Genome-wide screen identifies *Runx2* as a novel regulator of hematopoietic stem cell expansion and T-cell commitment

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Abstract

Self-renewing multipotent hematopoietic stem cells (HSCs) are a rare but important cell population which can reconstitute the entire blood and immune system following transplantation. Due to their rarity, it has been difficult to comprehensively study the mechanisms regulating HSC activity. However, recent improvements in hematopoietic stem and progenitor cell (HSPC) culture methods using polyvinyl alcohol-based media now facilitate large-scale *ex vivo* HSC expansion. Here we performed a genome-wide CRISPR knockout (KO) screen in primary mouse HSPCs to discover novel regulators of *ex vivo* expansion. The screen identified *Runx2* as a strong negative regulator of HSC expansion, which we validated using *ex vivo* and *in vivo* assays. Loss of *Runx2* increased the frequency of immunophenotypic HSCs in HSPC cultures by ~3-fold. Following expansion, these *Runx2*-KO HSCs engrafted at ~5-fold higher levels in transplantation assays. Non-cultured *Runx2*-KO HSCs also displayed enhanced reconstitution potential, but loss of *Runx2* did not alter blood parameters. Notably however, T-cell reconstitution was diminished from *Runx2*-KO HSCs, and we further validated an additional role for *Runx2* in T-cell commitment using *ex vivo* and *in vivo* assays. In summary, we have identified a multifaceted role for *Runx2* in HSCs, as a negative regulator of HSC self-renewal and as a facilitator of T-cell commitment. These results will contribute to our understanding transcriptional regulation of hematopoiesis and improve HSC therapies.

Key findings

- *Runx2*-deficient HSCs have a fitness advantage *ex vivo* and *in vivo* via increased self-renewal
- *Runx2* is necessary for normal T-cell commitment
- *Runx2* is not required for steady-state hematopoiesis but loss promotes HSC fitness in the context of expansion and regeneration

Introduction

Life-long hematopoietic system homeostasis relies on a rare population of self-renewing multipotent hematopoietic stem cells (HSCs), which are primarily found within the bone marrow[1,2]. It is estimated that just ~100,000 HSCs sustain the human hematopoietic system[1]. HSCs are also therapeutically important because HSCs will reconstitute the blood and immune system following transplantation into a bone marrow-ablated recipient. This is the basis for stem cell transplantation and related gene therapies. Stem cell transplantation is used clinically to treat a range of blood disorders including leukemias, anemias, and immunodeficiencies[3].

Ex vivo HSC expansion has been proposed as a method to improve HSC therapies. Increasing the numbers of functional HSCs available for therapy could have significant impacts, particularly increasing treatment access and safety. However, stable HSC expansion conditions have long remained challenging. We previously developed a polyvinyl-alcohol (PVA)-based culture system that permits long-term *ex vivo* expansion of transplantable mouse HSCs[4,5]. Within these hematopoietic stem and progenitor cell (HSPC) cultures, transplantable HSCs are found within the $CD201^+CD150^+cKit^+Sca1^+Lineage^-$ ($CD201^+CD150^+KSL$) compartment[6,7], which can be genetically manipulated at high efficiency[8,9]. This polymer-based HSC culture method has recently been adapted for human HSCs[10], highlighting the potential clinical translation of this approach. However, a comprehensive understanding of the molecular regulators of *ex vivo* HSC expansion remains lacking.

The *Runx* transcription factor family consists of *Runx1*, *Runx2*, and *Runx3*[11]. Runx factors contain a conserved Runt protein domain which binds DNA by dimerizing with the common co-binding partner CBFβ[11]. *Runx1* is a well-described hematopoietic transcription factor, required for HSC formation during definitive hematopoiesis[12]. *Runx1* also regulates adult HSPC activity[11] and is commonly mutated in leukaemia[13]. While *Runx3* is less well studied in hematopoiesis, loss has been shown to promote a myeloproliferative phenotype[14] and it has also been shown to prime HSCs for lymphoid differentiation[15]. *Runx2* is primarily thought of as a key regulator of skeletal development[16] and bone cell differentiation[17]. To date, few studies have investigated its role in hematopoiesis. *Runx2* has been reported to display a HSPC-restricted expression pattern, and to regulate myelopoiesis in normal and leukemic contexts[16,18]. In particular, *Runx2* has also been shown to be important for the development of plasmacytoid dendritic cells (pDCs)[19]. Despite this, there are no reported roles for *Runx2* in HSC self-renewal or fitness.

