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DUSP16 is a regulator of human hematopoietic stem and progenitor cells and promotes their expansion *ex vivo*

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To the Editor:

Cord blood (CB) hematopoietic stem (HSC) and progenitor (HPC) cells are widely used in treatment of malignant and non-malignant blood disorders. However, limited numbers of HSC and HPC in single CB units collected at the birth of a baby have hampered full potential for the most efficacious use of CB for hematopoietic cell transplantation (HCT) [1–4]. Efforts to *ex-vivo* expand HSC and HPC, including clinical efforts in this area have been reported [4–6], but it is not yet clear which of these may eventually be used world-wide for clinical CB HCT.

Negative regulators act to prevent transcription or translation and play crucial roles in regulation of biological activities. Previous reports demonstrated that attenuation of representative signaling pathways through overexpression of specific negative regulators were associated with enhancement of ex-vivo expansion of HSC and HPC [7]. Based on shRNA screening systems, several regulators of HSC/HPC expansion were discovered [ref 5, 9]. However, this loss-of-function screen system is not useful for screening negative regulators that have benefit for HSC and HPC expansion. It is thus important to develop a system to screen potential additional regulators for HSC and HPC expansion in the group of negative regulators. Recently, the fast developing of clustered regularly interspaced short palindromic repeats (CRISPR) mediated gene activation system has provided a away to conduct a gain-of-function screen in HSC and HPC.

To further understand HSC and HPC regulation, we utilized a CRISPR-mediated activation system, conducting a gene screen in CB $CD34^+$ cells. We identified 26 representative negative regulators of signaling pathways expressed in CB HSC/HPC (Fig. S1a). A small sgRNA pool was established and every 3 sgRNAs were designed to specifically activate expression of every single identified negative regulator. $CD34^+$ CB cells were harvested by FACS and stimulated in medium supplemented with Cyclosporine H (CsH), TPO, Flt3L and SCF for 24 hours. Thereafter, viruses were incubated with cells for 24 hours and the cells

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Author Contributions

X.W. and H.E.B. conceived the project and designed the experiments. X.W. performed the experiments and analyzed the data. X.W. and H.E.B. wrote and edited the manuscript.

Conflict of interests

Neither Drs. Wang or Broxmeyer have any Conflicts of Interest.

cultured for another 10 days. Rigorously phenotyped Lin^- $CD34^+$ $CD90^+$ $CD49f^+$ $CD45RA^-$ $CD38^-$ GFP^+ HSC were harvested by FACS, genomic DNA was extracted from these cells (Fig. S1b; diagrammatic representation). SgRNAs targeting DUSP16 were identified in these cells by sequencing. DUSP16 is known to negatively regulate JNK and p38 pathway activities [11]. First, we investigated expression patterns of DUSP16 in CB cells. Fresh HSC, multipotential progenitors (MPP), common myeloid progenitors (CMP) and megakaryocyte erythroid progenitors (MEP) were sorted from non-cultured CB. Using real-time PCR, we noted that expression levels of DUSP16 mRNA were in descending order from HSC to MPP, CMP and MEP (Fig. 1a). This result was confirmed by high-throughput data (Fig. S1c). Based on screening results and expression patterns of DUSP16 in HSC, MPP, CMP and MEP, we speculated that DUSP16 played a role in regulating HSC and HPC and their expansion.

