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SHORT COMMUNICATION

Preferential expression of cytochrome CYP CYP2R1 but not CYP1B1 in human cord blood hematopoietic stem and progenitor cells

Shuoqi Xu[a], Zhihua Ren[b], Yanan Wang[c,*,†], Xinxin Ding[a,*,†], Yongping Jiang[a,b,*,†]

[a]*Biopharmaceutical R&D Center, Chinese Academy of Medical Sciences & Peking Union Medical College, Suzhou 215126, China*
[b]*Biopharmagen Corp., Suzhou 215126, China*
[c]*Department of Clinical Laboratory, Suzhou Municipal Affiliated Hospital of Nanjing Medical University, Suzhou 215002, China*

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KEY WORDS

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Abstract Cytochrome P450 (CYP) enzymes metabolize numerous endogenous substrates, such as retinoids, androgens, estrogens and vitamin D, that can modulate important cellular processes, including proliferation, differentiation and apoptosis. The aim of this study is to characterize the expression of CYP genes in CD34+ human cord blood hematopoietic stem and early progenitor cells (CBHSPCs) as a first step toward assessment of the potential biological functions of CYP enzymes in regulating the expansion or differentiation of these cells. CD34+ CBHSPCs were purified from umbilical cord blood *via* antibody affinity chromatography. Purity of CD34+ CBHSPCs was assessed using fluorescence-activated cell sorting. RNA was isolated from purified CD34+ CBHSPCs and total mononuclear cells (MNCs) for RNA-PCR analysis of CYP expression. Fourteen human CYPs were detected in the initial screening with qualitative RT-PCR in CD34+ CBHSPCs. Further quantitative RNA-PCR analysis of the detected CYP transcripts yielded evidence for preferential expression of CYP2R1 in CD34+ CBHSPCs relative to MNCs; and for greater expression of CYP1B1 in MNCs relative to CD34+ CBHSPCs. These findings provide the basis for further studies on possible functions of CYP2R1 and CYP1B1 in CBHSPCs'

Abbreviations: bp, base pair; Ct, threshold cycle; CBHSPCs, cord blood HSPCs; FACS, fluorescence-activated cell sorting; HSPCs, hematopoietic stem and early progenitor cells; kbp, kilobase pair; MNCs, mononuclear cells; OD, optical density; CYP, cytochrome P450; PCR, polymerase chain reaction; PE, R-phycoerythrin; RT, reverse transcription

*Corresponding authors. Tel./fax: +86 512 62831269.
E-mail addresses: wangyn1980@163.com (Yanan Wang), xding001@gmail.com (Xinxin Ding), yjiang@biopharmagen.com (Yongping Jiang).
†These authors contributed equally to this work.

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proliferation and/or differentiation and their potential utility as targets for drugs designed to modulate CD34+ CBHSPC expansion or differentiation.

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1. Introduction

Cytochrome P450 (CYP) enzymes play important roles in the biotransformation of numerous endogenous compounds as well as drugs and chemical carcinogens[1,2]; in humans, 57 functional or potentially functional CYP genes have been identified[3]. CYP genes show varying degrees of tissue selective expression with some expressing widely in many tissues, whereas others showing stringent tissue specificity. The collection of CYP enzymes expressed in a given tissue can have a significant impact on the metabolome and biological function of that tissue.

The aim of this study is to identify CYP genes selectively expressed in human hematopoietic stem and early progenitor cells (HSPCs). In contrast to the large amounts of data available regarding CYP expression, regulation and function in the liver and other major extrahepatic organs such as lung and intestine[4], relatively little is known about the expression and function of CYP genes in stem or progenitor cells. We speculate that the HSPCs may suppress the expression of CYP genes that support differentiation, while enhancing the expression of CYP genes that are important for the maintenance of the pluripotency. CYP enzymes may modulate these biological functions through metabolism of various endogenous substrates, such as retinoids, androgens, estrogens, vitamin D, and bile acids. Therefore, it is worthwhile to identify the CYP genes that are preferentially expressed in HSPCs, as a first step toward characterization of their possible biological functions in these important cells and their potential utility as targets for drugs that are designed to modulate HSPC expansion or differentiation.

