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RXR negatively regulates *ex vivo* expansion of human cord blood hematopoietic stem and progenitor cells

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Abstract

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Authors' contributions

BG and HEB conceived the research, designed and supervised the experiments, interpreted data and wrote the manuscript. RJ supervised data analysis and wrote the manuscript. YJ and JH designed and performed experiments, analyzed data and wrote the manuscript. QW collected CB samples and prepared samples for transcriptome analysis. JFH and YT isolated stem cells, analyzed phenotyping data and wrote the manuscript.

Conflicts of interest/Competing interests (include appropriate disclosures)

Dr. Broxmeyer is a member of the Scientific Advisory Board of Elixell, a stem cell expansion company. There are no conflicts of interest for other authors.

Ethics approval (include appropriate approvals or waivers)

All animal experiments followed protocols approved by The Institutional Animal Care and Use Committee of SJTUSM.

Consent for publication (include appropriate statements)

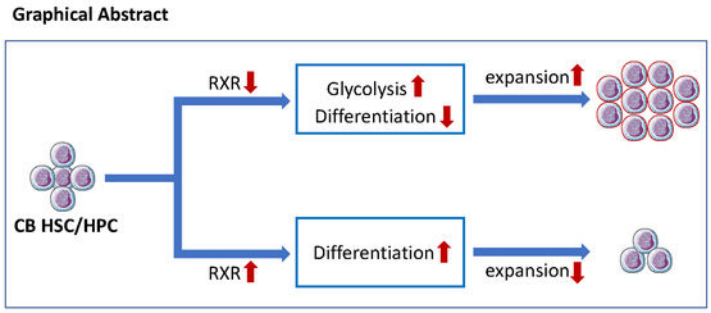
The authors are alone responsible for the content and writing of the paper. All authors reviewed and approved the final version of the manuscript.

Availability of data and material (data transparency)

Raw data will be provided upon reasonable request.

Ex vivo expansion of human cord blood (CB) hematopoietic stem cells (HSCs) is one approach to overcome limited numbers of HSCs in single CB units. However, there is still no worldwide acceptable HSC *ex vivo* expansion system. A main reason is that we still have very limited knowldege regarding mechanisms underlying maintenance and expansion of CB HSCs. Here we report that retinoid X receptor (RXR) activity is of significance for CB HSC *ex vivo* expansion. RXR antagonist HX531 significantly promoted *ex vivo* expansion of CB HSCs and progenitor cells (HPCs). RXR agonist Bexarotene notably suppressed *ex vivo* expansion of CB HSCs. Activation of RXR by Bexarotene significantly blocked expansion of phenotypic HSCs and HPCs and expressed increased functional HPCs as assessed by colony formation induced by UM171 and SR1. *In vivo* transplantation experiments in immune-deficient mice demonstrated that HX531 expanded CB HSCs possess long-term reconstituting capacities, and Bexarotene treatment inhibited expansion of functional CB HSCs. RNA-seq analysis revealed that RXR regulates expression of FBP1 (a negative regulator of glucose metabolism) and many genes involved in differentation. ECAR analysis showed that HX531 significantly promoted glycolytic activity of CB $CD34^+$ HSCs and HPCs. Our studies suggest that RXR is a negative regulator of *ex vivo* expansion of CB HSCs and HPCs.

Graphical Abstract



Keywords

Cord blood; Hematopoietic stem and progenitor cells; *Ex vivo* expansion; Retinoid X receptor

1 Introduction

Cord blood (CB) is a reliable source of engraftable hematopoietic stem cells (HSCs) for clinical therapies in hematopoietic cell transplantation [1]. Compared to bone marrow (BM) or mobilized peripheral blood (mPB) derived HSCs, CB HSCs possess advantages including

that it is readily available, and has a lower incidence of graft-versus-host diseases (GvHD), and less relapse among patients with minimal residual diseases (MRD) [2]. However, the use of CB HSCs in clinical transplantation is still somewhat limited, especially compared with that of mPB derived HSCs [3], in part is due to limited HSC numbers in single CB Units. Studies have demonstrated that mitigating extra physiological oxygen shock/stress (EPHOSS), *ex vivo* expansion or improving homing efficiency of CB HSCs may broaden the use of CB for hematopoietic cell transplantation [4–9].

Nuclear receptors (NRs) generally function as transcription factors which regulate transcription of target genes in multiple physiological processes [10]. The structure of NRs is composed of a trans-activation domain, DNA binding domain, nuclear localization signal and ligand binding domain [10,11]. NRs are usually activated by steroid or non-steroid hormones and metabolites *in vivo*. Upon ligands binding, NCoR1 (nuclear receptor corepressor) and SMRT (silencing mediator of retinoic acid and thyroid hormone receptor corepressor complex) disassociate from NRs, and transcription activation components with histone acetyltransferase (HAT) activity are recruited, thus facilitating transcription of specific target genes [10,11]. About 48 NRs in human cells have been characterized, and half of them are orphan receptors which lack traditional ligands [10]. Dimerization including homodimerization and heterodimerization plays an important role in NRs mediated regulation of gene transcription [10].