Here, we used a genome-wide CRISPR screen approach to identify *Runx2* as a potent negative regulator of HSC expansion *ex vivo*. Alongside boosting HSC expansion *ex vivo*, we also discovered that expanded *Runx2*-KO HSCs more efficiently reconstitute the myeloid and B cell lineages *in vivo* following transplantation. We have also discovered that *Runx2* is required for the normal development and reconstitution of T-cells.

Materials and Methods

Reagents referenced in this methods section are detailed in **Table S1-2**.

Mouse models

All experiments were initiated with 8-12-week-old mice and were approved by the UK Home Office. This study used two different Cas9-expressing mouse lines purchased from the Jackson Laboratory and bred in house: (1) the doxycycline-inducible Cas9-expressing line, C57BL/6-Gt(ROSA)26Sor$^{tm1(rtTA*M2)Jae}$ Col1a1$^{tm1(tetO-cas9)Sho}$ (JAX:029415); and (2) the constitutive Cas9-expressing line, C57BL/6-Gt(ROSA)26Sor$^{tm1.1(CAG-cas9*,-EGFP)Fezh}$ (JAX: 024858). We also generated *Runx2*-KO mice by crossing *Runx2*$^{tm1.1Tok}$ (MGI:6874594[20]) with B6.Cg-*Commd10*$^{Tg(Vav1-icre)A2Kio}$/J (JAX:008610[21]) for hematopoietic-specific *Runx2* deletion. C57BL/6-CD45.1 and C57BL/6-CD45.1/CD45.2 were bred in house while C57BL/6-CD45.2 were purchased from Envigo.

Ex vivo HSPC cultures

Bone marrow was collected and stained with APC-c-Kit antibody and then with anti-APC magnetic beads, before MACS column enrichment[8]. Bone marrow c-Kit$^+$ HSPCs were plated into 24-well CellBIND plates in PVA-based media[4,8] described in **Table S3** and incubated at 37°C with 5% CO_2 and 5% O_2. Complete media changes were performed every 2-3 days. Cultured HSCs were transferred to fibronectin-coated plates for lentiviral transduction at indicated timepoints and then returned to CellBIND plates for further culture.

Ex vivo HSPC CRISPR screen

Following an initial 17-day *ex vivo* expansion, ~100 million constitutive Cas9-expressing HSPCs (with a CD201$^+$CD150$^+$KSL cell frequency at ~10%; **Figure S1A**) were transduced with the Gouda genome-wide lentiviral sgRNA KO library (Addgene #73633[22]). High titer lentivirus library was generated from HEK 293T cells (in DMEM supplemented with sodium 10 mM pyruvate, 2 mM GlutaMax, 4.5 mM sodium propionate, 2mM sodium butyrate, 1.5 mM caffeine, and 5% heat-inactivated FBS), concentrated by ultracentrifugation, and titrated using K562 cells[23]. HSPCs were transduced for 6 hours at an MOI of 0.3, then recovered for 48

hours before being puromycin selected (1 ug/ml) for 48-hours prior to collection of the t0 timepoint. Droplet digital PCR on genomic DNA collected following puromycin selection confirmed ~0.8 virus integration per genome (**Figure S1B**). This genome-wide HSC KO library was split into two technical replicates and expanded another 21-days, with samples collected at t7, t14, and t21. Following gDNA extraction, sgRNAs were PCR amplified for next generation sequencing. The MAGeCK pipeline[24] was used to analyze the dataset.

Lentiviral gene knockouts

Guide sequences (**Table S4**) were acquired from the Bassik lab CRISPR knockout library[25] or designed using the WGE Sanger tool[26] and cloned into the pMCB306 lentiviral plasmid (Addgene #89360). As controls, a *Rosa26*-targeting sgRNA and a non-targeting sgRNA were used. Lentivirus was then produced by transfection of HEK293T cells and HSPCs were transduced on day 14 of culture. For experiments using dox-inducible Cas9 cells, Cas9 was induced by the addition of 500 ng/ml doxycycline for 48-hours at 48-hours post-transduction. HSPCs were collected at indicated time points for assays detailed below.