Several studies have reported detection of CYP expression in HSPCs. CYP1B1 and CYP2E1 (mRNA and possibly also protein), but not CYP2C9 or CYP3A4, were detected in human CD34+ hematopoietic stem and progenitor cells (>96% pure) isolated from peripheral blood[5,6]. CYP1B1 mRNA was also detected in human cord blood-derived CD34+ cells, and its levels were suppressed by treatment with SR1, an agent that increased *ex vivo* expansion of these cells[7]. In a more recent study, CYP1A1, 2B6, 2E1 and 3A4 were apparently detected in cord blood-derived CD34+ cells (80–85% pure) on day 0 of differentiation and they showed time-dependent increases in expression during differentiation into neuron-like cells, although it was not clear whether the basal expression on day 0 of differentiation was in the stem cells or in the contaminating mononuclear cells[8]. However, none of these studies determined whether the detected CYPs were preferentially expressed in the CD34+ cells, and the expression of the other human CYPs in HSPCs has not been examined.

In this study, we first performed a global analysis, using qualitative RNA-PCR, of all 57 human CYP genes for their possible expression in CD34+ human cord blood HSPCs (CBHSPCs). Following sequence validation of the detected CYP transcripts in CD34+ CBHSPCs, we further compared their expression levels to those in total mononuclear cells from the same donors using quantitative RNA-PCR. Our results indicate that CYP2R1 is preferentially expressed in CD34+ CBHSPCs relative to MNCs, whereas CYP1B1 is more abundantly expressed in MNCs than in isolated CD34+ CBHSPCs.

2. Materials and methods

2.1. Materials

CD34 MicroBead kit, magnetically assisted chemical separation (MACS) unit, human CD34-PE antibody and mouse IgG2a-PE antibody were purchased from Miltenyi Biotec (Shanghai, China). Ficoll solution was purchased from Huajing Bioproduct (Shanghai, China). Trizol reagent was supplied by Invitrogen (Carlsbad, CA, USA). Deoxy-ribonucleoside triphosphate (dATP, dCTP, dGTP and dTTP) for polymerase chain reaction (PCR), Taq polymerase, $MgCl_2$ and molecular weight standards for electrophoresis (DL500) were purchased from Sangon Biotech (Shanghai, China). Ultrapure agarose was supplied by Life Technologies (Paisley, UK). Oligonucleotide primers were synthesized by BioSune (Shanghai, China). RNase-free DNase I, PrimeScript II High fidelity RT-PCR kit and agarose gel DNA extraction kit were supplied by Takara Biotechnology (Dalian, China). Fast SYBR green master mix was purchased from Applied Biosystems (Foster City, CA). Other chemical reagents were purchased from Sigma-Aldrich.

2.2. Preparation of CD34+ CBHSPCs

Anonymous human cord blood donated by pregnant mothers who gave birth to healthy babies at full term was collected at Suzhou Municipal Hospital, Suzhou, China. All studies involving human tissue samples were approved by the local Institutional Review Board. Anticoagulated cord blood was diluted with phosphate-buffered saline (PBS), and then subjected to density-gradient centrifugation as described below. Diluted blood layered on top of a 75% Ficoll solution was centrifuged at 500*g*, at 4 °C, for 30 min. MNCs in the interphase were collected and washed with PBS. Cell numbers were manually counted using a hemacytometer. CD34+ CBHSPCs were purified from the MNCs using the CD34 MicroBead kit with FcR blocking reagent and a MACS unit based on two-step magnetic bead cell sorting.

Briefly, the MNCs were washed and resuspended in PBS buffer containing 1% bovine serum albumin and 2 mmol/L edetic acid (EDTA). Cells were first incubated with monoclonal mouse anti-human CD34 antibodies labeled with MACS microbeads, in the presence of human IgG as blocking reagent, at 4 °C, for 30 min. Labeled cells were loaded onto a column installed in a magnetic field, and CD34− cells, which were not conjugated with the microbeads, were removed as unbound fraction. Trapped CD34+ cells were eluted after removing the column from the magnet according to the instructions of the CD34 MicroBead Kit.