To explore the function of DUSP16 in CB $CD34^+$ cells, a lentivirus based system was used to overexpress DUSP16 in HSC and HPC (Fig. S1d; diagrammatic representation). Freshly isolated $CD34^+$ cells were stimulated in medium supplemented with cyclosporine H, TPO, Flt3L and SCF for 24 hours and then transduced with the DUSP16 overexpression virus. At Day 4, $CD34^+$ and GFP+ cells were sorted by FACS and cultured for another 4 days. At day 8, cells were harvested, and percentages and numbers of HSC determined by flow cytometry (Fig. 1b; diagram of culture times). Overexpression of DUSP16 upregulated DUSP16 expression levels (Fig. S1e) and resulted in a 2.2-fold increase in total granulocyte macrophage (CFU-GM) and multipotential (CFU-GEMM) colonies, relative to control cells, primarily due to a 3.1-fold increase in CFU-GEMM colonies. (Fig. 1c). Although no significant increases of total cell numbers were detected by DUSP16 overexpression in CB cells (Fig. S1f), the $CD34^+$ $CD38^-$ $CD45RA^-$ subset and rigorously defined HSC population ($CD34^+$ $CD38^-$ $CD45RA^-$ $CD49f^+$ $CD90^+$) were 5.7- and 9-fold more abundant than control cells at Day 8 (Fig. 1d). We also explored effects of short hairpin (sh) RNA-induced down regulation of DUSP16 on CB $CD34^+$ cells (Fig. S1g, h). DUSP16 knock-down decreased numbers of CFU-GEMM 2.1-fold, and total CFU numbers 1.7-fold (Fig. S1i). Knock-down of DUSP16 affected total cell expansion more than 2-fold and the $CD34^+$ $CD38^-$ $CD45RA^-$ and $CD34^+$ $CD38^-$ $CD45RA^-$ $CD49f^+$ $CD90^+$ subsets were respectively 2.1- and 1.5-fold decreased. (Fig. S1j, k). To further elucidate connections between DUSP16 and HSC/HPC activity, we induced site-specific mutations in the DUSP16 protein. DUSP16 includes one nuclear localization signals (NLS) and one nuclear export signal (NES) motif [12]. Four representative mutant isoforms of DUSP16 were constructed. Overexpression of NES, NLS or NES and NLS double mutant isoforms induced comparable enhanced expansion of HSC (Fig. S1l) and HPC (Fig. S1m) in CB $CD34^+$ cells similar to that of wildtype DUSP16. However, the catalytic domain mutant isoform had no effects on HSC and HPC expansion. The results suggest that DUSP16-promoted CB HSC and HPC expansion is dependent on its dephosphorylation activity. Overexpression and down-regulated expression data indicated that DUSP16 plays a role in regulation of phenotypic HSC and functional HPC in vitro.

Phenotype of HSC populations does not always recapitulate their functional activities [13]. Hence, we assessed whether *ex vivo* expansion of functional human CB HSC was enhanced by DUSP16 overexpression. We performed limiting dilution assays (LDA) to assess

numbers of functional HSC and calculated numbers of SCID repopulating cells (SRC) in order to verify results of the HSC phenotype data. Day 4 DUSP16 virus transduced GFP^+ $CD34^+$ cells were sorted by FACS and cultured *ex vivo* for another 4 days. Cells were then transplanted into sublethally irradiated immunodeficient NOD-SCID IL-2rγnull (NSG) mice (Fig. 1b; diagrammatic representation). LDA results demonstrated that DUSP16 overexpressing cells displayed enhanced engraftment relative to recipients of control cells, both in peripheral blood (PB) at 2 and 4 months respectively (Fig. 1e, f) and bone marrow (BM) at 4 months (Fig. 1g). DUSP16 overexpression did not induce changes in myeloid/lymphoid ratios of donor engrafting cells. (Fig. 1h). Numbers of BM SRCs were calculated and compared with the control group, DUSP16 overexpression had an increased SRC frequency from 1/10580 to 1/3289, a 3.2-fold enhancement above that of the control group (Fig. 1i, j). Long-term reconstitution and self-renewal capability of DUSP16 overexpression CB $CD34^+$ cells were confirmed by secondary transplantation. Respective 5.4-fold and 7.8-fold increases in chimerism rates of DUSP16 overexpression CB $CD34^+$ cells at month 4 PB and BM was detected (Fig. 1k). No significant changes in ratios of myeloid/lymphoid cells were detected in both PB and BM by overexpression of DUSP16 in secondary transplants (Fig. 1l). Thus, overexpression of DUSP16 enhanced *ex vivo* expansion of functional HPC (Fig. 1c) and self-renewing HSC (Fig. 1k, i).

