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Research Article

Proteomic Profiling of *Ex Vivo* Expanded CD34-Positive Haematopoetic Cells Derived from Umbilical Cord Blood

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Ex vivo expansion of haematopoetic cells by application of specific cytokines is one approach to overcome boundaries in cord blood transplantation due to limited numbers of haematopoetic stem cells. While many protocols describe an effective increase of total cell numbers and the amount of CD34-positive cells, it still remains unclear if and how the procedure actually affects the cells' properties. In the presented publications, CD34-positive cells were isolated from cord blood and expanded for up to 7 days in media supplemented with stem cell factor (SCF), thrombopoietin (THPO), interleukin 6 (IL-6), and fms-related tyrosine kinase 3 ligand (FLT3lg). At days 3 and 7, expanded cells were harvested and analyzed by flow cytometry and quantitative proteomics. 2970 proteins were identified, whereof proteomic analysis showed 440 proteins significantly changed in abundance during *ex vivo* expansion. Despite the fact that haematopoetic cells still expressed CD34 on the surface after 3 days, major changes in regard to the protein profile were observed, while further expansion showed less effect on the proteome level. Enrichment analysis of biological processes clearly showed a proteomic change toward a protein biosynthesis phenotype already within the first three days of expression.

1. Introduction

Although several groups in preclinical and clinical settings have attempted *ex vivo* expansion of the cord blood (CB) product in order to increase haematopoetic progenitor and granulocyte numbers and to reduce the duration of posttransplant neutropenia (summarized in [1]), the implementation of protocols applying for instance several cytokines has been complicated by the following facts.

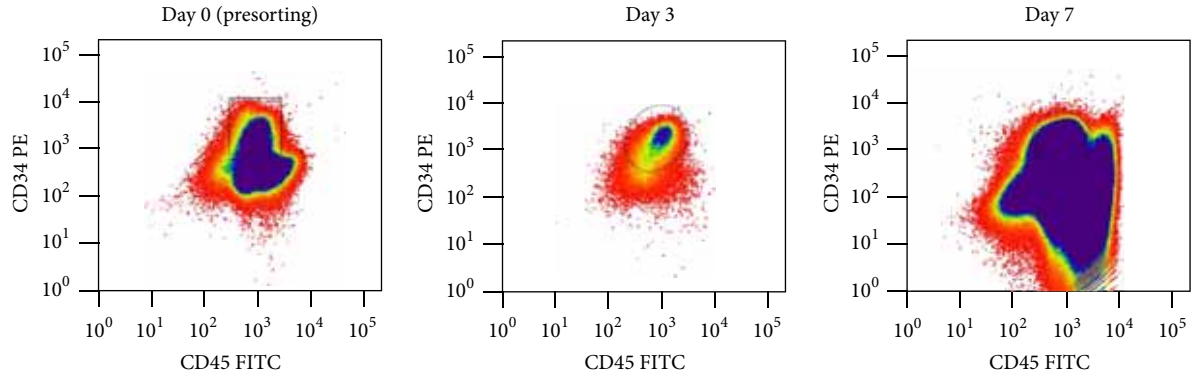
(1) CB transplants are frozen in the majority of banks in a single bag. Clinical trials were performed with only a fraction of CB unit expanded *ex vivo* with the larger remainder infused unmanipulated. Therefore, the expanded product usually could be infused only 10–14 days after transplantation. Alternative approaches focus on the expansion of one CB unit together with a second nonmanipulated unit.

(2) Clinical grade growth factors are only available for a limited number of cytokines and are expensive.

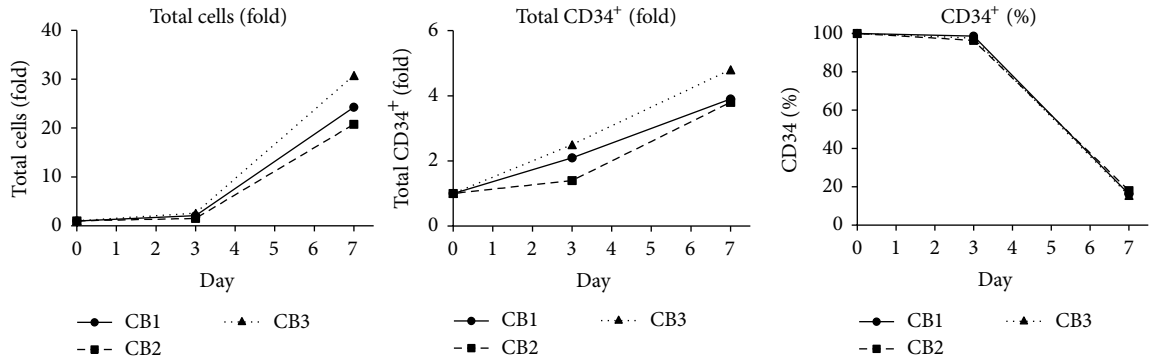
(3) Moreover, none of the clinical experiences could unequivocally document a clear benefit of infusion of such *ex vivo* cytokine expanded components.

Since cytokine-driven *ex vivo* expansion of $CD34^+$cells from CB is being discussed controversially, other ways to improve haematopoetic engraftment time and reconstitution after CB transplantation are being explored including double CB transplants (summarized in [2]) and cotransplantation of a single CB unit together with highly purified $CD34^+$ mobilized peripheral blood stem cells from a haploidentical or related or completely unrelated donor as a very promising clinical approach [3].

Recently Csaszar et al. presented an interesting approach for rapid expansion of human HSC by automated control



(a) Representative development of CD34-expression during expansion. On day 0, cells were enriched by MACS (one column) and sorted for $CD34^{high}/CD45^{low}$-expression (gate R4). Isolated cells were expanded on 6-well plates in cytokine-supplemented media with SCF, TPO, FLT3-lg, and IL-6. After 3 days, most cells still expressed significant amounts of CD34 on the surface while after 7 days, less than 20% could be classified as $CD34^{high}/CD45^{low}$.