2.3. Flow cytometric analysis

The purity and the number of freshly isolated CD34+ CBHSPCs were assessed *via* fluorescence-activated cell sorting (FACS) using human CD34-PE antibody and isotype-matched mouse IgG2a-PE. Cells were incubated with either antibody at room temperature for 15 min in the dark. After the incubation, the cells were washed once in PBS. FACS

analysis was performed on a BD Accuri® C6 flow cytometer equipped with a 488-nm laser. At least 5000 events were acquired to analyze the abundance of total MNCs or CD34+ CBHSPCs in each sample. Further analysis was performed to assess the purity of cells after gating on the MNC region, which was defined based on forward and side scatter profiles. The percentage of CD34+ CBHSPCs among total MNCs was assessed after correcting for the percentage of cells reactive with the isotype control IgG2a. Purified cells were frozen at −80 °C until used for RNA extraction.

2.4. *Isolation of total RNA and cDNA synthesis*

Total RNA was isolated from MNCs and CD34+ CBHSPCs using Trizol reagent according to the manufacturer's instructions. RNA preparations (10–100 μg) were subsequently incubated with 10 units of RNase-free DNase I for 30 min at 37 °C in order to remove any contaminating genomic DNA. RNA quality was assessed spectrally by the A260/A280 ratio using the One-Drop Spectrophotometer (Nanodrop, Nanjing, China) and visually following agarose gel electrophoresis. First-strand cDNA was synthesized with 0.5–1.0 μg of total RNA using the PrimeScript II High Fidelity RNA-PCR kit. The reverse transcriptase was omitted in negative control reactions.

2.5. *Qualitative RNA-PCR*

Each 20-μL amplification reaction mixture contained an amount of reverse transcription (RT) product equivalent to 50 ng of input RNA, 2 μL of 10× Taq buffer, 3 mmol/L $MgCl_2$, 0.2 mmol/L (each) dATP, dGTP, dTTP and dCTP, 0.5 μmol/L each primer, and 2 units of Taq polymerase. Conditions for PCR amplification on a GeneAmp 9700 thermocycler (Applied Biosystems) were as follows: 3 min of denaturation at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 56 °C, 30 s at 72 °C; followed by a final extension for 5 min at 72 °C. To monitor potential contamination of reagents, negative control reactions, in which water was used in place of cDNA, were included with each batch of PCR analysis.

Resected normal, adjacent liver biopsy tissue from an anonymous adult patient with liver cancer was used for RNA preparation. The liver RNA sample was used as a positive control for detection of several CYPs using RNA-PCR.

PCR primers (Supplemental Table 1) were designed using source DNA and RNA sequences obtained from GenBank database. All primer sets had a calculated annealing temperature of 56 °C. PCR products were analyzed by agarose gel electrophoresis with ethidium bromide staining. The bands corresponding to each specific PCR product were purified from the gels using TaKaRa mini BEST agarose gel DNA extraction kit and further analyzed by DNA sequencing at Sangon Biotech in order to confirm identity of the PCR products.

2.6. *Quantitative RNA-PCR*

Real-time PCR analyses were carried out using the StepOne-Plus PCR sytem (Perkin-Elmer Applied Biosystems). Each reaction mixture (20 μL) contained an amount of RT product equivalent to 100 ng of input RNA, 0.25 μmol/L each PCR primer (Table 1) and standard amounts of kit components in the fast SYBR green master mix. The reaction was monitored for 45 PCR cycles. At the end of the PCR cycles, melting curve analysis was performed by heating to 95 °C, then cooling to 65 °C, and finally heating to 92 °C at 0.2 °C/s. For each primer pair, RT products were serially diluted, so that an amount of first-strand cDNA equivalent to 1/3 - 1/729 of the initial amount of total RNA used in the RT step was used to perform quantitative PCR. Threshold cycle (Ct) values were plotted as a function of input RT-product amounts (or of the dilution factor of the RT product, as a surrogate) in order to establish the linear range of PCR amplification for each primer pair and for constructing standard curves. The relative amounts of CYP1B1 or CYP2R1 mRNAs in two different sample types (MNCs and CD34+ CBHSPCs) were determined by comparing to a common set of standard curves and by further normalizing the results by the amounts of *β*-actin in the same samples. Standard curves were constructed using RNA from either MNCs (for genes expressed at higher levels in MNCs than in CD34+ CBHSPCs) or CD34+ CBHSPCs (for genes expressed at higher levels in CD34+ CBHSPCs than in MNCs). No-RT negative controls were routinely included for each sample.