NRs including Retinoid acid receptor (RAR) and peroxisome proliferator-activated receptor gamma (PPARG) play an important role in human CB HSC maintenance and *ex vivo* expansion [11]. RARs signaling regulate many processes which includes development, cell growth, cell differentiation, and cell metabolism [12]. Retinoid acid receptor (RAR) antagonist promotes CB HSC *ex vivo* expansion by repressing differentiation [13]. Inhibition of retinoid acid production by suppressing aldehyde dehydrogenase (ALDH) activity is sufficient to expand functional CB HSCs and HPCs [13]. PPARG is one of the three subtypes of the PPAR family [11]. PPARG is mainly involved in adipogenesis, lipid metabolism, and glucose homeostasis [11]. PPARG antagonism enhances glucose metabolism of CB HSCs and expands CB HSCs and progenitor cells (HPCs) by preventing differentiation and promoting glycolysis [5]. PPARG antagonist GW9662 down-regulates several differentiation associated genes including CD38, CD1d, HIC1, FAM20C, DUSP4, DHRS3, and ALDH1A2, and a negative regulator of glycolysis, FBP1[5,11].

Retinoid X receptor (RXR) dimerizes with multiple nuclear receptors including RAR, PPAR, Vitamin D receptor (VDR), Liver X receptor (LXR) and so on [10]. However, the exact role of RXR in CB HSC expansion is still unknown. Here we report that RXR antagonist HX531 significantly promoted *ex vivo* expansion of rigorously defined phenotypic CB HSCs, while RXR agonist Bexarotene suppressed their *ex vivo* expansion. Moreover, Bexarotene treatment blocked *ex vivo* expansion of phenotypic CB HSCs and hematopoietic progenitor cells (HPCs) induced by SR1 and UM171. *In vivo* transplantation analysis demonstrated that HX531 expanded CB HSCs were functional after transplantation into immune-deficient NSG mice, and Bexarotene blocked functional HSC *ex vivo* expansion. RNA-seq analysis demonstrated that HX531 treatment downregulates expression of *FBP1* and also several differentiation associated genes, and significantly enhances

glycolysis of CB CD34$^+$ HSCs and HPCs. These findings suggest that RXR may function as a switch for CB HSC *ex vivo* expansion.

2 Methods and Material

2.1 Mice

NM-NSG (NOD-PrkdcscidIL2rgem1B2mem1/Smoc) mice (6–8 weeks old) were ordered from Shanghai Model Organisms and maintained in the Laboratory Animal Center of the Shanghai Jiao Tong University School of Medicine (SJTUSM). All animal experiments followed protocols approved by The Institutional Animal Care and Use Committee of SJTUSM.

2.2 CB CD34$^+$ cells isolation and culture

Cord blood samples were obtained from Affiliated Sixth People's Hospital, Shanghai Jiao Tong University. Mononuclear cells were isolated by density gradient centrifugation with Ficoll-Paque Plus (GE Healthcare, Piscataway, NJ, USA). CD34$^+$ cells were enriched with immunomagnetic selection kit (Miltenyi Biotec, Auburn, CA, USA) following manufacturer's instructions. CB CD34$^+$ cells were cultured in RPMI-1640 medium (Lonza, 12–702Q) with 10% fetal bovine serum (GE Healthcare HyClone. SH30071.03), 100 ng/mL stem cell factor (SCF) (R&D Systems, #7466-SC-010/CF), thrombopoietin (TPO) (R&D Systems, #288-TP-200/CF), Fms-like tyrosine kinase 3 ligand (FL) (BioLegend, # 710802).

2.3 Immunostaining and Flow cytometry

Immunostaining and flow cytometry analysis were performed as previously described [5]. CD34$^+$ Cells were stained with fluorescence conjugated antibodies at 4°C for 30 min. Cells were washed with cold PBS and cell pellets were fixed with 1% formaldehyde. Samples were analyzed on an LSRII flow cytometer (BD Biosciences). The following antibodies from BD Bioscience (San Jose, CA, USA) were used for cell surface staining (1:200 dilution): FITC anti-human Lineage Cocktail 1 (CD3, CD14, CD16, CD19, CD20, CD56), CD34-APC (581), CD38-PE (HIT2), CD45RA-PE-CF594 (HI100), CD90-PEcy7 (5E10), CD49f-PerCPcy5.5 (GoH3), and CD45-APC (HI30).

2.4 Colony-forming unit assay

CFU assay was performed as previously reported [5]. Human cord blood CD34$^+$ cells were plated in semi-solid methylcellulose culture medium in the presence with 30% fetal bovine serum (FBS) (GE Healthcare HyClone. SH30071.03), 2 mM L-glutamine (Lonza, 17–605E), 100 μM β-mercaptoethanol (Sigma, M6250), 1U/mL Erythropoietin (EPO) (R&D Systems, 287-TC-500), 50 ng/mL SCF (R&D Systems, #7466-SC-010/CF), 10 ng/mL IL-3 (R&D Systems, 203-IL-050/CF) and 10 ng/mL GM-CSF (R&D Systems, 7954-GM-010), and were cultured in a 5% O_2/5% CO_2 humidified incubator. Colonies were scored with an inverted microscope 14 days after seeding.