(b) Expansion rates of total and CD34-positive cells. Within the first 3 days, total cell count increased 2.07-fold (±0.32) while only a slight change in CD34-expression could be observed, hence the amount of $CD34^+$ cells also expanded by factor 2.00 (±0.32). Within further 4 days of cytokine-driven expansion, the total cell count reached a value of 25.30-fold (±2.93) while expression of CD34 decreased dramatically, resulting in only a 4.17-fold (±0.32) expansion of cells classified as CD34 positive.

Figure 1: Umbilical cord blood cells were enriched by MACS and sorted for $CD34^{high}/CD45^{low}$-expression.

of inhibitory feedback mechanism [4], whereas the group of Delaney et al. is focusing on Notch-mediated expansion [5]. Although the $CD34^+$ expanded cell populations were well characterized, no clear protein data are available to define the changes in the $CD34^+$ population in the expanded product.

Mass spectrometry (MS)-based proteomics allows rapid assessment of changes in protein expression [6]. However, in spite of its potential, only few studies applied proteomics to obtain insights into biological processes within cord-blood-derived $CD34^+$ cells [7–12]. With a broad spectrum of different techniques, analysis of cell lysate led to the identification of up to 370 proteins [10] in native $CD34^+$ cord blood cells. Nevertheless, biological processes occurring during *ex vivo* expansion need further elucidation.

While these studies were limited to a detection of qualitative description of native $CD34^+$ cells, emerging techniques are in the position to identify more than thousands of proteins within a cell and also to quantify these proteins in parallel. In contrast to labeling-based quantification techniques such as ICAT, iTRAQ, TMTs or SILAC, label-free quantification avoid any additional sample preparation step and allows direct analysis of clinical specimen [13–15]. Therefore, a label-free approach was chosen to profile expression changes of *ex vivo* expanded $CD34^+$ haematopoetic stem/progenitor cells derived from umbilical cord blood.

This is the first report applying label-free proteomics to reveal proteomic changes during *ex vivo* expansion of $CD34^+$ haematopoetic stem/progenitor cells derived from umbilical cord blood. The results clearly document significant changes towards a protein biosynthesis phenotype already 3 days after expansion not reflected by the immunophenotype.

2. Material and Methods

2.1. Collection of Cord Blood. CB was collected with the informed consent of the mother according to established methods [16]. Briefly, after delivery of the baby, the cord was doubly clamped and transected, and the blood was collected in special collection bags containing citrate-phosphate dextrose. In the experiments performed, only CB units not suitable for banking due to exclusion criteria, such as low cell number or low volume, were used (ethics committee approval no. 2975).

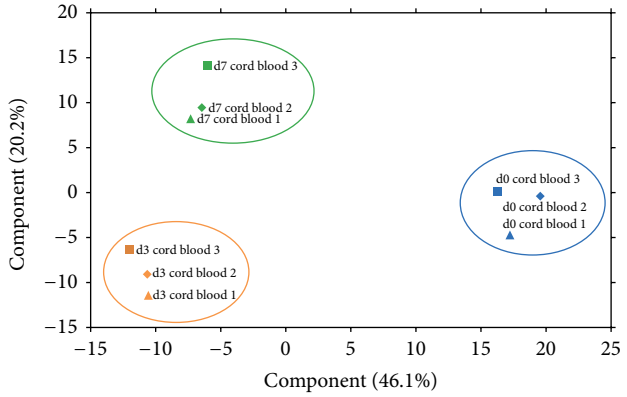


Figure 2: Principal component analysis (PCA) of $CD34^+$cells, showing that the three time points are distinct. Based on protein expression data, the largest variance was observed between day 0 and day 3; compared to this, the variation between day 3 and day 7 is smaller. Only little variances were observed between the three different cord blood donors.

Table 1: Number of proteins with expression profiles from day 0 to day 7. Discrimination between early, late, and long-term changes as well as between transient and permanent regulations shows that most significant changes occur early and remain regulated permanently.

Expression profile			Proteins
Early transient	Up		17
	Down		25
Early permanent	Up		167
	Down		151
Late	Up		38
	Down		6
Long term	Up		21
	Down		15
No regulation			903
	In total		1343

2.2. Isolation of $CD34^+$-Positive Haematopoetic Cells. CD34-positive cells were isolated using a two-step procedure of magnetic activated cell sorting (MACS) and fluorescence activated cell sorting (FACS).

Firstly, mononuclear cells were isolated using Ficoll-based density centrifugation (Biochrom; Berlin, Germany) as described in previous publications [17]. Remaining erythrocytes were removed by ammonium-chloride lysis (10 min.; 4°C), and cells were washed twice with phosphate buffered saline. Subsequent enrichment of $CD34^+$cells was performed by antibody labeling with paramagnetic beads against the CD34-epitope (Indirect CD34 Micro Bead Kit) and by application of LS-columns and MIDI-MACS magnets (all Miltenyi; Bergisch Gladbach, Germany) according the manufacturer's instructions.

Secondly, in order to achieve 100% purity, the enriched cells (after one MACS-column) were stained with antibodies against CD34 (PE-conjugated) and CD45 (FITC-conjugated; both BD Biosciences; San Jose, USA) and sorted for the surface expression of $CD34^{high}/CD45^{low}$ using a MoFlo XDP cell sorter (Beckman Coulter; Fullerton, USA) at the Core Flow Cytometry Center at the Düsseldorf University Medical Centre.

2.3. Expansion of $CD34^+$-Positive Haematopoetic Cells. The isolated $CD34^+$cells were seeded at a density of 2^*10^5/3 ml/well on 6-well plates and incubated at standard cell culture conditions (37°C, 5% CO_2, humidified air) in expansion media consisting of DMEM (Lonza; Basel, Switzerland) with 30% fetal calve serum (Gibco; Karlsruhe, Germany), penicillin/streptomycin/glutamine (all Lonza), and cytokines (50 ng/mL SCF, 20 ng/mL FLT3 lg, 50 ng/mL IL-6, 10 ng/mL TPO; all R&D Systems; Minneapolis, USA). On days 3 and 7, respectively, total cells were harvested, enumerated by Neubauer-chamber, and, after washing with PBS two times, used for protein isolation. In parallel, an aliquot of app. 5^*10^4 cells was stained against CD34/CD45 for flow cytometric analysis (FACSCanto; BD Biosciences).

