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Microarray profiles of *ex vivo* expanded hematopoietic stem cells show induction of genes involved in noncanonical Wnt signaling

D.L. Zanette[1,2], J.C.C. Lorenzi[1,2], R.A. Panepucci[1,2], A.R.D. Santos[1,2], G.A. Molfetta[1,2], A.G. Araujo[2,3], W.A. Silva Junior[1,2] and M.A. Zago[2,3]

[1]Departamento de Genética, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brasil
[2]Centro de Terapia Celular, Centro Regional de Hemoterapia, Ribeirão Preto, SP, Brasil
[3]Departamento de Clínica Médica, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brasil

Corresponding author: D.L. Zanette
E-mail: zanette@usp.br

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ABSTRACT. The low number of hematopoietic stem cells (HSC) in umbilical cord blood (UCB) is directly related to increased risk of transplant failure. Effective *ex vivo* expansion of HSC has been tried for many years, with conflicting results because of the inability to reproduce *in vitro* HSC proliferation in the same way it occurs *in vivo*. We compared freshly isolated HSC with their expanded counterparts by microarray analysis and detected activation of the noncanonical Wnt (wingless-type MMTV integration site family) pathway. Study of early alterations during *ex vivo* UCB-HSC expansion could contribute to improvement of *ex vivo* expansion systems.

Key words: Umbilical cord blood; Hematopoietic stem cell; Microarrays; *Ex vivo* expansion

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INTRODUCTION

Bone marrow hematopoietic stem cells (HSC) are traditionally used in hematopoietic transplantation, but HSC obtained from umbilical cord blood (UCB) (Kelly et al., 2009) have some advantages, such as availability from cryopreserved units and decreased risk of graft-versus-host disease. However, the low number of HSC in UCB is directly related to increased risk of graft failure and delayed engraftment and immune reconstitution. Effective *in vitro* expansion of HSC has been long tried, with conflicting reports of success (Gilmore et al., 2000; McNiece et al., 2000; Pranke et al., 2005; Walenda et al., 2011; Auvray et al., 2012). The failure of these protocols may be due to the inability to reproduce *in vitro* the combination of factors that occurs *in vivo*, where simultaneous HSC proliferation and maintenance of long-term hematopoietic reconstitution is achieved (Astori et al., 2001; Blank et al., 2008).

The transplantation of two UCB units, one *ex vivo* expanded and one unmanipulated, has proven to be effective. A promising clinical trial using *ex vivo* expanded UCB-HSC in the presence of the Notch ligand Delta1 results in enhanced initial myeloid engraftment, suggesting that these cultured cells promote long-term engraftment of the unmanipulated UCB unit that is concomitantly infused (Delaney et al., 2010).

The summarized gene expression profiles of expanded HSC and their unmanipulated counterparts reported here suggest activation of the noncanonical Wnt pathway. Study of these early alterations during expansion may contribute to a better understanding of HSC biology and to improvement of *ex vivo* expansion systems.

MATERIAL AND METHODS

$CD34^+$ cells were obtained from 13 UCB samples using magnetic beads (MACS, Miltenyi Biotec), after informed consent of the mother. Samples were split in 2 freshly isolated $CD34^+$ cells (t0) and 3-day *ex-vivo* expanded cells (t3), which were used for flow cytometry and RNA extraction. A FACScan (Becton Dickinson, San Diego, CA, USA) system was used to determine the starting $CD34^+$ purity (always >88%), $CD34^+/CD90^+$, $CD34^+/CD38^{neg}$, and cell viability by annexin-V and propidium iodide. All analyses were performed at t0 and t3, counting 10,000 events, with the Cellquest software (Becton Dickinson).

Cells were cultured under serum-free conditions, in FreeStyle 293 Expression Medium (Invitrogen, Carlsbad, CA, USA), supplemented with 1% human albumin and a cocktail of cytokines, based on previous reports (Pranke et al., 2005; Kelly et al., 2009): 50 ng/mL flt-3 ligand, 35 ng/mL thrombopoietin, 50 ng/mL stem cell factor, and 10 ng/mL interleukin-6.

Total RNA was isolated with TRIzol® LS Reagent (Invitrogen), following manufacturer recommendations. Gene expression profiles were obtained using Amersham CodeLink UniSet Human I BioArrays (GE Amersham Biosciences, Piscataway, NJ, USA) platform. We used 2 pools of unmanipulated $CD34^+$ cells (t0) and 2 pools of expanded cells (t3), each pool consisting of 4 different samples. The fluorescence values were normalized (75th percentile) and the fold-changes were averaged between the 2 pools from t3 and t0.