2.5 In vivo transplantation

50, 000 $CD34^+$ CB cells treated with vehicle, HX531 or Bexarotene for 4 days were intravenously transplanted into sublethally irradiated primary NSG recipient mice (350 cGy; 137^{Cs} source, single dose). The percentage of human $CD45^+$ cells in the bone marrow was checked by immuno-staining and flow cytometry at month 4 after transplantation.

2.6 RNA sequencing

Vehicle, HX531 or Bexarotene treated CB $CD34^+$ cells were lysed and RNA was extracted using RNeasy Mini Kit (QIAGEN, Valencia, CA, USA). RNA sequencing analysis was performed at SeqWright Genomic Services (GE Healthcare) as previously reported.

2.7 Seahorse Extracellular Flux Assay

The extracellular acidification rate (ECAR) of human CB $CD34^+$ cells was determined by using Seahorse XF Extracellular Flux Analyzer (Agilent Technologies) as previously indicated [5]. Briefly, 100, 000 purified $CD34^+$ cells were added into each well of cell culture plates. Plates were centrifuged for 10 min, at 1, 000 g. During this process, glucose (Sigma, G8644), oligomycin (Sigma, #75351), 2-DG (Sigma, D8375) medium was sequentially (A, B, C) added into the utility plate for ECAR analysis.

2.8 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0. Data are shown as mean values ± standard deviation (SD), unless stated otherwise as standard error of the mean (SEM). Two-tailed Student's t-tests were performed for statistical analysis between two groups. One-way analysis of variance (ANOVA) was used to compare differences in means between more than two groups where indicated.

3 Results and Discussion

A role for PPARG (peroxisome proliferator-activated receptor gamma) and RAR (retinoid acid receptor) in *ex vivo* expansion of CB HSCs has been reported [5,10]. However, if RXR activity is involved in CB HSC *ex vivo* expansion remains unknown. In order to answer this question, we performed an *ex vivo* expansion experiment using the well-established RXR antagonist HX531 (1 μM) and RXR agonist Bexarotene (100 nM) or LG268 (100 nM). All expansion experiments were performed with RPMI-1640 medium containing 10% fetal bovine serum with cytokines (SCF, TPO, Flt3L). We found that RXR antagonism by HX531 significantly promoted *ex vivo* expansion of rigorously defined CB HSCs (Lin $^-CD34^+CD38^-CD45RA^-CD49f^+CD90^+$) and multiple potent progenitors (MPPs; Lin $^-CD34^+CD38^-CD45RA^-CD49f^-CD90^-$), while Bexarotene notably suppressed their *ex vivo* expansion (Fig. 1a–c). The effect of HX531 on *ex vivo* expansion of CB HSCs and HPCs was reversed by Bexarotene (Fig. 1a–c). Another RXR agonist LG268 showed similar effects to that of Bexarotene (Fig. 1b and c). These data suggest that RXR negatively regulates *ex vivo* expansion of rigorously-defined phenotypic CB HSCs and MPPs.

Next, we assessed how RXR activation might affect *ex vivo* expansion of CB HSCs induced by SR1 and UM171. UM171 or SR1 significantly promoted *ex vivo* expansion of

$CD34^+CD38^-$ cells, rigorously-defined phenotypic HSCs and MPPs, an effect completely suppressed by RXR agonist Bexarotene (Fig. 2a–d).

To determine a role for RXR in *ex vivo* expansion of functional HPCs, we performed colony-forming unit (CFU) assay. CB CFU-granulocyte-macrophage (GM) and granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM) after 4 day of *ex vivo* expansion (Fig. 3a). HX531 treatment significantly increased numbers of CFU-GM and CFU-GEMM. Bexarotene or LG268 did not significantly affect numbers of CFU-GM and CFU-GEMM, but greatly suppressed increased CFU numbers induced by HX531(Fig. 3a). Consistent with the HSC and MPP phenotyping data, Bexarotene significantly blocked expansion of functional HPCs induced by SR1 and UM171 as revealed by CFU assay (Fig. 3b). HX531 has no additive effect with SR1 and UM171 on expansion of CFU-progenitor cells (Fig. 3c).

In order to check the effect of RXR antagonism or activation on functional HSCs with long-term repopulating capacity, we transplanted vehicle (DMSO), HX531 or Bexarotene expanded CB $CD34^+$ cells into immunocompromised recipient mice. Four months after transplantation, we checked human cell chimerism in the BM of recipient mice, human $CD45^+$ cell chimerism significantly increased in the HX531 group compared with that of the vehicle group (Fig. 4a and b). In contrast, engraftment was notably lower in the Bexarotene group than those cultured with vehicle (Fig. 4a and b). This demonstrates that RXR negatively regulates *ex vivo* expansion of functional HSCs.