Transgene based therapies induce concerns, and small molecule based methods may be safer and more convenient. We thus explored whether we could find small molecules to upregulate DUSP16 expression in HSC and HPC. Histone acetylation is regulated by HDAC family members. HDACs can classified in four classes depending on sequence homology [14]. Previous reports showed that the regulatory elements of DUSP16 are sensitive to histone acetylation [15]. We thus constructed 8 shRNAs to knock-down expression of individual HDACs in CB $CD34^+$ cells. Real-time PCR analysis demonstrated that knock-down of HDAC1 and HDAC3 resulted in upregulated DUSP16 expression levels (Fig. 2a, b). Consistent with this, HSC numbers were increased (Fig. 2c), demonstrating that expression of DUSP16 in CB $CD34^+$cells is regulated by HDAC1 and HDAC3. HDACis are widely used in regulation of HDAC activity. We tested several common HDACis and by use of real-time PCR, found that the HDAC1 and HDAC3 inhibitors that include Trichostatin A (TSA), m-Carboxycinnamic acid bis-hydroxamide (CBHA), Trapoxin, Chlamydocin, and Valproic Acid (VPA) upregulated DUSP16 expression after 24 hours treatment of CB $CD34^+$ cells (Fig. 2d). Inhibitors of HDAC6 or HDAC8 including Nexturastat A, HPOB, BRD73954 and PCI34051 had no effect on DUSP16 expression. Consistent with DUSP16 expression level upregulation, numbers of HSC were increased after treatment by TSA, CBHA, Trapoxin and Chlamydocin (Fig. 2e). To further confirm whether HDACis promoted HSC expansion through upregulation of DUSP16, we used small molecule BCI, an inhibitor of DUSP family members, to down-regulate DUSP16 expression. Real-time PCR showed that BCI treatment decreased upregulation of DUSP16 in CB $CD34^+$ cells by CBHA treatment (Fig. 2f). Consistent with the block of DUSP16 upregulation, HSC numbers were decreased 2.2-fold after BCI treatment in CB $CD34^+$ cells. (Fig. 2g and Fig. S2). This suggests that HDACis that target HDAC1 and HDAC3, such as CBHA and TSA activate expression of DUSP16 and enhance HSC expansion *ex vivo*.

Small numbers of HSC in CB have limited their full potential use in human transplant protocols for adults. We demonstrated that enforced expression of DSUP16 in CB $CD34^+$ cells enhanced *ex vivo* expansion of functional HPC and self-renewing HSC. HDACis upregulate DUSP16 expression through inhibition of HDAC1/HDAC3 and functionally mimic DUSP16 overexpression. Our work thus provides a new and novel target to enhance production of human CB HSC/HPC *ex vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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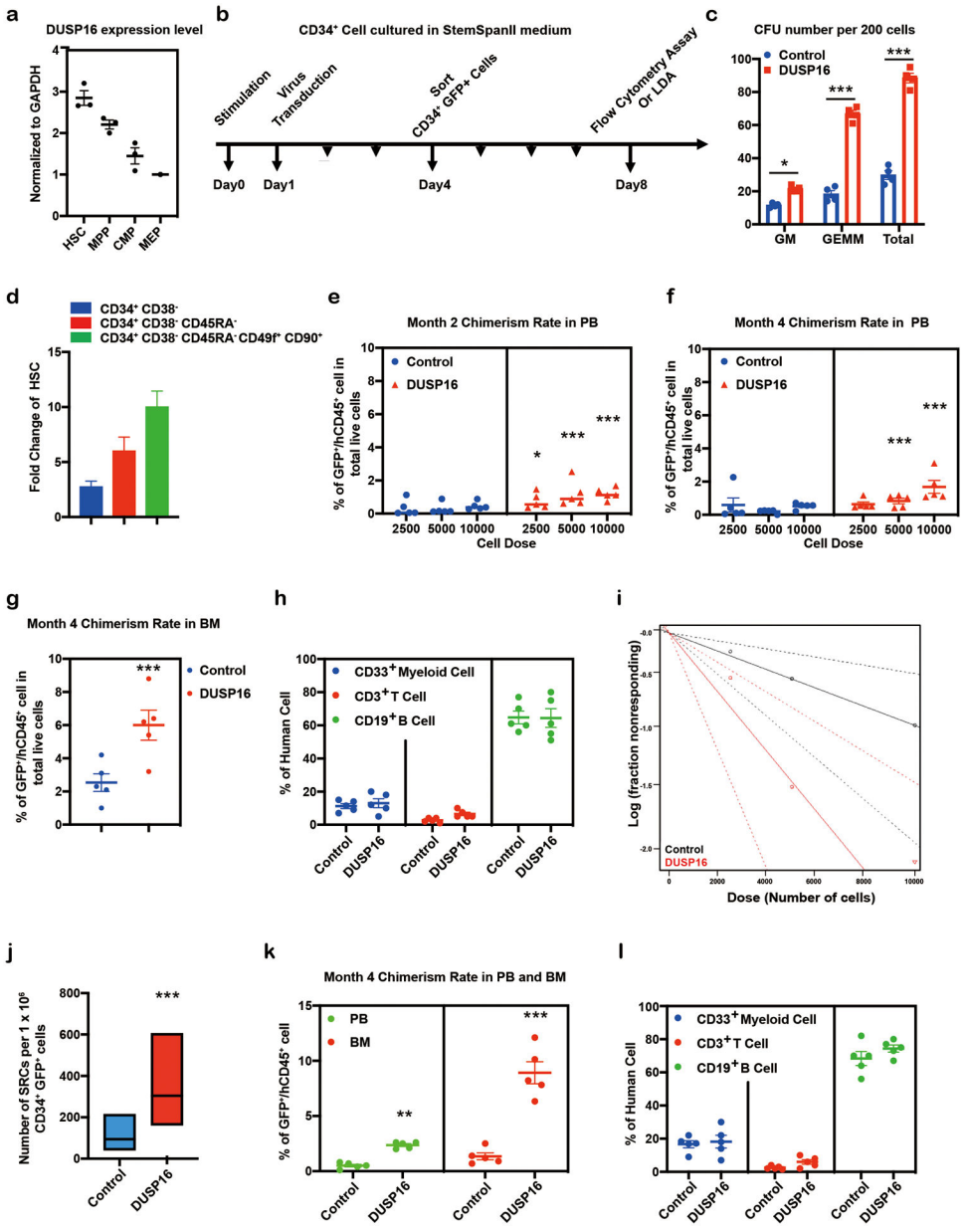