2.4. Cell Lysis and Sample Preparation. For protein extraction, cells were lysed and homogenised in lysis buffer (2 M thiourea, 7 M urea, 30 mM Tris-HCl, pH 8.0). After determination of protein concentration using Bradford assay 10 μg of proteins were loaded on a SDS-PAGE. After complete entry into the gel, the SDS-PAGE was stopped after 5 min. Gels were silver stained according to [18] and one band per sample was cut out. Bands were destained and washed, and proteins were digested with trypsin (Promega, Mannheim, Germany).

2.5. Mass Spectrometric Analysis. Extracted peptides were analysed in a shuffled batch design with a HPLC (RSLCnano U3000, Thermo Fisher Scientific, Bremen, Germany) online coupled to a LTQ Orbitrap Elite (Thermo Fisher Scientific) mass spectrometer.

Samples were loaded on a trap column (Acclaim PepMap C18; 2 cm × 100 μm × 5 μm, 100 Å, Thermo Fisher Scientific) and washed with 0.1% TFA for 10 minutes. Trap column was switched online with separation column (Acclaim PepMap RSLC C18; 25 cm × 75 μm × 2 μm, 100 Å, Thermo Fisher Scientific) and peptides were separated for 120 minutes using a gradient of A: 0.1% FA and B: 0.1% FA, 84% ACN. The gradient started with 4% B and rose up to 40% B, followed by a washing step with 95% B for 5 minutes. Peptides were ionized via electrospray (1.2 keV). During chromatographic separation, the LTQ Orbitrap Elite was operated in a TOP20 data-dependent mode. MS-spectra were measured in the Orbitrap, mass range 350–1700 m/z, polysiloxane (445.120030 Da) as lock mass. From up to 20 most intense ions MS/MS spectra after collision induced dissociation in the iontrap were acquired. Dynamic exclusion of already measured ions was enabled.

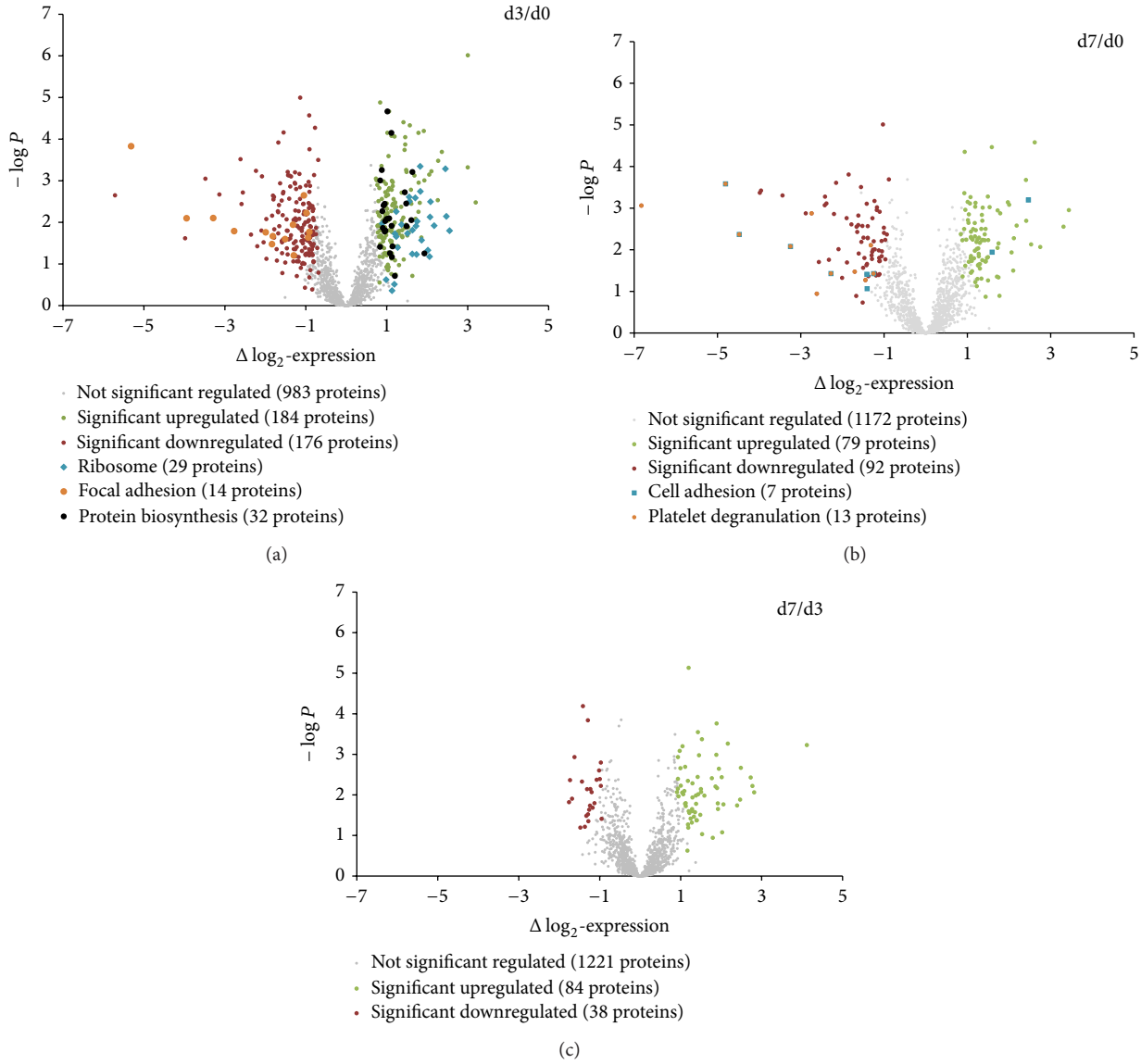


FIGURE 3: Volcano plot of pairwise differences in protein abundance. Significant regulated proteins must show differences in $\log_2$-differences in protein abundance (x-axis) and in the –log, P value (y-axis). Proteins with biological processes like focal adhesion or protein biosynthesis showed a strong regulation between day 0 and day 3.