To gain some mechanistic insight into how RXR regulates *ex vivo* expansion of CB HSCs and HPCs, we performed RNA sequencing analysis. We found that RXR antagonism by HX531 significantly downregulated the expression of *HIC1, DHRS3, DUSP4, CD38, CD1D* (Fig. 4c). These genes are involved in cell differentiation [5, 14–17], suggesting that HX531 may promote expansion of CB HSCs partly by suppressing differentiation. Of note, HX531 significantly suppressed expression of *FBP1* (Fig. 4c), which is a negative regulator of glycolysis regulated by PPARG in CB $CD34^+$ cells. RXR activation by Bexarotene increased expression of *HIC1, DHRS3, DUSP4, CD38, CD1D,* and also other genes involved in differentiation including *FAM20C* and *ALDH1A2* (Fig. 4d) [18,19]. Since FBP1 has been identified as a negative regulator of glucose metabolism and expansion of CB HSCs and HPCs [5], we did ECAR analysis using a Seahorse XF extracellular flux instrument. HX531 significantly enhanced the glycolytic activity of CB $CD34^+$ HSCs and HPCs (Fig. 4e). Thus, these data suggest that RXR may govern *ex vivo* expansion of CB HSCs and HPCs by modulating glycolysis and cell differentiation.

Nuclear receptors usually function as transcription factors which play important roles in cellular processes [10]. We and others previously demonstrated that PPARG and RAR are involved in CB HSC *ex vivo* expansion [5,11,13]. PPARG and RAR can dimerize with RXR [10]. PPARG antagonism enhances expansion of CB HSCs and HPCs, while PPARG activation has no significant effect on HSC expansion [5]. RAR antagonism significantly increases expansion of CB HSCs, and RAR activation induces differentiation of CB HSCs [13,20]. Taken together, PPARG and RAR may be both involved in enhanced expansion of CB HSCs by RXR antagonism, while only RAR is involved in decreased expansion of CB HSCs induced by RXR agonists. RXR forms heterodimers with many other nuclear

receptors, and further studies are needed to address the potential role of these receptors in CB HSC and HPC expansion and maintenance.

In the present study, we demonstrate that RXR antagonist HX531 treatment downregulates the expression of *HIC1, DHRS3, DUSP4, CD38, CD1D* in CB $CD34^+$ HSCs and HPCs. HIC1 is a tumor suppressor which is involved in granulocytic differentiation, and *HIC1* hypermethylation has been frequently observed in leukemia and solid tumors [16]. RXR antagonism may promote *ex vivo* expansion of CB HSCs and HPCs by preventing granulocytic differentiation. It would be very interesting to know if hypermethylation mediated silence of *HIC1* exists in CB HSCs. DHRS3 mediates retinoic acid synthesis and thus is involved in retinoids induced differentiation [21]. Since RAR has been identified as a regulator of CB HSC expansion, HX531 may block trans-activation activity of RXR-RAR heterodimers to facilitate expansion of CB HSCs. DUSP4, a phosphatase that inactivates p38 MAPK signaling transduction. Loss of *DUSP4* suppresses megakaryocyte differentiation of human $CD34^+$ cells induced by TPO [16]. Inhibition of DUSP4 may promote expansion of CB HSCs and HPCs by partly blocking megakaryocyte differentiation [16]. CD38 and CD1D are both cell surface markers which are associated with hematopoietic cell differentiation [11]. RXR antagonist HX531 significantly downregulates expression of *CD38* and *CD1D*, while RXR agonist Bexarotene notably increases expression of *CD38* and *CD1D*. Taken together, RXR regulates *ex vivo* expansion of CB HSCs at least partly by transiently repressing hematopoietic cell differentiation. In addition, HX531 significantly downregulates *FBP1*. Inhibition or knockdown of *FBP1* enhances glycolysis of CB HSCs and HPCs, and is sufficient to promote *ex vivo* expansion of functional CB HSCs and HPCs [5]. Consistently, HX531 notably enhances glycolytic activity of CB HSCs and HPCs, suggesting that RXR antagonism facilitates CB HSC expansion by promoting glucose metabolism. RXR agonist Bexarotene did not affect FBP1-glycolysis axis of CB HSCs and HPCs. In summary, RXR may regulate *ex vivo* expansion of CB HSCs and HPCs by orchestrating trans-activation activity of RXR-RAR and RXR-PPARG heterodimers, of which cell differentiation and glucose metabolism are involved.