Validation of microarray results was performed with reverse transcribed cDNAs and TaqMan® probes in an ABI Prism 7500 Detection System (Applied Biosystems). Normalization was based on the geometric mean of the Ct values for 2 endogenous genes, ACTB and GUSB. The $2^{\Delta\Delta Ct}$ method (Pfaffl, 2001) was used to compare gene expression profiles of t3 and t0 cells,

with the latter used as the calibrator. Statistical analyses were performed using the GraphPad software (nonparametric, one-tailed Mann-Whitney test, 95% confidence interval, $P < 0.05$).

RESULTS

The expansion protocol resulted in an average 2.5-fold increase in the absolute number of $CD34^+$ cells ($P = 0.0001$; Figure 1A) and most cells preserved the surface $CD34^+$ expression in t3 (Figure 1B). The absolute number of $CD34^+/CD38^-$ cells increased 7.2-fold ($P = 0.0001$; Figure 2A). The percentage of $CD34^+/CD38^-$ cells was also increased ($P = 0.012$; Figure 2B). The absolute number of $CD34^+/CD90^+$ cells showed an average 5.9-fold increase, although not significant ($P = 0.15$) and there was no induction of cell death at t3 (data not shown).

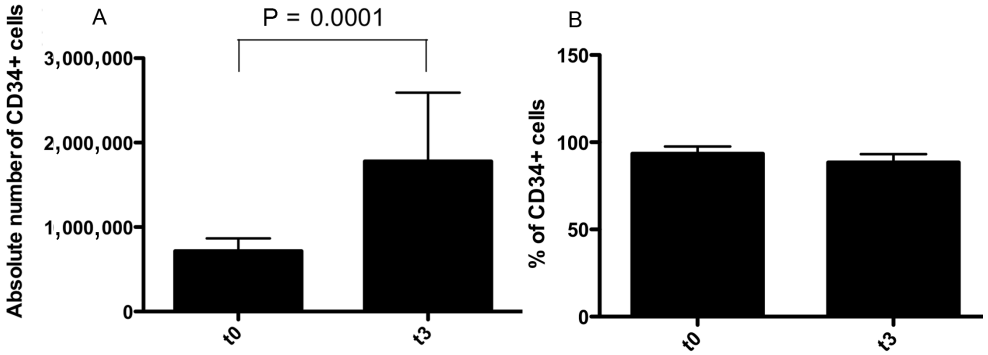

Figure 1. $CD34^+$ contents in unmanipulated (t0) and expanded (t3) cells and validation of microarray expression data of t0 and t3 cells. **A.** Absolute number of $CD34^+$ cells at t0 and t3 ($P = 0.0001$). **B.** Percentage of CD34+ cells at t0 and t3 ($P = 0.006$).

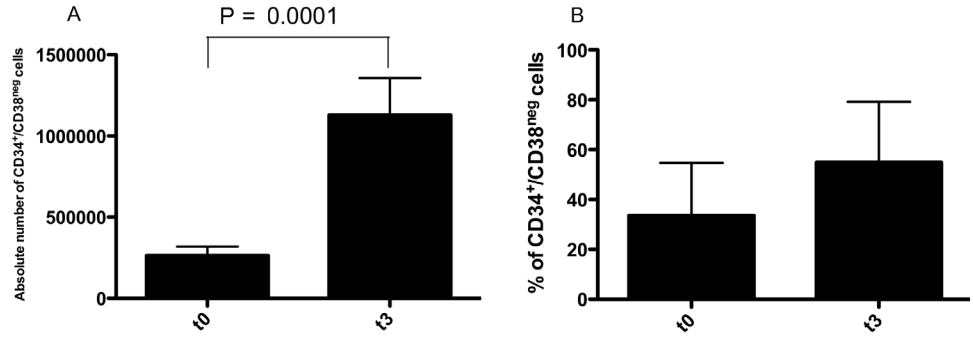

Figure 2. $CD34^+/CD38^{neg}$ contents in unmanipulated (t0) and expanded (t3) cells and validation of microarray expression data of t0 and t3 cells. **A.** Absolute number of $CD34^+/CD38^{neg}$ at t0 and t3 ($P = 0.0001$). **B.** Percentage of $CD34^+/CD38^-$ cells at t0 and t3 ($P = 0.012$).