2.6. Identification and Quantification of Proteins. For peptide and protein identification as well as for label-free quantification we used MaxQuant (Version 1.2.7.4) associated with Andromeda [19, 20]. MS/MS spectra were searched against the Uniprot/Swissprot-Database (human entries only, date 13/06/2012) with a mass tolerance of 10 ppm or 0.5 Da in MS and MS/MS mode, respectively. Oxidation of methionine as well as N-terminal acetylation as variable modifications were considered during database search. The false discovery rate (FDR) on peptide and protein level was below 1%. Known contaminants and reverse entries were removed from the protein list.

A minimum of 2 unique peptides were required for identification as well as for quantification. All proteins were quantified only based on unique peptides. Furthermore, only proteins identified in all experiments were regarded for quantification.

Statistical analysis was performed using Perseus as part of MaxQuant. R-based two sample t-tests (FDR <0.05, $S0$ = 0.8) between all conditions were used to declare a protein regulation as significant. $\log_2$-expression values were plotted against the P value for graphical clarification of significant protein regulation (Figure 3). Fisher's exact test was used to extract enriched GO-Terms, KEGG-Pathways, and Uniprot-Keywords (P value < 0.001, enrichment >2.3).

A list with all identified proteins, peptides (included modified peptides), and label-free quantification values with detailed quantification parameter is presented in Supplementary Table 2 (see Supplementary Materials available online at http://dx.doi.org/10.1155/2013/245695).

Table 2: Examples of enriched biological processes in significant regulated proteins.

Category	Number of proteins regulated (in total)	Enrichment factor	P value	Regulated proteins
Early permanent: upregulated				
Serine family amino acid metabolic process[1]	4 (4)	8.04	$2.3E-04$	PHGDH; PSPH; SHMT1; SHMT2
Translational initiation[1]	28 (34)	6.62	$3.7E-21$	EIF2B2; EIF2B4; EIF3B; EIF3E; EIF3H; EIF3K; EIF4G1; EIF4G2; EIF5B; PABPC1; RPL12; RPL13; RPL14; RPL17; RPL18; RPL22; RPL27; RPL3; RPL38; RPL4; RPL7; RPL7A; RPL9; RPLP0; RPLP0P6; RPS10; RPS10P5; RPS13; RPS16; RPS18; RPS19; RPS2; RPS25; RPS3; RPS3A; RPS4X; RPS7; RPS8; RPSA; RPSAP58
Ribosome[2]	27 (33)	6.58	$2.9E-20$	PWP2; RPL12; RPL13; RPL14; RPL17; RPL18; RPL22; RPL27; RPL3; RPL38; RPL4; RPL7; RPL7A; RPL9; RPLP0; RPLP0P6; RPS10; RPS10P5; RPS13; RPS16; RPS18; RPS19; RPS2; RPS25; RPS3; RPS3A; RPS4X; RPS7; RPS8; RPSA; RPSAP58
Translational elongation[1]	28 (35)	6.43	$1.7E-20$	GFM1; RPL12; RPL13; RPL14; RPL17; RPL18; RPL22; RPL27; RPL3; RPL38; RPL4; RPL7; RPL7A; RPL9; RPLP0; RPLP0P6; RPS10; RPS10P5; RPS13; RPS16; RPS18; RPS19; RPS2; RPS25; RPS3; RPS3A; RPS4X; RPS7; RPS8; RPSA; RPSAP58
Cellular process involved in reproduction[1]	29 (42)	5.55	$3.4E-18$	CDK1; RPL12; RPL13; RPL14; RPL17; RPL18; RPL22; RPL27; RPL3; RPL38; RPL4; RPL7; RPL7A; RPL9; RPLP0; RPLP0P6; RPS10; RPS10P5; RPS13; RPS16; RPS18; RPS19; RPS2; RPS25; RPS3; RPS3A; RPS4X; RPS7; RPS8; RPSA; RPSAP58; UBAP2L
Protein targeting to ER[1]	27 (40)	5.43	$1.4E-16$	RPL12; RPL13; RPL14; RPL17; RPL18; RPL22; RPL27; RPL3; RPL38; RPL4; RPL7; RPL7A; RPL9; RPLP0; RPLP0P6; RPS10; RPS10P5; RPS13; RPS16; RPS18; RPS19; RPS2; RPS25; RPS3; RPS3A; RPS4X; RPS7; RPS8; RPSA; RPSAP58
Initiation factor[3]	17 (29)	4.71	$2.4E-09$	EIF2A; EIF2B2; EIF2B4; EIF2S1; EIF2S2; EIF3A; EIF3B; EIF3C; EIF3CL; EIF3D; EIF3E; EIF3EIP; EIF3L; EIF3H; EIF3K; EIF4A1; EIF4G1; EIF4G2; EIF5B
Protein biosynthesis[3]	31 (64)	3.90	$2.9E-13$	AARS; CARS; EEF1A1; EEF1A1P5; EEF1A2; EEF1D; EEF1G; EEF2; EIF2A; EIF2B2; EIF2B4; EIF2S1; EIF2S2; EIF3A; EIF3B; EIF3C;EIF3CL; EIF3D; EIF3E; EIF3EIP; EIF3L; EIF3H; EIF3K; EIF4A1; EIF4G1; EIF4G2; EIF5B; EPRS; ETF1; GARS; GFM1; IARS; MARS; NARS; TARS
Ribonucleoprotein[3]	28 (78)	2.89	$2.2E-08$	MRPS27; RPL12; RPL13; RPL14; RPL17; RPL18; RPL22; RPL27; RPL3; RPL38; RPL4; RPL7; RPL7A; RPL9; RPLP0; RPLP0P6; RPS10; RPS10P5; RPS13; RPS16; RPS18; RPS19; RPS2; RPS25; RPS3; RPS3A; RPS4X; RPS7; RPS8; RPSA; RPSAP58
RNA transport[2]	22 (66)	2.68	$3.4E-06$	EEF1A1; EEF1A1P5; EEF1A2; EIF2B2; EIF2B4; EIF2S1; EIF2S2; EIF3A; EIF3B; EIF3C; EIF3CL; EIF3D; EIF3E; EIF3H; EIF4A1; EIF4G1; EIF4G2; EIF5B; ELAC2; GEMIN5; NUP93; PABPC1; PABPC4; RANGAP1; XPOT
Cellular macromolecule catabolic process[1]	43 (145)	2.38	$2.0E-09$	CDK1; EIF3E; EIF4A1; EIF4G1; ETF1; FEN1; PABPC1; PABPC4; PSMC1; PSMC2; PSMD1; PSMD2; PSMD3
ncRNA metabolic process[1]	21 (71)	2.38	$4.3E-05$	AARS; CARS; ELAC2; EPRS; FTSJ3; GARS; GEMIN5; IARS; MARS; NARS; NOP2; PDCD11; RPL14
Early permanent: downregulated				
Platelet degranulation[1]	13 (21)	5.43	$2.8E-08$	ACTN1; CALM2; F13A1; FLNA; ITGA2B; ITGB3; PECAM1; PPBP; THBS1; TLN1; TUBA4A; VCL; WDR1
Focal adhesion[2]	13 (22)	5.19	$6.2E-08$	ACTN1; CRKL; FLNA; FLNB; ITGA2B; ITGB3; MYL12A; PPP1R12A; RAP1B; THBS1; TLN1; VASP; VCL
Exocytosis[1]	14 (24)	5.12	$2.3E-08$	ACTN1; CALM2;CALM1; F13A1; FLNA; ITGA2B; ITGB3; PECAM1; PLEK; PPBP; SCRN1; SNCA; THBS1; TLN1; TUBA4A; VCL; WDR1
Calmodulin binding[3]	6 (11)	4.79	$5.2E-04$	ADD3; IQGAP2; PPP3CA; SPTAN1; SPTBN1; STRN