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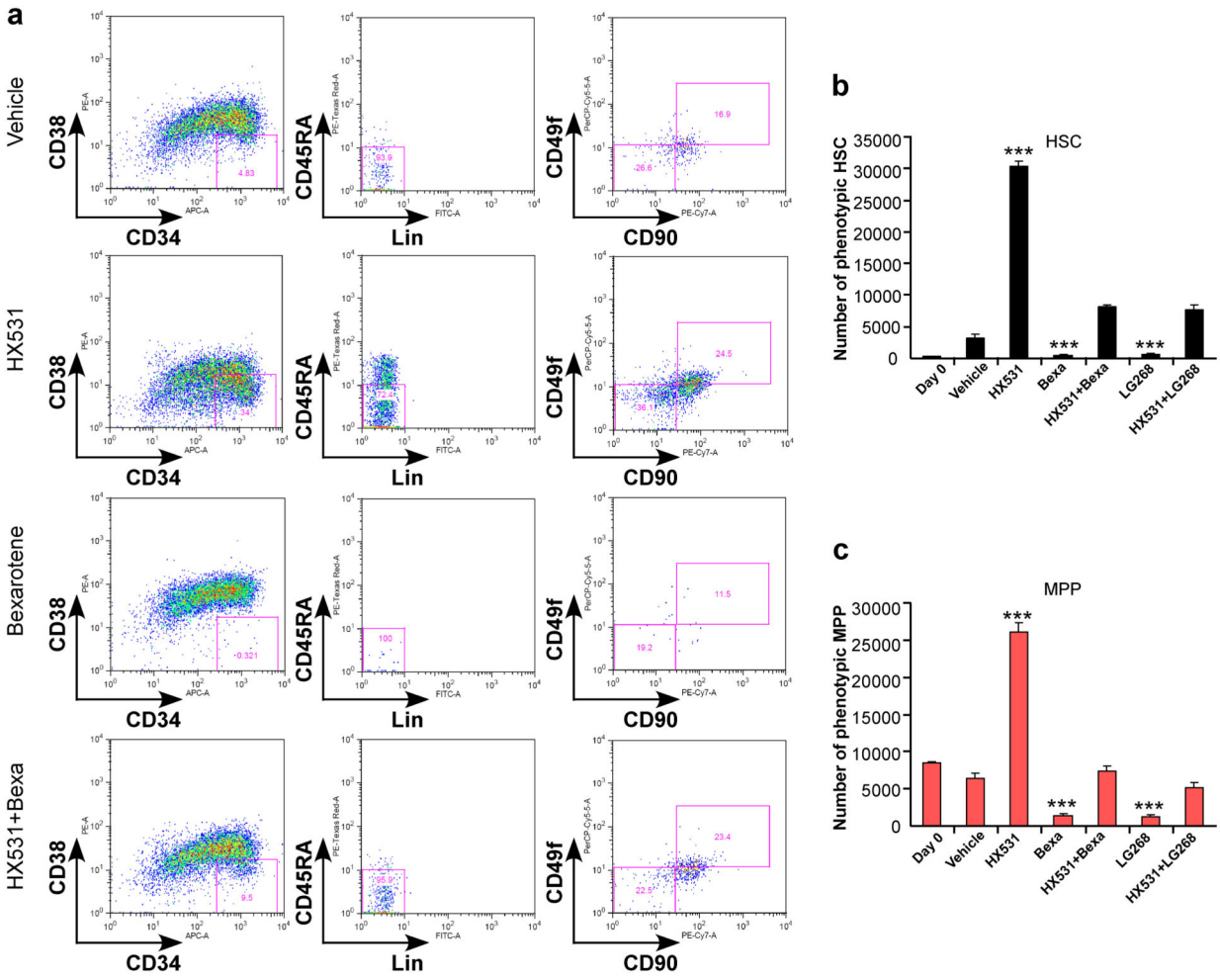

Figure 1

Figure 1. RXR regulates *ex vivo* expansion of human CB HSCs and HPCs.

a Flow plots showing effects of RXR antagonist HX531 (1 μM) and agonist Bexarotene (100 nM) on *ex vivo* expansion of human CB HSCs and HPCs. HSC (Lin$^-$CD34$^+$CD38$^-$CD45RA$^-$CD49f$^+$CD90$^+$) expansion was determined by FACS analysis.

b Quantification of phenotypic HSC after culturing CB CD34$^+$ cells with Vehicle, HX531, Bexarotene, HX531+Bexarotene, LG268 (RXR agonist), and HX531+LG268. Data are shown as mean±S.E.M. One-way ANOVA test, ***p<0.001.

c Quantification of phenotypic MPP (Lin$^-$CD34$^+$CD38$^-$CD45RA$^-$CD49f$^-$CD90$^-$) after culturing CB CD34$^+$ cells with Vehicle, HX531, Bexarotene, HX531+Bexarotene, LG268 (RXR agonist), and HX531+LG268. Data are shown as mean±S.E.M. One-way ANOVA test, ***p<0.001.