Flow cytometry and sorting

HSPC cultures, peripheral blood, bone marrow, thymi, spleens and T-cell differentiations were stained using the relevant antibody cocktails (**Tables S5-17**) and then analyzed on a BD Fortessa or sorted on a BD FACS Fusion. For intracellular flow cytometry, cultured HSPCs were fixed and permeabilized using the BD Pharmigen Transcription Factor Buffer Set and then antibody stained for 40 minutes with anti-RUNX2-PE or an IgG isotype control.

HSC transplantation

For CRISPR *Runx2*-KO experiments, C57BL/6-CD45.1 mice were lethally irradiated (12 Gy) and received tail vein injections of 2000 $CD201^{+}CD150^{+}$KSL cells and 1 million whole bone marrow competitor cells. For *Runx2*-flox KO experiments, lethally irradiated C57BL/6-CD45.1 mice received either 100 fresh bone marrow $CD150^{+}CD34^{-}$KSL HSCs and 1 million whole bone marrow competitor cells, or 10,000 day-14 cultured cells (initiated from $CD150^{+}CD34^{-}$ KSL HSCs) and 1 million whole bone marrow competitor cells. Peripheral blood was taken every 4-weeks and analyzed via flow cytometry using the panel in **Table S9**. At the endpoint, bone marrow cells were collected and stained with the panels in **Table S10-11** for flow cytometry. Secondary transplant assays were performed using 5 million whole bone marrow from primary recipients.

CUT&Tag chromatin profiling

CUT&Tag was performed as described previously[27] using anti-RUNX2 and anti-H3K4me3 antibody (**Table S1**). Libraries were 150bp paired-end sequenced with a NovaSeq (Illumina, Novogene). Quality checking of FastQ files was performed using FastQC (v0.12.1)[28]. The reads were trimmed using Trim Galore (v0.6.10)[29]. Bowtie2 (v2.5.1) was then used to map the trimmed reads to the mm39 reference genome[30]. Bigwigs were produced using deeptools bamCoverage (v3.5.1)[31] and visualised using computeMatrix and plotHeatmap (v3.5.1)[31]. Peaks were called using LanceOtron callPeaks[32]. De novo motif discovery was performed using Streme (v5.5.3)[33], and enriched motifs were compared to the HOCOMOCO (v12) database using Tomtom (v5.5.3)[33,34]. Raw motif analysis data can be found in **Table S18.**

Transcriptomic analysis

RNA from FACS-isolated $CD201^+CD150^+$KSL immunophenotypic HSCs (n=3) and $CD201^-CD150^-$KSL hematopoietic progenitor cells (HPC; n=3) were extracted using the Direct-zol RNA Microprep kit. Libraries were 150bp paired-end sequenced with a NovaSeq (Illumina, Novogene). Quality checking of FastQ files was performed using FastQC (v0.12.1)[28]. The reads were trimmed using Trim Galore (v0.6.10)[29]. STAR (v2.7.10b) was then used to map the trimmed reads to the mm39 reference genome[35]. Quantification of gene expression was performed using featureCounts (v2.0.3)[36]. Differential gene analysis was performed using DESeq2 (v1.40.2)[37]. GO over representation analysis was performed using Cluster Profiler (v4.10.1)[38]. Raw datasets can be found in **Tables S20-S22**.

Ex vivo T-cell differentiation

Biotinylated recombinant human DLL4 was incubated with streptavidin-coated polystyrene particles at 30 minutes at room temperature to generate DLL4-µBeads. $1\text{-}20\times10^3$ cells from indicated 14-day HSPC cultures were differentiated with 1×10^5 DLL4-µBeads in the media described in **Table S23**. After 7 days incubation at 37°C in 5% CO_2, cells were harvested for flow cytometry using the panel in **Table S10**.

