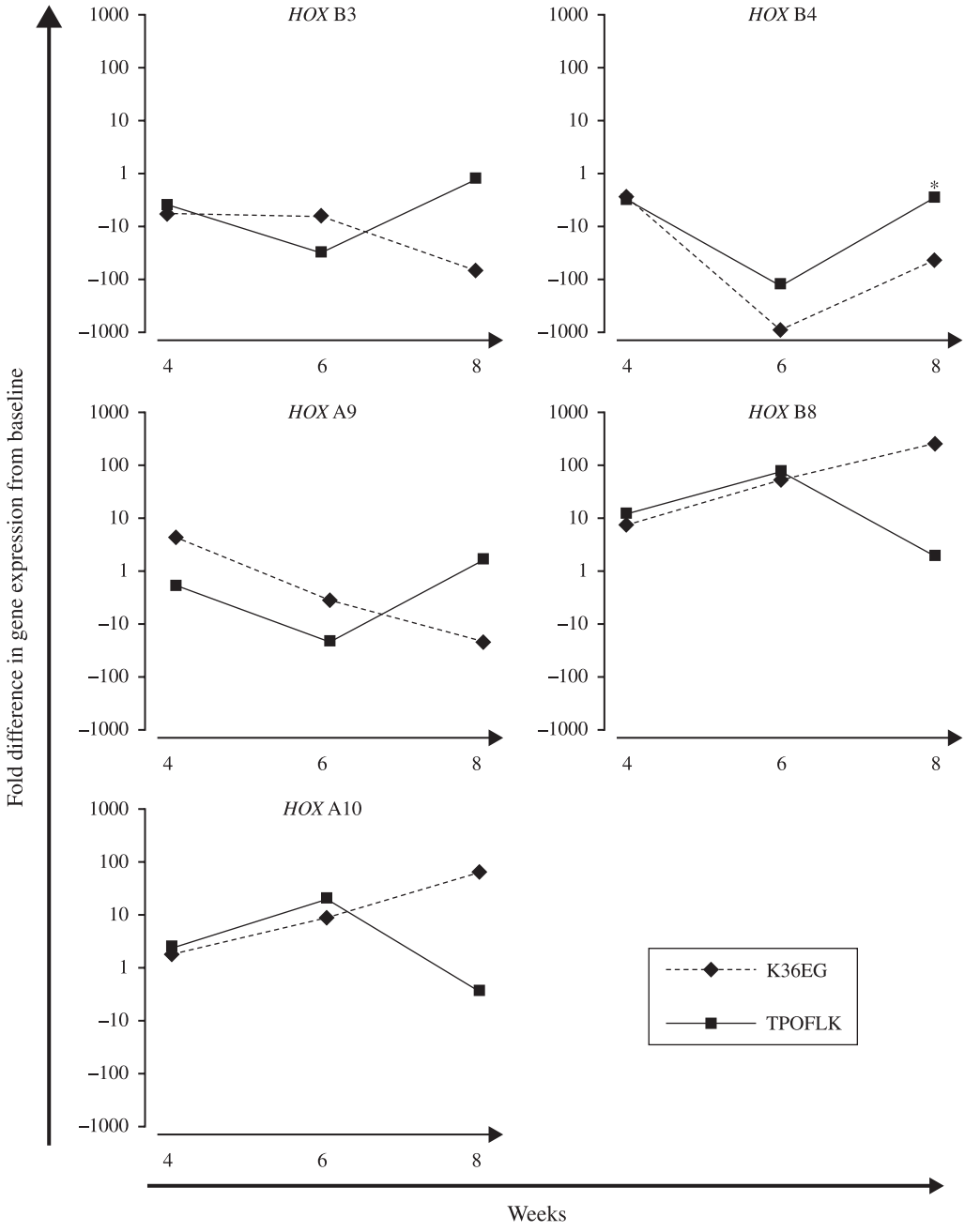

Figure 4. Quantitative RT-PCR analysis of *HOX* gene expression in TPOFLK- and K36EG-stimulated cord blood CD133$^+$ cells. The effect of TPOFLK and K36EG stimulations on the expression of several *HOX* genes in expanding CD133$^+$ cell was analysed by quantitative RT-PCR by measuring the relative fold difference with day 0 *HOX* gene expression. At week 8 TPOFLK induced more expression of *HOX B3*, *HOX B4* and *HOX A9* genes known to be preferentially expressed in primitive HSPC than K36EG, which favoured expression of *HOX B8* and *HOX A10* associated with commitment to the myeloid lineage.

Restricted amounts of RNA available from expanding CD133$^+$ cells limited SMART-*HOX* PCR analysis to five carefully selected *HOX* genes.