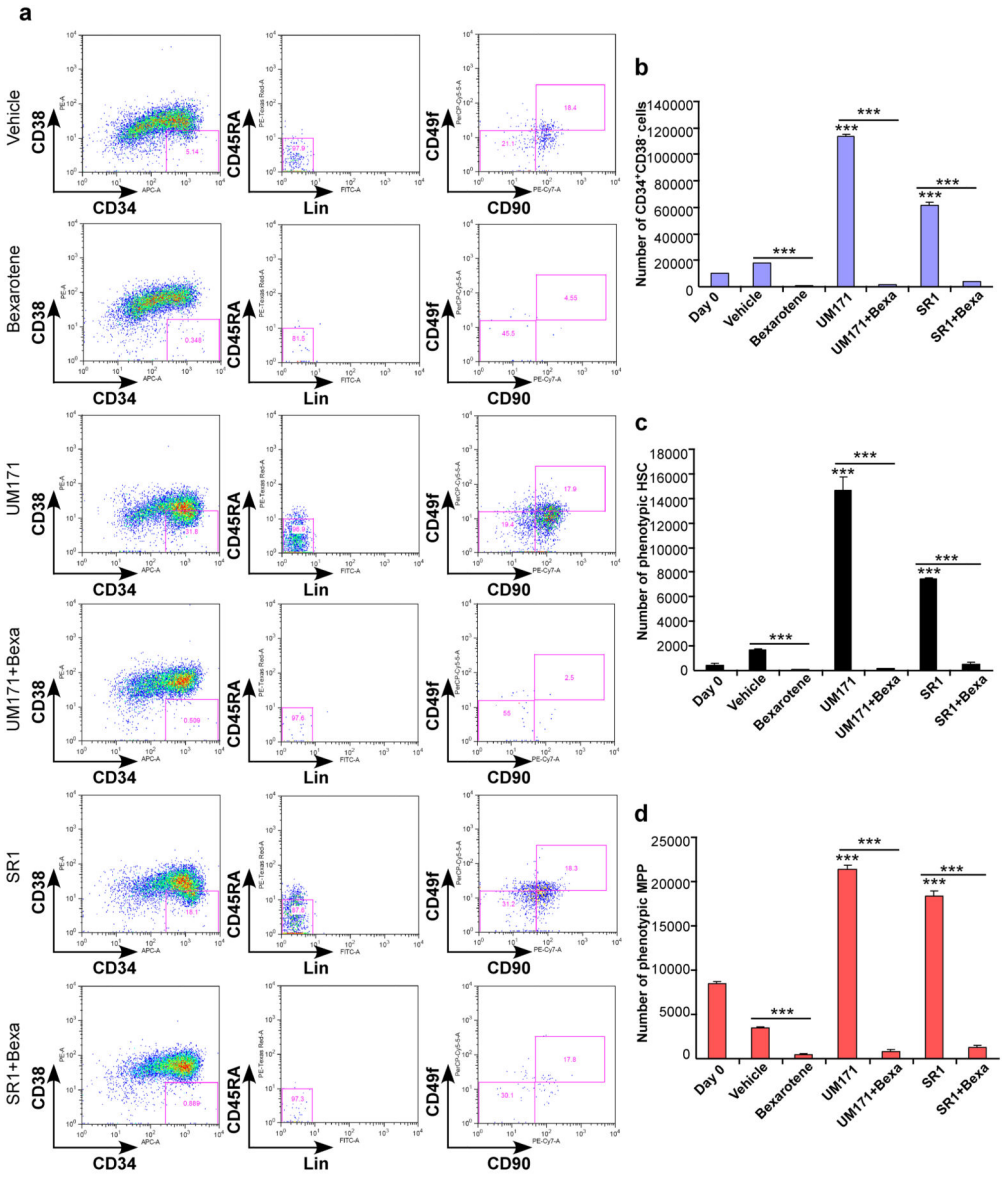

Figure 2

Figure 2. RXR blocks *ex vivo* expansion of human CB HSCs and HPCs induced by UM171 and SR1

a Flow plots showing effects of RXR agonist Bexarotene on *ex vivo* expansion of human CB HSCs and HPCs induced by UM171 and SR1. HSC ($Lin^-CD34^+CD38^-CD45RA^-CD49f^+CD90^+$) expansion was determined by FACS analysis.

b Quantification of $CD34^+CD38^-$ cells after culturing CB $CD34^+$ cells with Vehicle, Bexarotene, UM171, UM171+Bexarotene, SR1, and SR1+ Bexarotene. Data are shown as mean±S.E.M. One-way ANOVA test, ***p<0.001.

c Quantification of phenotypic HSCs after culturing CB $CD34^+$ cells with Vehicle, Bexarotene, UM171, UM171+Bexarotene, SR1, and SR1+ Bexarotene. Data are shown as mean±S.E.M. One-way ANOVA test, ***p<0.001.

d Quantification of phenotypic MPPs ($Lin^{-}CD34^{+}CD38^{-}CD45RA^{-}CD49f^{-}CD90^{-}$) after culturing CB $CD34^{+}$ cells with Vehicle, Bexarotene, UM171, UM171+Bexarotene, SR1, and SR1+ Bexarotene. Data are shown as mean±S.E.M. One-way ANOVA test, ***p<0.001.