Results

An ex vivo CRISPR KO screen identifies putative regulators of HSC expansion

To comprehensively identify the regulators of *ex vivo* HSC expansion potential, we performed a genome-wide CRISPR KO screen during a PVA-based mouse HSPC expansion culture (**Figure 1A**). Whilst these cultures are heterogenous, they enable cell culture scales sufficient for a genome-wide screen. Following CRISPR library transduction and puromycin selection, we collected samples weekly for 3-weeks (t0-t21) for sgRNA sequencing. We detected essentially all sgRNAs throughout the 21-day screen, although the library compositions slowly

changed over time (**Figure S1C**). We performed MAGeCK analysis[24] to identify statistically-significant regulators of HSPC expansion in these *ex vivo* conditions.

From this screen analysis, we identified both putative essential genes with a negative effect (beta) score (where sgRNAs abundance decrease over time) and putative negative regulators with a positive effect score (where sgRNA abundance increases over time) (**Figure 1B; Table S24**). Initially focusing on the 2305 statistically-significant essential genes (FDR<10%), we compared them with the Achilles common essential list[39] (containing 1552 genes). While 995 were also found in the Achilles list, 1310 appeared to be HSPC-specific essential genes (**Figure 1C**). Several of these are previously reported regulators of HSPC activity (e.g. *Gata2*[40,41], *Lmo2*[41,42]). However, the list also contains various novel putative essential genes for the growth and/or survival of HSPCs in PVA-based cultures.

Given our interest in identifying mechanisms to boost HSC expansion, we focused on the putative negative regulators of ex vivo expansion. Our screen identified 92 statistically significant (FDR<10%) hits (**Figure 1B**). This again included reported HSC negative regulators (e.g. *Trp53*[43], *Pten*[44]) and novel putative regulators. To better understand the potential biological networks negatively regulating HSPC expansion, we performed STRING analysis[45] (**Figure 1D**). The transcription factor *Runx2* appeared in this analysis as a well-connected node, but one that had not to our knowledge been previously implicated in regulating HSC expansion. We confirmed that *Runx2*-targeting sgRNAs increased ~5-fold during the screen (**Figure 1E**). This was notably higher than the other Runx family members, *Runx1* and *Runx3*, which appeared to be more modest negative regulators of HSPC expansion (~3-fold) and did not reach statistical significance (**Figure 1E**). These results suggested *Runx2* as a novel negative regulator of *ex vivo* HSC expansion.

Loss of Runx2 enhances HSC fitness but inhibits T-cell output

Given that *Runx2* was one of the top putative negative regulators in our HSPC expansion screen, yet played an unknown role in HSC activity, we sought to validate this hit. First, we validated that RUNX2 was expressed in the majority of our cultured HSCs via flow cytometry (**Figures S2A-B)**. RUNX2 expression in *ex vivo* HSCs was similar to freshly-isolated HSCs, but lower than in pDCs, which are known to highly express RUNX2[19] (**Figure S2C**). Next, we performed targeted deletion of *Runx2* in our *ex vivo* HSPC cultures by transducing dox-inducible Cas9-expressing HSCs with lentivirus encoding *Runx2*-targeting sgRNAs or control sgRNAs (*Rosa26*-targeting sgRNA, non-targeting sgRNA) (**Figures 2A-B**). We confirmed that these sgRNAs induced loss of RUNX2 protein expression (**Figure 2C)**. We then tracked the

frequency of immunophenotypic CD201^{+}CD150^{+}KSL (p)HSCs[6,46] (**Figure 2D-E; Figure S2D**) and KSL (p)HSPCs for 3-weeks following dox-mediated Cas9 induction (**Figure S2E**). Loss of *Runx2* progressively increased the pHSC frequency to ~3-fold by day 21 (**Figure 2E**). These results confirmed our CRISPR screen identification of *Runx2* as a negative regulator of pHSC expansion.

We were next interested in determining the functional consequences of *Runx2* loss. We performed HSC transplantation assays in CD45.1^{+} lethally irradiated mice. To account for the increased frequency of pHSCs in the *Runx2*-KO cultures, we sorted and transplanted 2000 CD45.2^{+} pHSCs from 14-day *Runx2*-KO and control HSC cultures per recipient alongside 1 million CD45.1^{+}CD45.2^{+} whole bone marrow competitor cells (**Figure 2F**). Donor chimerism was then tracked monthly in the peripheral blood (**Figure S2F**). Given the similar phenotypes between the two control sgRNAs and the two *Runx2*-targeting sgRNAs, the results have been merged for ease of visualization.