2.7. *Data analysis*

Statistical significance of differences between the two groups was determined using Student's *t*-test.

3. Results

3.1. *Global analysis of CYP mRNA expression in CD34+ CBHSPCs by RNA-PCR*

Initial analysis was focused on identifying those CYP mRNAs that are detectable in CD34+ CBHSPCs. PCR primers were designed for each of the 57 potentially functional human CYPs. The purified CD34+ CBHSPC preparations from each donor typically contained over 1.5×10^6 cells, and were more than 94% pure, as illustrated in Fig. 1. Not all primer pairs used in the initial screening skipped an intron; therefore, the RNA samples were treated with RNase-free DNase I to minimize amplification of genomic DNA.

Table 1 Primers used for quantitative RNA-PCR analysis.[a]

Gene	Primer	Sequence (5′-3′)	Product size (bp)	NCBI accession no.	Location
CYP1B1	Sense Antisense	gctgcagtggctgctcct cccacgacctgatccaattct	81	NM_000104.3	Exons 2, 3
CYP2R1	Sense Antisense	cagcctcatccgagcttc ccacagttgatatgcctcca	96	NM_024514.4	Exons 1, 2
β-actin	Sense Antisense	agagctacgagctgcctgac cgtggatgccacaggact	114	NM_001101.3	Exons 4, 5

[a]Primers were designed according to the sequences in GenBank.

Following several trials to optimize PCR conditions, 14 of the 57 CYPs were consistently detected in purified CD34+ CBHSPCs as a single band of expected size, as shown in Fig. 2. These included CYP1A1, CYP1B1, CYP2E1, CYP2C18, CYP2C19, CYP2J2, CYP2R1, CYP2S1, CYP2U1, CYP4F2, CYP4V2, CYP20A1, CYP27A1 and CYP51A1. Notably, for these CYPs, the amplified cDNA sequence did span at least one intron–exon junction, making it unlikely that they were amplified from genomic DNA. Furthermore, in other experiments (data not shown), the target bands were not detected when reverse transcriptase was omitted during the RT step. The specificity of the PCR detection was confirmed by sequencing analysis of the detected mRNA-derived PCR products; the detected DNA sequences of the PCR products were 95% to 100% identical to reference sequences in Genbank (data not shown).