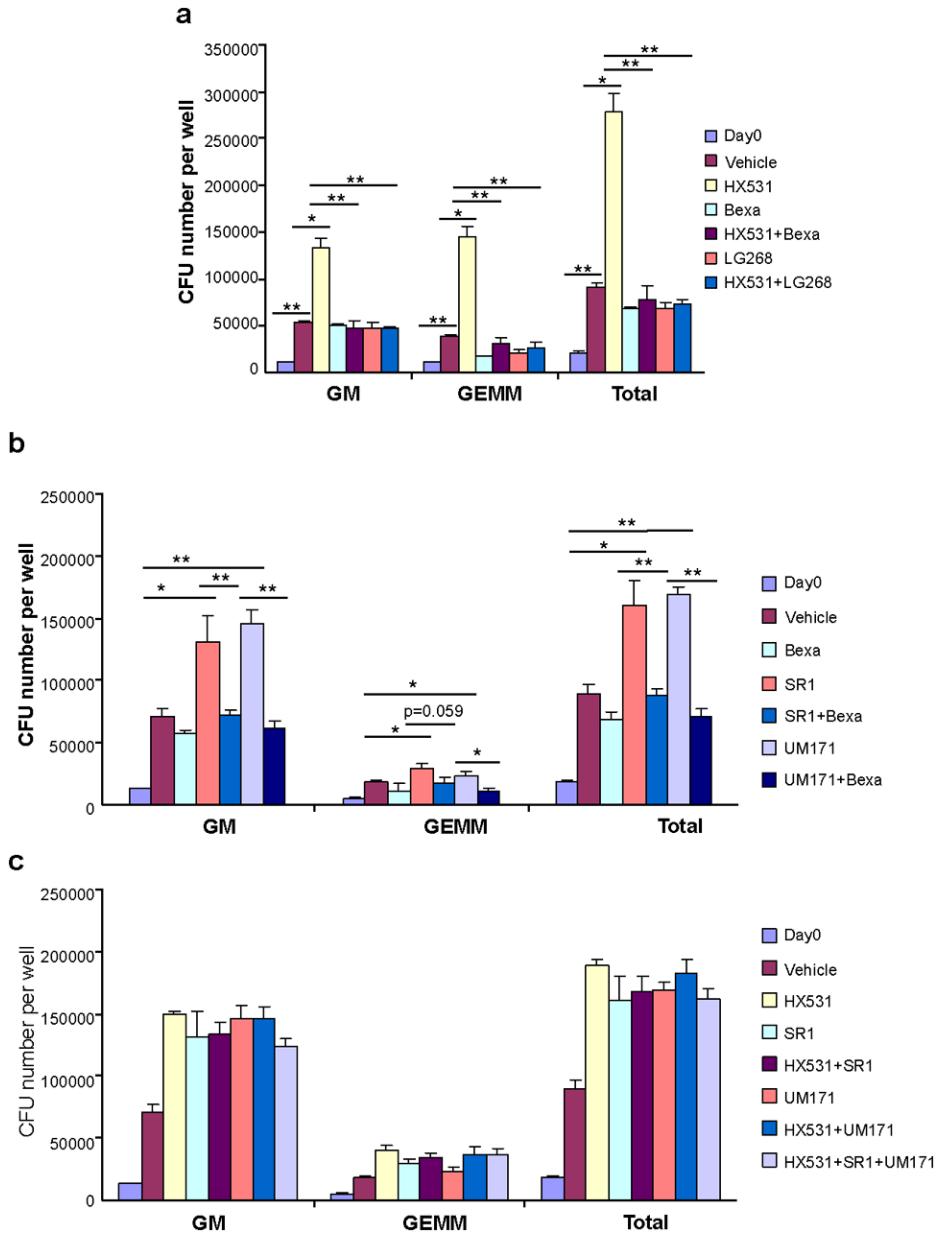

Figure 3. RXR regulates expansion of functional CB HPCs

a Bar graph showing the effect of RXR antagonist HX531 or RXR agonist (Bexarotene and LG268) on *ex vivo* expansion of functional HPCs determined by CFU assay. Data are shown as mean±S.E.M. One-way ANOVA test, *p<0.05; **p<0.01.

b Quantification of CFU number per well after culturing CB $CD34^+$ cells with Vehicle, HX531, Bexarotene, HX531+Bexarotene, UM171, UM171+Bexarotene, SR1, and SR1+ Bexarotene. Data are shown as mean±S.E.M. One-way ANOVA test, *p<0.05; **p<0.01.

c Quantification of CFU number per well after culturing CB $CD34^+$ cells with Vehicle, HX531, SR1, SR1+HX531, UM171, UM171+HX531, and UM171+SR1+HX531. Data are shown as mean±S.E.M.