The top 25 down- and up-modulated genes found in t3 expanded cells, according to the microarrays, were determined (Tables 1 and 2). Among the top 25 up-regulated genes, there were genes involved in cell division and proliferation, while among down-regulated genes, we

found modulators of homing, such as chemokine CXCR4 (C-X-C motif receptor 4), HIF1A (hypoxia inducible factor 1 alpha subunit), SERPINA1 (serpin peptidase inhibitor 1), PDE4B (phosphodiesterase 4B, cAMP-specific), and RGS1 (regulator of G-protein signaling 1).

Table 1. Top 25 of negatively regulated genes in expanded cells (t3) compared to unmanipulated cells (t0).

Gene symbol	Description	Fold-change t3/t0
TWF1	PTK9 protein tyrosine kinase 9 (PTK9)	-502.1
KIFC3	kinesin family member C3	-161.2
IFI44L	interferon-induced protein 44-like	-61.9
TPPP3	tubulin polymerization-promoting, member 3	-56.7
FBXW7	F-box and WD-40 domain protein 7 (FBXW7), transcript var. 1	-51.1
RGS1	regulator of G-protein signalling 1	-49.5
CXCL10	chemokine (C-X-C motif) ligand 10	-97.9
SIK1	salt induced kinase 1	-37.4
SERPINA1	serine (or cysteine) proteinase inhibitor, member 1	-24.9
MS4A1	membrane-spanning 4-domains, subfamily A, member 1	-19.9
PDE4B	phosphodiesterase E4 dunce homolog, *Drosophila*	-16.1
ELAVL4	ELAV (embryonic lethal, abnormal vision, *Drosophila*)-like 4	-15.5
VPREB3	pre-B lymphocyte gene 3	-15.2
CLEC4E	C-type lectin domain family 4, member E	-13.9
S100A12	S100 calcium binding protein A12 (calgranulin C)	-13.7
TMSB15A	thymosin beta 15A	-13.6
KLF2	Kruppel-like factor 2	-13.3
CD69	CD69 antigen	-13.3
RAG2	recombination activating gene 2	12.5
HIST2H2BE	histone 2, H2be	-12.5
STK17B	serine/threonine kinase 17b	-12.2
PLBD1	phospholipase B domain containing 1	-10.9
XAF1	XIAP associated factor-, transcript var. 1	-10.8
DUSP1	dual specificity phosphatase 1	-9.9
CXCR4	chemokine (C-X-C motif) receptor 4 transcript var. 2	-9.2

Table 2. Top of 25 positively regulated genes in expanded cells (t3) compared to unmnaipulated cells (t0).

Gene symbol	Description	Fold-change t3/t0
ADAM18	a disintegrin and metalloproteinase domain 18	44.1
PHLDA1	apoptosis-associated nuclear protein PHLDA1	42.1
BBC3	BCL2 binding component 3	33.7
KIF2C	kinesin family member 2C	24.9
TAS2R1	taste receptor, type 2, member 1	21.5
STAT2	signal transducer and activator of transcription 2	21.1
INMT	indolethylamine N-methyltransferase	19.7
AFG3L2	AFG3 ATPase family gene 3-like 2	19.6
WNT5B	wingless-type MMTV integration site family, member 5B	17.7
ITIH1	inter-alpha (globulin) inhibitor H1	17.1
XPO7	exportin 7	14.2
SMAD6	SMAD, mothers against DPP homolog 6	12.1
GAL	galanin	11.9
PCK2	phosphoenolpyruvate carboxykinase 2 (mitochondrial)	11.5
PLK1	polo-like kinase 1 (*Drosophila*)	10.8
PHLDA2	pleckstrin homology-like domain, family A, member 2	10.6
CYLC1	*H. sapiens* cylicin mRNA	10.2
C1orf106	chromosome 1 open reading frame 106	9.3
LAT	linker for activation of T cells	9.3
MT1F	metallothionein 1F (functional)	9.1
RBP4	retinol binding protein 4, plasma	8.9
AURKB	aurora kinase B	8.8
IL9R	interleukin 9 receptor (IL9R)	8.7
HIG2	hypoxia-inducible protein 2	8.5
HBD	hemoglobin, delta	8.3

Twelve genes were validated by qPCR: five genes of the Wnt pathway: WNT5A, WNT5B, WNT8A (wingless-related MMTV integration site 5A, 5B and 8A), GSK3B (glycogen synthase kinase 3 beta) and DKK1 (dickkopf 1 homolog). Among the down-regulated genes, CXCR4, HOXB4 (homeobox B4) and BMI1 (polycomb ring finger oncogene) were validated. Expression of SMAD6 (SMAD family member 6), a negative regulator of BMP and TGF-beta/activin-signaling that was up-regulated in the expanded cells, was also validated. Finally, cell cycle-related genes, BIRC5 (baculoviral IAP repeat containing 5) and CDC25C (cell division cycle 25 homolog C), were also validated and were significantly up-regulated in expanded cells (Figure 3).