Fig 1. DUSP16 overexpression enhanced *ex vivo* expansion of functional human CB HSC/HPC.

a. DUSP16 expression level (n = 3 independent pooled Lin- CB samples). DUSP16 expression across the human haematopoietic hierarchy. Each circle represents an independent gene expression data set. **b**. The experimental strategy for virus transduction and transplantation of CB HSC and HPC. **c**. Colony output of transduced GFP^+ and $CD34^+$ cells (n = 4 cultures from 2 experiments). **d**. Fold change of HSC in the sorted cells at day 8 after transduced with DUSP16 overexpression vector. **e** and **f**. Percentage of human $CD45^+$ and GFP^+ cells in PB at 2 and 4 months after transplantation of NSG mice with different dose of transduced cells. (n = 5 mice per group). **g**. Percentage of human $CD45^+$ and GFP^+ cells in BM at 4 months after transplantation of NSG mice with 10000 virus transduced cells. **h**. Myelo-lymphopoiesis in mice that received 10000 virus transduced cells. **i**.

Frequency of human SRCs in CB $CD34^+$ cells by overexpression of DUSP16 or control vector. **j**. HSC frequencies (line in the box) and confidence intervals (box) presented as numbers of SRCs in 1 × 106 $CD34^+$cells. **k**. Percentage of human $CD45^+$ and GFP^+ cells in PB and BM at 4 months in secondary recipients. **l**. Myelo-lymphopoiesis in secondary recipients in month 4. Data are shown as mean ± s.e.m. *P < 0.05; **P < 0.01; ***P < 0.001.