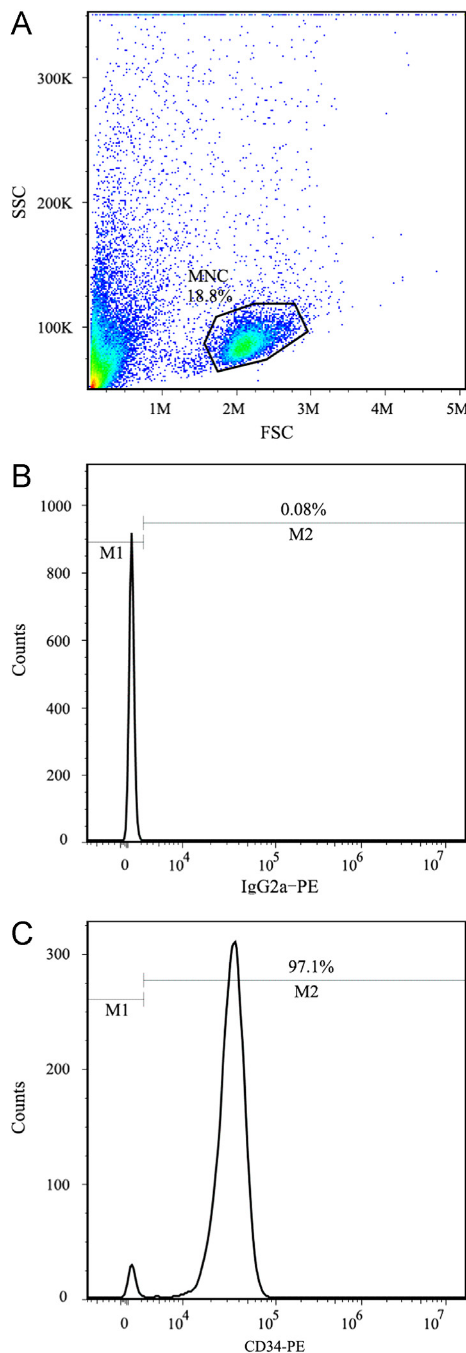

Figure 1 Purity of isolated CD34+ CBHSPCs. CD34+ CBHSPCs were isolated *via* magnetic bead-based antibody affinity purification as described in Section 2. (A) Scatter diagram of a representative purified CBHSPC preparation. Forward scatter (FSC) and side scatter (SSC) were used to define gate MNC (the 'lymphoid gate'). (B) Negative control. Signal was detected using a PE fluorescence-labeled, isotype-matched monoclonal mouse IgG2a control antibody. (C) Fractions of CD34− (M1) and CD34+ (M2) cells within gate MNC. Cells were incubated with a PE fluorescence-labeled monoclonal mouse anti-CD34 antibody.

The other 43 human CYPs were not detected in our study under the conditions used. To validate the primers used, we also attempted to detect these CYPs in a human liver RNA sample, used as a positive control. As shown in Fig. 3, 12 of the 43 remaining human CYPs (CYP1A2, CYP2A6, CYP2A7, CYP2B6, CYP2C9, CYP3A4, CYP3A5, CYP4A22, CYP4F11, CYP4F12, CYP7B1 and CYP11A1) were detected in the liver, though they were not detected in CD34+ CBHSPCs in the same experiment. The remaining 31 CYPs were not detected in either sample (data not shown). These data served to validate the negative results for the 12 CYPs that were detected in human liver in this study, while casting doubt on the validity of the negative results on the remaining 31 CYPs.

3.2. *Relative expression levels of the detected CYPs in MNCs and CD34+ CBHSPCs*

Preliminary semiquantitative studies (data not shown) were conducted on three paired samples of CD34+ CBHSPCs and total MNCs (aliquot saved before purification of CD34+ CBHSPCs). Quantitative RT-PCR was conducted for all 14 detected CYP genes, as well as for CD34 and β-actin (as controls); but a standard curve was not constructed. The results indicated that, in each sample, while some CYPs were more abundant, others were less abundant (when normalized to β-actin) in MNCs than in CD34+ CBHSPCs. However, when comparing the results from the three samples, the trend (relative levels in MNCs *vs*. CD34+ CBHSPCs) was consistent only for some of the CYPs, among which CYP2R1 had the greatest enrichment, whereas CYP1B1 had the least enrichment in the CD34+ CBHSPCs.

Subsequent studies were focused on determining relative expression levels of CYP1B1 and CYP2R1 in MNCs and CD34+ CBHSPCs. A total of five MNC and CD34+ CBHSPC samples (each pair from a single donor) were analyzed by quantitative RNA-PCR. As shown in Fig. 4, the relative mRNA levels for CYP1B1 (normalized by β-actin levels) were consistently lower (by 0.12-fold) in CD34+ CBHSPCs than in MNCs, whereas the normalized mRNA levels for CYP2R1 were consistently higher (by about 17-fold) in CD34+ CBHSPCs than in MNCs. The specificity of all primers (Table 1) used for the quantitative RT-PCR was confirmed by melting curve analysis and by the detection of a single band of expected size on agarose gels (data not shown). In negative control reactions in which the reverse transcriptase was omitted during cDNA synthesis, no signals were detected; Ct values obtained from triplicate reactions were consistent, and standard curves showed good linearity; the Ct values of the samples all fell in the linear range of the standard curves (not shown).