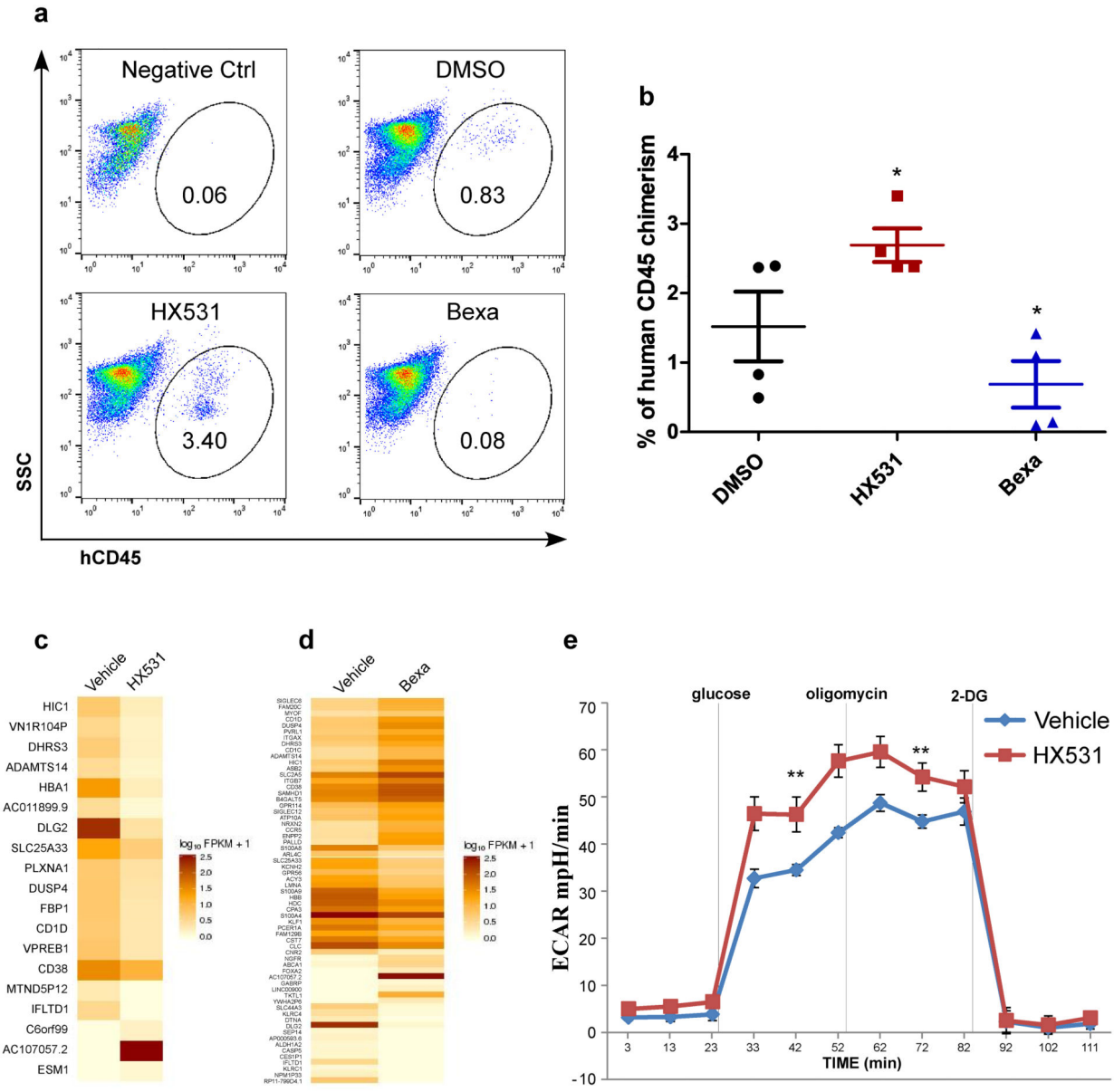

Figure 4. RXR regulates expansion of functional CB HSCs by modulating cell differentiation and glycolysis

a Flow plots showing effects of RXR antagonist HX531 or RXR agonist (Bexarotene and LG268) on *ex vivo* expansion of functional HSCs determined by *in vivo* transplantation experiment for 4 months. 50,000 CB $CD34^+$ cells were plated at day 0 and *ex vivo* cultured with Vehicle, HX531 or Bexarotene for 4 days. Human CD45 cell chimerism in recipient mice was analyzed 4 months after transplantation.

b Quantification of human CD45 cell chimerism in the bone marrow of recipient mice of each groups after 4 months transplantation. Data are shown as mean±S.E.M. n=4 mice per group. *p<0.05.

c RNA-seq analysis of Vehicle and HX531 expanded CB $CD34^+$ HSCs and HPCs. CB $CD34^+$ cells were cultured with Vehicle (DMSO), HX531 for 4 days. $CD34^+$ cells were enriched and then analyzed by RNA-seq.

d RNA-seq analysis of Vehicle and Bexarotene expanded CB $CD34^+$ HSCs and HPCs. CB $CD34^+$ cells were cultured with Vehicle (DMSO), Bexarotene for 4 days. $CD34^+$ cells were enriched and then analyzed by RNA-seq.

e Extracellular Acidification Rate (ECAR) was measured in purified CB CD34+ cells following a 4-day culture with vehicle or HX531. Data pooled from three independent experiments are shown as mean mean±S.D. (n=3 independent experiments). **p<0.01.