Table 2: Continued.

Category	Number of proteins regulated (in total)	Enrichment factor	*P* value	Regulated proteins
Organic acid catabolic process[1]	14 (38)	3.23	2.4*E* − 05	ACADM; ALDH6A1; BCKDHA; CPT2; DDAH2; DECR1; DLST; ECI1; DCI; ETHE1; GLUD1; GLUD2; HADHB; HIBCH; IVD; MCCC1
Cell activation[1]	16 (48)	2.93	2.6*E* − 05	ACTN1; ADA; CALM2; CALM1; F13A1; FLNA; ITGA2B; PECAM1; PLEK; PPBP; PPP3CA; RAP1B; RAP1A; THBS1; TLN1; TUBA4A; VCL; WDR1; YWHAZ
Late regulated: upregulated				
Cell adhesion molecules (CAMs)[2]	3 (5)	21.21	2.0*E* − 04	HLA-DRA; ITGAL; ITGB2;
Glycoprotein[3]	11 (76)	5.12	2.9*E* − 06	ASAH1; CD74; GLB1; HLA-DRA; ITGAL; ITGB2; MPO; PLD3; PRTN3; RNASET2; VIM;
Signal[3]	9 (67)	4.75	5.0*E* − 05	ASAH1; IL-25; CD74; GLB1; HLA-DRA; ITGAL; ITGB2; MPO; PLD3; PRTN3; RNASET2
Late regulated: downregulated				
No significant enriched GO-Terms due to protein number <30				
Long term: up- and downregulated				
No significant enriched GO-Terms due to protein number <30				
Early transient: up- and downregulated				
No significant enriched GO-Terms due to protein number <30				

Annotations for biological processes (GOBP[1]) KEGG[2], and Uniprot Keywords[3] of regulated proteins were compared with proteins in the whole dataset. Enriched categories have at least an enrichment factor >2.3 and a *P* value of less than 0.001. Due to multiple functionalities of proteins, some proteins are listed in multiple categories.

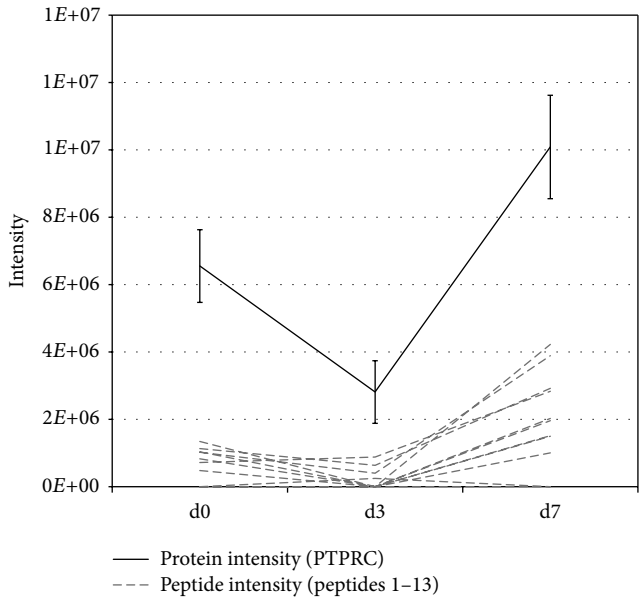


Figure 4: Expression of PTPRC. The labelfree intensity of PTPRC (also known as CD45) is quantified based on 13 unique peptides with similar expression profiles. A significant down regulation with a fold change of −2.33 was observed between day 0 and day 3. From day 3 to day 7 a significant up regulation with a fold change of +3.6 was observed.