4. Discussion

To our knowledge, this is the first comprehensive analysis of the expression of human CYP genes in isolated HSPCs. Of the 57 human CYPs, only 14 were specifically detected by qualitative RNA-PCR in CD34+ CBHSPCs. Transcripts for 12 other CYP genes, including CYP1A2, CYP2A6, CYP2A7, CYP2B6, CYP2C9, CYP3A4, CYP3A5, CYP4A22, CYP4F11, CYP4F12, CYP7B1 and CYP11A1, were not detected in CD34+ CBHSPCs (though they were detected in the liver as a positive control), a result indicating that they are not expressed in CD34+ CBHSPCs, or are expressed at levels below the detection limit of the experimental conditions used. For the remaining CYP genes, the data are not conclusive regarding whether they were expressed or not in the CD34+ CBHSPCs, given the lack of a positive control for the effectiveness of the PCR assays, even though the PCR primers were all expected to work. Overall, our data provide the first unequivocal evidence for the presence of transcripts of multiple CYP genes (CYP2J2, CYP2R1, CYP2S1, CYP2U1, CYP4F2, CYP4V2, CYP20A1, CYP27A1 and CYP51A1) and confirm the presence of transcripts for several other CYP genes (CYP1A1, CYP1B1, CYP2C18, CYP2C19 and CYP2E1) in purified preparations of (undifferentiated) human HSPCs. These results provide the foundation for further studies to characterize the expression of the corresponding proteins and their functions.

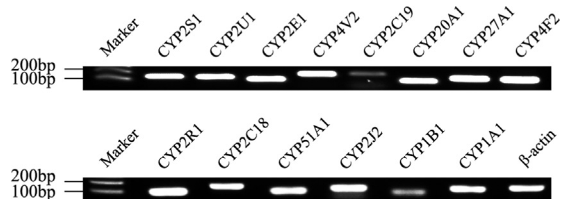

Figure 2 RNA-PCR detection of CYP expression in isolated CD34(+) CBHSPCs. PCR was performed with primer pairs shown in Supplemental Table 1. PCR products (10 μL each) were analyzed on an ethidium bromide-stained agarose gel. RNA-PCR products of 14 detected CYPs and β-actin (control) are shown. Marker: DL500.

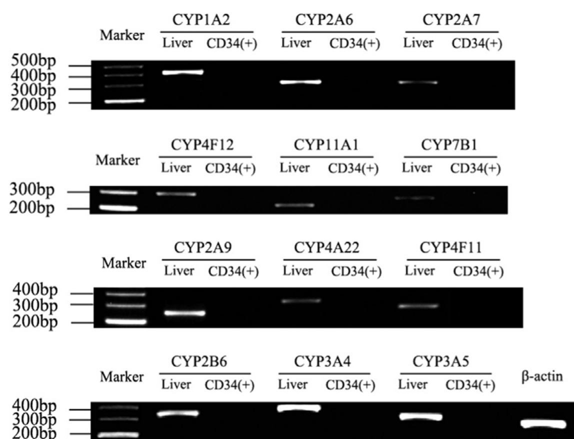

Figure 3 Positive control for selected CYP genes not detected in CD34(+) CBHSPCs. RNA from a human liver was used as a positive control. PCR was performed with primer pairs shown in Supplemental Table 1. PCR products (10 μL each) were analyzed on an ethidium bromide-stained agarose gel. Marker: DL500. PCR products of expected sizes were detected in liver RNA, but not in RNA from CD34(+) CBHSPCs.