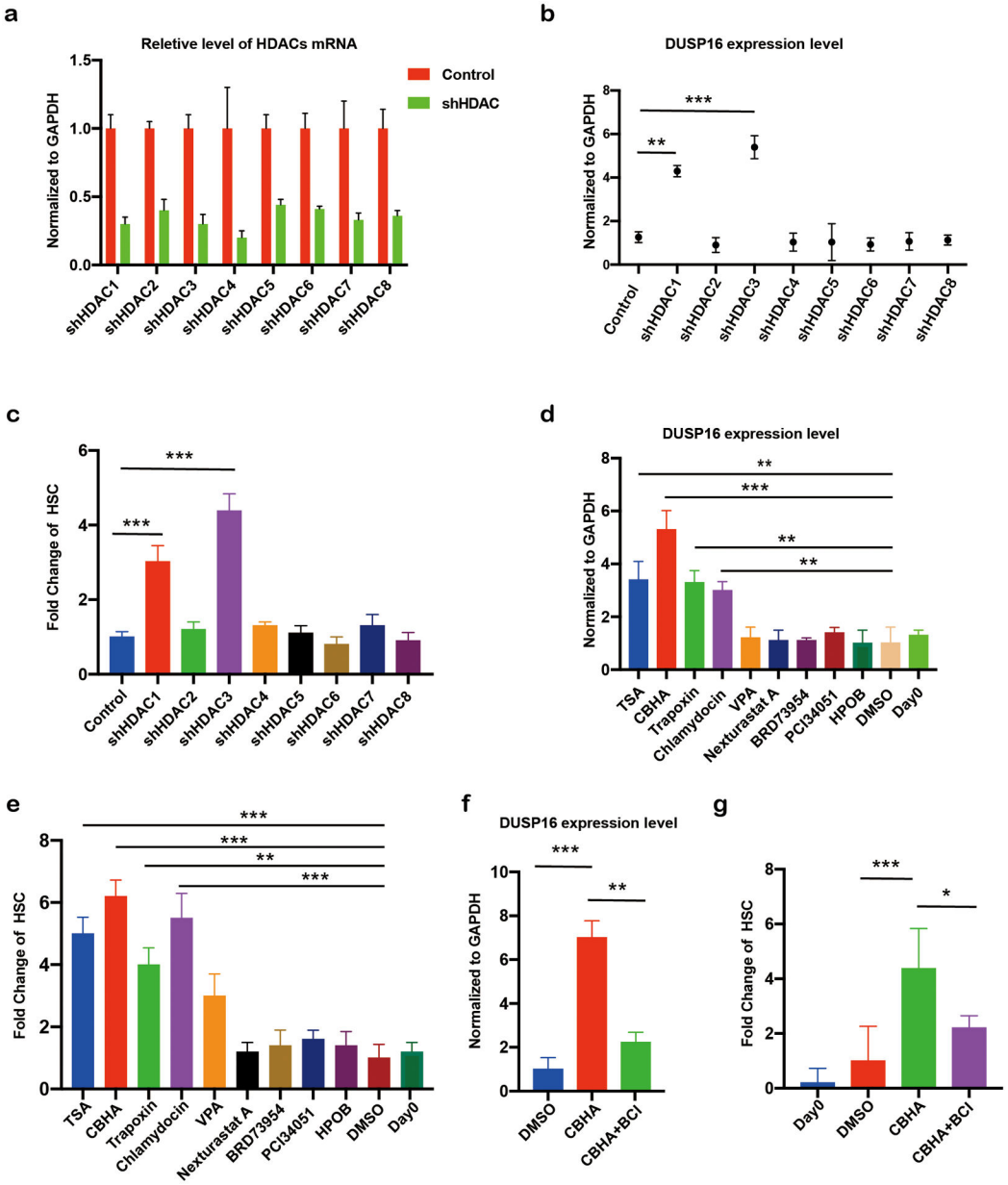

Fig 2. HDACi upregulates DUSP16 expression through inhibition of HDAC1/HDAC3, and functionally mimics DUSP16 overexpression.

a. Expression level of HDACs after knock-down by shRNA in CB CD34$^+$ cells. Data are shown as relative mRNA levels normalized to the level in control, which was set to 1. n=3 for each group. **b**. Fold change of DUSP16 expression after HDACs knock-down by shRNA in CB CD34$^+$ cells. n = 3 per group. **c**. Fold expansion of HSC from CB CD34$^+$ cells after HDACs knock-down by shRNA. n = 3 per group. **d**. Fold change of DUSP16 expression level after HDACi treatment in CB CD34$^+$ cells. n=3 per group. **e**. Fold expansion of HSC from CB CD34$^+$ cells after HDACi treatment. n = 3 per group. **f**. Fold change of DUSP16 expression level after CBHA and CBHA plus BCI treatment in CB CD34$^+$ cells. n = 3 per

group. **g**. Fold *ex vivo* expansion of CB HSC after CBHA and CBHA plus BCI treatment. n= 3 per group. Data are shown as mean ± s.e.m. *P < 0.05; **P < 0.01; ***P < 0.001.