3. Results

The application of cord blood cells for clinical trials and transplantation is often limited by low amount of $CD34^+$ haematopoetic stem cells [1]. Therefore, *ex vivo* expansion is often used to increase the number of $CD34^+$cells. To monitor changes of $CD34^+$cells during *ex vivo* expansion we chose a proteomic approach allowing us to profile changes on protein level and give insight into altered biological processes. Here, we analysed $CD34^+$cells isolated under GMP-conditions and *ex vivo* expanded for up to seven days. Whole cell lysates of FACS-isolated $CD34^+$cells (day 0) and subsequently expanded cells were analysed using label-free MS analysis of day 3 and day 7, respectively.

3.1. Ex Vivo Expansion of $CD34^+$ Haematopoetic Stem/Progenitor Cells. Following expansion in cytokine-supplemented media (SCF, TPO, FLT3-lg, IL-6) cell numbers of $CD34^+$ haematopoetic stem/progenitor cells increased by factors 2.1 and 4.2 after 3 and 7 days, respectively (Figure 1(b)). It is interesting to note that after 3 days the $CD34^+$cells mainly dominate the cell population (amount of $CD34^+$cells: 97.5%), whereas the $CD34^+$cells reflected by a total increase of cell number by factor 25.3 were underrepresented after 7 days (amount of $CD34^+$cells: 16.6%). As preliminary experiments revealed, appliance of this expansion protocol also increased the amount of colony-forming units approximately by factor 2 on day 3 and by factor 6 on day 7, respectively (Supplementary Figure 1).

3.2. Proteome Profiling of Ex Vivo Expansion. For proteome profiling we exploit the advantage of label-free proteome analysis without prior protein labelling allowing us to quantify and identify complex protein mixtures on the same analytical platform. The whole cell lysates of three CB donors isolated at day 0 (after withdrawal and FACS enrichment), day 3, and day 7 after *ex vivo* expansion were analysed. Altogether we identified 2970 unique proteins. Detailed Gene Ontology (GO) annotation showed that we identified proteins from almost all cellular compartments (nucleus, cytosol, Golgi apparatus, cytoskeleton, membrane, extracellular region, and more) involved in 65 different biological processes (based on GO slim terms, data not shown). For further analysis of proteome changes during *ex vivo* expansion we only considered 1343 proteins quantified in all three donors and time points.

Principal compound analysis (PCA) clearly demonstrates that the greatest variance of the datasets was obtained from the analysed time points and not from individual donors (Figure 2). By analysing the reproducibility of the LC-MS system we could exclude system inherent influence. The Pearson correlation of $\log_2$-expression value between the technical replicates on day 0 was 0.967.

Statistical analysis of protein abundance by ANOVA analysis of 1343 proteins revealed that 903 of the identified proteins (67.2%) showed no significant regulation at any time point, whereas 440 proteins showed significant changes along the analysed expansion time (Figure 3). In agreement with the PCA, we observed the greatest number of expression changes already after 3 days (time point d3) in an early stage of *ex vivo* expansion (Table 1). In total 360 proteins were significantly regulated after 3 days. From these proteins 318 proteins were also found significantly changed on day 7 and exhibited an "early-permanent" effect during *ex vivo* expansion. In comparison to this group of proteins we observed 42 proteins with "early-transient" abundance change. From day 3 to day 7 a small group of 80 proteins showed alterations in abundance level in late stage of *ex vivo* expansion.

To describe the changes on protein level, we defined five different expression profiles (Table 2): "Early-transient" and "early-permanent" reflect early changes in the proteome of $CD34^+$ haematopoetic stem/progenitor cells, while "late" as well as "long term" reflect changes during longer expansion time. Within the group of early transient upregulated proteins (in total 17 proteins) we found the transferrin receptor protein 1 (CD71) 7.6-fold upregulated on day 3 and 2.3-fold downregulated on day 7. An equal number of 36 proteins were detected to be long-term regulated (21 up, 15 down). Representative proteins are the actin-binding protein tropomodulin-3, the cyclin-dependent kinase 6 (both long term downregulated), or two tRNA-ligases responsible for amino acid activation (VARS and WARS, both long-term upregulated).

3.3. Biological Processes Involved in Ex Vivo Expansion. Next we were interested to extract the biological information of the proteome changes upon *ex vivo* expansion. Therefore, we searched for enriched Gene Ontology terms (molecular function and biological processes), KEGG-Pathways, and Uniprot Keywords within the regulated groups of proteins (Table 2, Figure 3).

Within the group of early permanent upregulated proteins biological processes linked to translation (initiation/elongation), ribosomal activity, and protein biosynthesis are significantly enriched. From 1343 proteins, 33 are linked with the KEGG-pathway "ribosome," whereof 27 are upregulated with a fold change of 1.85 to 5.51. Significant enrichment could also be found for the Uniprot Keyword "protein biosynthesis." 31 proteins out of 64 proteins in the whole dataset showed a significant up-regulation. We identified several translation initiation factors (e.g., EIF2A, EIF3A, EIF4A, and EIF5B) as well as 10 tRNA-ligases in the aminoacyl-tRNA-biosynthesis pathway (e.g., GARS, MARS, and NARS) being permanently upregulated.

The proteins which were found permanently downregulated during *ex vivo* expansion revealed an enrichment of the KEGG-pathway focal adhesion (e.g., integrin α-2b/β-3 and filamin A/B), the GO-Term exocytosis as well as platelet degranulation (e.g., platelet basic protein).

As the number of proteins regulated between days 3 and 7 was smaller compared to day 0/day 3 (Figure 3), less categories with proteins regulated only in a later stage during *ex vivo* expansion were significantly enriched. We observed cell adhesion molecules (HLA-DRA, ITGAL—known as CD11a, ITB2 - known as CD18) as well as the general term of "glycoproteins" (e.g., CD74 and VIM) being enriched in the group of late time up-regulated proteins.