For the CYP transcripts detected in the purified CD34+ CBHSPC preparations, it remained to be confirmed whether they were expressed in the CD34+ cells or in the small fraction ($<6\%$ of total) of contaminating CD34− MNCs. Due to limitations in the amounts of RNA samples available from each donor for real-time PCR analysis, we were not able to resolve this question for all 14 of the detected CYP transcripts. However, we were successful in demonstrating a large enrichment of the CYP2R1 transcript in purified CD34+ CBHSPC preparations compared to total MNCs, a result strongly supporting the notion that CYP2R1 transcript was selectively present in the CD34+ stem/progenitor cells. In contrast, CYP1B1 transcript was selectively excluded by the cell isolation process, a result indicating that they were enriched in CD34− MNCs.

The remarkable and opposing cell specificity of CYP1B1 and CYP2R1 mRNA expression in CD34+ CBHSPCs and CD34− MNCs may have interesting implications for possible functions of these CYP enzymes in hematopoietic stem cell proliferation and/or differentiation. CYP1B1, which is expressed in a number of human tissues, can metabolize both xenobiotic (*e.g.*, polycyclic aromatic hydrocarbons) and endogenous compounds (including estrogen, retinol, retinal and arachidonic acid)[9,10]. CYP1B1 is overexpressed in tumor cells[11]. Mutations of CYP1B1 have been linked to the etiology of glaucoma[10] and the initiation or progression of tumorigenesis[11]. Of particular interest, CYP1B1 is

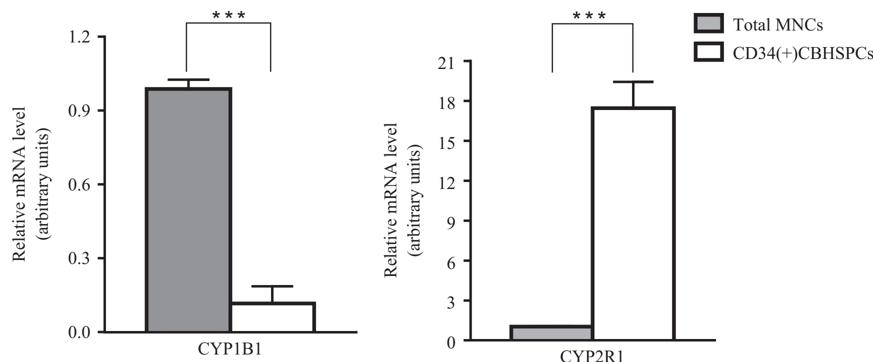

Figure 4 Quantitative RT-PCR analysis of CYP1B1 and CYP2R1 in MNCs and CD34(+) CBHSPCs. Quantitative RT-PCR was performed as described in Section 2. The relative expression levels of CYP1B1 and CYP2R1 mRNAs in the two sample types were normalized by the levels of β-actin. The levels in MNCs were arbitrarily set to 1. (***$P<0.001$, means±SD; $n=5$; Student's t-test).

a target gene for the aryl hydrocarbon receptor; the latter appears to function as a suppressor of CD34+ CBHSPC expansion[7].

CYP2R1, which has not been as intensely studied as CYP1B1 has, appears to have stricter substrate specificity. It is the major vitamin D 25-hydroxylase in human liver[12]. Mutations of CYP2R1 gene were associated with vitamin D deficiency. CYP2R1 has also been detected in extrahepatic tissues, including prostate and ovarian cancer cells[13,14]. It is conceivable that CYP2R1, perhaps through its metabolism of vitamin D or other yet unknown endogenous substrates, may play an important role in maintaining the CD34+ CBHSPCs in an undifferentiated and pluripotent state, whereas CYP1B1 may have an opposite role. Further studies to test this hypothesis are warranted. Additionally, the differential expression of CYP1B1 and CYP2R1 also suggests differences in the expression/function of the nuclear receptors that regulate their expression; further studies to examine the status of these regulatory molecules may also shed light on the functional significance of the differential expression of CYP1B1 and CYP2R1. Confirmation of an important role of one or both of these CYPs in modulating CD34+ CBHSPC expansion or differentiation would support their potential utility as drug targets for diseases or therapies that adversely affect CBHSPCs.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.apsb.2014.10.003.

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