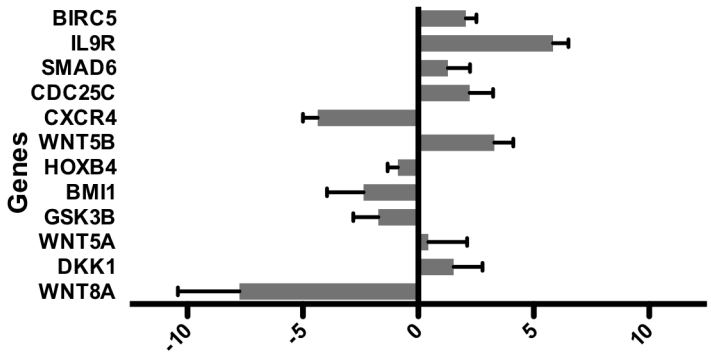

Figure 3. Validation of microarray data by qPCR. Y-axis = gene names. X-axis = fold-change values, with t0 as the calibrator samples.

DISCUSSION

As expected for a short-term expansion culture, the increase in the absolute number of $CD34^+$ cells was modest, albeit significant. Most cells retained expression of the $CD34^+$ marker, and there was a significant increase in the $CD34^+/CD38^-$ cell population. Despite the known association of the $CD34^+/CD38^-$ phenotype with a primitive HSC phenotype, it is highly heterogeneous (da Silva et al., 2009).

WNT5B gene expression was increased in expanded HSC, while its paralog, WNT5A, was not significantly altered, suggesting they do not have overlapping functions in this scenario. WNT5B is a noncanonical WNT ligand that inhibits activation of β-catenin induced by WNT3A (Kanazawa et al., 2005). The only canonical WNT ligand found to be modulated here was WNT8A, which was strongly down-regulated at t3. The down-regulation of canonical ligands and up-regulation of noncanonical ligand WNT5B, WNT receptor FZD9 and canonical inhibitor DKK1, led us to hypothesize that there is a skewing towards noncanonical Wnt signaling during early *ex vivo* expansion.

The canonical WNT pathway is fundamental for maintenance, expansion and lineage decisions in various tissues, including the hematopoietic system (Jeannet et al., 2008). While canonical WNT pathway is very well defined, regulation of the noncanonical pathway is less clear, with multiple β-catenin-independent pathways potentially existing side by side (van Amerongen et al., 2008). Regarding the role of canonical WNT pathway in HSC, there are conflicting reports. It has been shown that canonical WNT signaling regulates HSC self-re-

newal, promoting proliferation and inhibiting differentiation (Kirstetter et al., 2006), but it has also been shown to inhibit self-renewal, depending on the developmental stage (Kokolus and Nemeth, 2010). WNT5A was shown to increase the engraftment of UCB-HSCs by inhibiting WNT3A canonical signals, but it is not known which noncanonical pathways are used by WNT5A (Nemeth et al., 2007). A recent report also showed the importance of noncanonical Wnt for the maintenance of HSC in the niche and in *ex vivo* cultures in the presence of a supporting osteoprogenitor cell line (Sugimura et al., 2012). These and other conflicting reports reflect different influences and interactions of canonical and noncanonical WNT signals (Fleming et al., 2008), and most importantly, the intrinsic differences between the HSC populations studied and the conditions used in each study. Among these conditions, hypoxia has been recently shown to regulate WNT/β-catenin signaling (Kaufman, 2010), and absence of hypoxia during *ex vivo* expansion could explain the apparent induction of noncanonical WNT signaling. Nevertheless, it is very challenging to interpret WNT signals because they vary considerably depending on the cellular context (van Amerongen and Nusse, 2009).

Taken together, the changes in gene expression during early *ex vivo* expansion described here suggest that a shift towards noncanonical Wnt signaling is one of the factors involved in the loss of long-term reconstitution by *ex vivo* expanded UCB-HSC.

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