Furthermore, we considered the group of protein transiently or long-term changed during *ex vivo* expansion. Due to a lower number of proteins in these groups, a significant enrichment of biological processes was not observed. Nevertheless, proteins with this expression profiles can be relevant for the understanding of *ex vivo* expansion of $CD34^+$cells. For example, we quantified receptor-type tyrosine-protein phosphatase C (PTPRC), also known as CD45. PTPRC was quantified based on 13 peptides with similar expression profiles (Figure 4) falling into the category of early transient down-regulated proteins. This protein showed a significant lower expression (fold change −2.3) on day 3, but interestingly the abundance of CD45 in the day 7 population was 3.6-fold higher than on day 3 and therewith PTPRC almost reached a higher abundance than at day 0. These findings are consistent with the observed number of $CD45^+$ cells during FACS (Figure 1(a)), where the number of $CD45^+$ cells was lower on day 3 compared to day 0 as well as day 7.

3.4. Cytometric Validation of Selected Surface Marker. For the validation of identified candidate proteins we performed flow cytometry of selected surface markers. The data for CD13 as well as for CD71 can be found in supplementary Figure 2. $CD34^+$cells displayed a medium expression of CD13 already on day 0 which significantly increased by day 3 and then slightly declined by day 7. CD71, on the other hand, was only weakly expressed on day 0, increased by day 3, and stayed stable up to day 7. These observations are congruent to the intensity values obtained by label-free quantification

on proteome level, where CD13 shows an early upregulated profile and CD71 a long-term upregulated profile.

3.5. Influence of Cytokines Used for Ex Vivo Expansion. In our dataset we searched explicitly for proteins linked with the cytokines used for *ex vivo* expansion, in particular SCF, TPO, FLT3-lg, and IL-6. No permanent influence on direct interaction partners could be observed. We used STRING [21] and Ingenuity Pathway Analysis (Ingenuity systems, http://www.ingenuity.com/) to search for regulated downstream effectors or a general regulation of cytokine regulated pathways, but detected no significant changes on protein level, neither after 3 days nor after 7 days of expansion. The same is true for the NOTCH-signaling pathway, which is known to be linked with *ex vivo* expansion of $CD45^+$ cells [5], where no regulation was observed.

4. Discussion

Application of cytokine-supplemented media results in fast and effective expansion of haematopoietic cells [1]. In our experiments on the *ex vivo* expansion of cord-blood-derived $CD34^+$cells by application of cytokines, total cell count increased within the first 3 days by approximately factor 2. Nearly all cells still expressed the CD34-epitope on the surface, without any distinct morphological change or difference in flow cytometric analysis in comparison to native $CD34^+$cells.

In-depth proteomics identified 2970 unique proteins in cell lysates stemming from *ex vivo* expanded $CD34^+$cells derived from umbilical cord blood. This is—to our knowledge—by now the most detailed view on protein level for these cells along *ex vivo* expansion time of seven days. Of 290 proteins described by [12] in native $CD34^+$cells we confirmed 221 proteins. Label-free quantification of 1343 proteins indicates major changes in the proteome along the expansion time.

However, proteomic assessment revealed a significant change in the protein profile already at day three of expansion. In particular proteins that are related to the family of translation factors, ribosomal activity and cell cycle function are upregulated. This is in accordance with the increasing cell count observed. In contrast to this, early downregulated proteins are linked with specific focal adhesion/cytoskeleton remodelling functions (e.g., ACTN1, ITGB3, TLN1, and VASP). Although native $CD34^+$cells are of rather nonadherent character under *in vitro* culture conditions, they still bear the intrinsic potential to engraft into tissue (e.g., to home into bone marrow). Therefore, the decrease of proteins with extracellular matrix linkage might be explained by a loss of this property due to differentiation of the cells and subsequent reduced ability to engraft.

During continued expansion, the cell number increased further, but flow cytometric assessment on day 7 suggests a potential differentiation of the cells and showed that less than 20% of the cells still express CD34. Additionally the presence of a population of $CD34^{high}$-expressing cells (Figure 1), which is specific for most mature leukocytes, was observed. This variation is also reflected by a change in the CD45-protein expression profile (Figure 4). It is interesting to note that in PCA, this shift does not result in a higher difference for day 3/day 7 compared to day 0/day 3 (as depicted in Figure 2). Although the expansion rate of $CD34^+$cells from day 0 to day 3 is on the same level than of day 3 to day 7 (Figure 1(b)), we conclude that the day-3 cells are already undergoing severe changes. Furthermore, most proteins and associated pathways were permanently changed over the analysed expansion time, as only minor changes between later time points were observed. As no regulation of direct interaction partners or downstream effectors of the cytokines used during expansion could be observed, we propose that the described changes could be more independent from the used expansion protocol than believed so far. Further insight might be gained by applying targeted proteomics techniques with a higher sensitivity against special pathways (e.g., NOTCH pathway).

Late effects on the protein level are in particular the higher abundance of specialised cell adhesion molecules. While the change in the CD pattern observed in flow cytometry (CD45-expression) on day 7 is confirmed by proteomics (not only of CD45 and CD34, but also CD11, CD18, and CD74), assessment of protein expression clearly demonstrates that severe changes occur much earlier.

Interestingly, several proteins within the late regulated group of proteins are linked with leukocyte or lymphocyte activation (DUSP3, HLA-DRA, PAK1, CD45, and SYK). Since a potential differentiation might result in an impaired ability to engraft in a patient, but might otherwise lead to a faster reconstitution, further examination of these effects is highly recommended. Future aspects should involve assessment of CD34 subpopulations, such as $CD34^+/CD38^-$cells, which are considered as the most primitive haematopoetic progenitor, by resorting after expansion. It also should be examined, whether variations in cytokines applied can direct predifferentiation into specific directions (e.g., either myeloid or lymphoid).

5. Conclusion

Ex vivo expansion of cord-blood-derived $CD34^+$cells by application of cytokine-supplemented media results in fast and effective expansion of haematopoetic cells and therefore is an interesting approach that is intensively pursued. Proteomic profiling of expanded cells revealed early upregulation of proteins related to protein biosynthesis but downregulation of focal adhesion, exocytosis proteins within three days.