In both liquid culture systems (TPOFLK and K36EG), *HOX B3*, *HOX B4* and *HOX A9* gene expression levels were below those of baseline after 4 weeks of culture, which may be explained by the impact of artificial expansion methods on the cells' gene expression profile. However, after 8 weeks, the higher cell proliferation and CFC output from CD133$^+$ cells caused by TPOFLK, was concomitant with a higher gene expression (between 10- and 100-fold difference and back to baseline level) of *HOX B3*, *HOX B4* and *HOX A9* than for cells stimulated by K36EG. Interestingly, *HOX B3*, *HOX B4* and *HOX A9* gene expression was previously associated with early HSPC (Lawrence *et al*. 1996; Van Oostveen *et al*. 1999). Such patterns correlated with down-regulation of *HOX B8* and *HOX A10* gene expression (conventionally linked with committed myeloid progenitors; Krishnaraju *et al*. 1997; Taghon *et al*. 2002) in cells at week 8 under K36EG stimulation. In this 8-week CD133$^+$ cell expansion study, TPOFLK maintained a higher transcriptional activity of *HOX B3*, *HOX B4* and *HOX A9* genes than K36EG, which favoured *HOX B8* and *HOX A10* expression. Interestingly, ectopic *HOX B8* expression was reported to induce maintenance of myeloid progenitor (Perkins & Cory 1993) and *HOX A10* expression is known to be expressed in committed myeloid cells and to be important in regulating haemopoietic lineage determination (Thorsteinsdottir *et al*. 1997; Taghon *et al*. 2002).

External HSPC stimulation by cytokines activates a series of signal transduction events mediating intrinsic activation of complex transcription factor pathways (Shivdasani & Orkin 1996). The differential expression of the particular *HOX* genes investigated here may be linked with different cell cycle dynamics induced by K36EG and TPOFLK, respectively. Quesenberry and colleagues suggested that HSPC could respond differently to external stimuli at diverse points in the cell cycle causing a shift in chromatin and variation in gene expression (Quesenberry *et al*. 2002). *HOX* transcription factors have been reported as master regulators of the haemopoietic system (Magli *et al*. 1997). A study by Sauvageau *et al*. (1994) established that at least 22 of the 39 known *HOX* genes were expressed at various levels in five different CD34$^+$ human bone marrow subsets. *HOX* gene products were later confirmed to play a central role in the proliferation and the differentiation of early HSPC (Giampaolo *et al*. 1994; Sauvageau *et al*. 1997). Upon growth factor stimulation, Giampaolo *et al*. (1994) noticed rapid up-regulation of *HOX B3* expression followed by a delayed up-regulation of *HOX B4*. This may be the result of *HOX B3* directly activating downstream *HOX B4*. Expression of *HOX A9* followed similar patterns to *HOX B3* and *HOX B4* gene expression. *HOX A9* was previously linked by several studies to influence general haemopoiesis including maintenance of the erythroid, myeloid and lymphoid lineages (Lawrence *et al*. 1997). Hox A9 transcription factor's mode of action, however, could have more to do with HSPC proliferation modulation than their differentiation *per se* (Izon *et al*. 1998; Lawrence *et al*. 1999).

From week 6 in TPOFLK-supplemented CD133$^+$ cells, early HSPC-associated *HOX* gene (*HOX B3*, *HOX B4* and *HOX A9*) expression appeared to be rapidly up-regulated (although those levels were still below those in baseline freshly isolated CD133$^+$ cells) which correlated with the highest CFC output. Interestingly, the reduction in CFC output at week 8 of TPOFLK-stimulated culture may account for the maintenance of primitive HSPC as it was concomitant with a significant down-regulation of *HOX B8* and *HOX A10* (preferably expressed in myeloid progenitors and precursors). Taken together these data thus confirmed TPOFLK's adequacy for HSPC expansion. It may be explained by converging studies demonstrating that thrombopoietin's activation of the p38/MAPK signal transduction pathway resulted in activation of upstream stimulation factor 1 and 2 (USF1 and USF2) transcription factors known to bind to the *HOX B4* gene promoter region triggering its transcription (Giannola *et al*. 2000; Galibert *et al*. 2001; Kirito *et al*. 2003).

Work of this nature described here is difficult because of the extreme rarity of the $CD133^+$ HSPC population in cord blood. Furthermore, as a result o f the limited number of colonies that are generated from liquid delta cultures, a compromise must be reached between functional characterization and genetic expression analysis. However, this study is important because, although little is known of the potential of immature cord blood populations beyond haemopoietic use, reports are emerging, including from our laboratory, concerning the potential of cord blood for multiple non-haemopoietic tissue regeneration (Forraz *et al*. 2004).

In conclusion, TPOFLK demonstrated potential for cord blood $CD133^+$ HSPC *ex vivo* expansion inducing slow proliferation, maintenance of early HSPC as well as promoting HSPC survival status. TPOFLK notably induced the maintenance of primitive CFC possibly by modulating transcriptional activation of *HOX B3*, *HOX B4* and *HOX A9* genes. Taken together this study suggested that HSPC *ex vivo* expansion strategy development could further evolve by simultaneously optimizing cytokine synergistic action as well as by better understanding the intrinsic transcriptional pathways (like the HOX family) which balance HSPC self-renewal and differentiation.

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