Though, it can be argued that this procedure may alter the properties of the haematopoetic stem/progenitor cells. While a direction on day 3 could not be distinguished based on our data, there are indications for a general leukocyte and/or lymphocyte differentiation in further cultivated cells, which is not accessible by flow cytometry.

Taking this into account, questions arise concerning the effectiveness of the expansion in regard to maintenance of the undifferentiated state of the haematopoetic cells.

Authors' Contribution

H. Falkenberg and T. F. Radke contributed equally to the paper.

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References

[1] S. S. Kelly, C. B. S. Sola, M. De Lima, and E. Shpall, "Ex vivo expansion of cord blood," *Bone Marrow Transplantation*, vol. 44, no. 10, pp. 673–681, 2009.

[2] J. E. Wagner and E. Gluckman, "Umbilical cord blood transplantation: the first 20 years," *Seminars in Hematology*, vol. 47, no. 1, pp. 3–12, 2010.

[3] M. N. Fernández, "Improving the outcome of cord blood transplantation: use of mobilized HSC and other cells from third party donors," *British Journal of Haematology*, vol. 147, no. 2, pp. 161–176, 2009.

[4] E. Csaszar, D. C. Kirouac, M. Yu, W. Wang et al., "Rapid expansion of human hematopoietic stem cells by automated control of inhibitory feedback signaling," *Cell Stem Cell*, vol. 10, no. 2, pp. 218–229, 2012.

[5] C. Delaney, S. Heimfeld, C. Brashem-Stein, H. Voorhies, R. L. Manger, and I. D. Bernstein, "Notch-mediated expansion of human cord blood progenitor cells capable of rapid myeloid reconstitution," *Nature Medicine*, vol. 16, no. 2, pp. 232–236, 2010.

[6] M. Bantscheff, S. Lemeer, M. M. Savitski, and B. Kuster, "Quantitative mass spectrometry in proteomics: critical review update from 2007 to the present," *Analytical and Bioanalytical Chemistry*, vol. 404, no. 4, pp. 939–965, 2012.

[7] C. Zenzmaier, M. Kollroser, B. Gesslbauer, A. Jandrositz, K. H. Preisegger, and A. J. Kungl, "Preliminary 2-D chromatographic investigation of the human stem cell proteome," *Biochemical and Biophysical Research Communications*, vol. 310, no. 2, pp. 483–490, 2003.

[8] C. Zenzmaier, B. Gesslbauer, N. Grobuschek, A. Jandrositz, K. H. Preisegger, and A. J. Kungl, "Proteomic profiling of human stem cells derived from umbilical cord blood," *Biochemical and Biophysical Research Communications*, vol. 328, no. 4, pp. 968–972, 2005.

[9] W. Tao, M. Wang, E. D. Voss et al., "Comparative proteomic analysis of human CD34+ stem/progenitor cells and mature CD15+ myeloid cells," *Stem Cells*, vol. 22, no. 6, pp. 1003–1014, 2004.

[10] F. Liu, J. Lu, H. H. Fan et al., "Insights into human CD34+ hematopoietic stem/progenitor cells through a systematically proteomic survey coupled with transcriptome," *Proteomics*, vol. 6, no. 9, pp. 2673–2692, 2006.

[11] A. D'Alessandro, G. Liumbruno, G. Grazzini, S. Pupella, L. Lombardini, and L. Zolla, "Umbilical cord blood stem cells: towards a proteomic approach," *Journal of Proteomics*, vol. 73, no. 3, pp. 468–482, 2010.

[12] A. D'Alessandro, G. Grazzini, B. Giardina, and L. Zolla, "In silico analyses of proteomic data suggest a role for heat shock proteins in umbilical cord blood hematopoietic stem cells," *Stem Cell Reviews and Reports*, vol. 6, no. 4, pp. 532–547, 2010.

[13] K. A. Neilson, N. A. Ali, S. Muralidharan et al., "Less label, more free: approaches in label-free quantitative mass spectrometry," *Proteomics*, vol. 11, no. 4, pp. 535–553, 2011.

[14] M. C. Wiener, J. R. Sachs, E. G. Deyanova, and N. A. Yates, "Differential mass spectrometry: a label-free LC-MS method for finding significant differences in complex peptide and protein mixtures," *Analytical Chemistry*, vol. 76, no. 20, pp. 6085–6096, 2004.

[15] B. Sitek, D. M. Waldera-Lupa, G. Poschmann, H. E. Meyer, and K. Stühler, "Application of label-free proteomics for differential analysis of lung carcinoma cell line A549," *Methods in Molecular Biology*, vol. 893, pp. 241–248, 2012.

[16] G. Kögler, J. Callejas, P. Hakenberg et al., "Hematopoietic transplant potential of unrelated cord blood: critical issues," *Journal of Hematotherapy*, vol. 5, no. 2, pp. 105–116, 1996.

[17] G. Kögler, T. F. Radke, A. Lefort et al., "Cytokine production and hematopoiesis supporting activity of cord blood-derived unrestricted somatic stem cells," *Experimental Hematology*, vol. 33, no. 5, pp. 573–583, 2005.

[18] M. V. Nesterenko, M. Tilley, and S. J. Upton, "A simple modification of Blum's silver stain method allows for 30 minute detection of proteins in polyacrylamide gels," *Journal of Biochemical and Biophysical Methods*, vol. 28, no. 3, pp. 239–242, 1994.

[19] J. Cox and M. Mann, "MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification," *Nature Biotechnology*, vol. 26, no. 12, pp. 1367–1372, 2008.

[20] C. A. Luber, J. Cox, H. Lauterbach et al., "Quantitative proteomics reveals subset-specific viral recognition in dendritic cells," *Immunity*, vol. 32, no. 2, pp. 279–289, 2010.

[21] L. J. Jensen, M. Kuhn, M. Stark et al., "STRING 8—a global view on proteins and their functional interactions in 630 organisms," *Nucleic Acids Research*, vol. 37, no. 1, pp. D412–D416, 2009.