Even from the earliest 4-week timepoint, the frequency of *Runx2*-KO HSC-derived cells was higher in the peripheral blood; ~75% compared to ~30% of control cells (**Figure 2G**). This enhanced donor chimerism was sustained in the peripheral blood (myeloid, T- and B-cells) throughout the 20-week time course in the *Runx2*-KO recipients, while donor chimerism in the control cohort gradually dropped to ~15% (**Figure 2G-H**). Endpoint bone marrow analysis also confirmed correspondingly high chimerism; ~90% donor chimerism in the *Runx2*-KO recipients as compared to ~10% in the controls (**Figure 2I; Figure S2F**). Within the bone marrow, we also observed a modest (~2-fold) increase in KSL frequency (**Figure S2G**), in line with the *ex vivo* phenotype.

Within the *Runx2*-KO HSC recipients, blood parameters appeared normal in complete blood count analysis, suggesting that loss of *Runx2* did not block blood production (**Figure S2H**). However, further investigation identified a lineage output bias from the *Runx2*-KO HSCs. Within the *Runx2*-KO HSC-derived peripheral blood cells, B-cell frequency was enhanced while T-cell frequency was correspondingly depleted (**Figure 2J**) compared to control HSCs. The frequency of myeloid cells was unaltered. Secondary transplantation assays confirmed that *Runx2*-KO HSCs sustained high peripheral blood and bone marrow chimerism long-term (**Figure S2I-J**). Together, these results suggest that loss of *Runx2* provides HSCs with a fitness advantage while inhibiting T-cell potential.

Heterozygous loss of Runx2 is sufficient to increase HSC expansion and engraftment

To further investigate the role of *Runx2* in hematopoiesis and to better understand the importance of *Runx2* dosage *in vivo*, we bred and characterized a *Runx2*^{flox} *Vav1-iCre* transgenic mouse line (**Figure 3A**). Analysis of the bone marrow, spleen and thymus from *Runx2*$^{flox/flox}$ *Vav1-iCre*$^{+}$ (*Runx2*-hom KO) and *Runx2*$^{flox/wt}$ *Vav1-iCre*$^{+}$ (*Runx2*-het KO) adult mice (8-12-weeks) revealed no gross changes associated with unperturbed hematopoiesis compared to wild-type (*Runx2*-wt) controls (**Figures S3A-G**). This was in line with our *Runx2*-KO transplant recipients and a previous report that did not observe changes in blood parameters[47].

To understand whether loss of *Runx2* altered HSC activity without *ex vivo* culture, we performed transplantation assays using freshly-isolated *Runx2*-hom, *Runx2*-het KO and *Runx2*-wt HSCs. *Runx2*-hom displayed higher long-term donor peripheral blood and bone marrow chimerism (**Figures 3B-C**). Again, the lineage output of the peripheral blood showed a decreased T-cell output in the *Runx2*-hom KO donor cells. To our surprise, *Runx2*-het KO HSCs largely recapitulated the boost in reconstitution potential, although they did not display reduced T-cell output (**Figure 3D**). No gross changes in hematopoiesis were observed in any of the recipient groups (**Figure S3H**).

We next investigated how *Runx2* dosage influenced HSC expansion. Both *Runx2*-hom KO and *Runx2*-het KO HSCs displayed a similar phenotype to our CRISPR KOs *ex vivo*, generating higher pHSC frequencies (**Figure 3E**). *Runx2*-hom and *Runx2*-het KO HSC cultures also engrafted at similar levels in transplantation assays, and significantly higher than *Runx2*-wt controls (**Figures 3F-H**). Again, the complete blood cell counts showed no gross changes in hematopoiesis (**Figure S3I**). Finally, we performed secondary transplant assays to evaluate long-term HSC activity for both the fresh and the cultured HSC transplants (**Figures S3J-M**). In both settings, *Runx2*-deficient donor chimerism remained 2-5-fold higher than the *Runx2*-wt controls. We conclude that even partial loss of *Runx2* is sufficient to significantly boost HSC expansion and long-term repopulation capacity.

Loss of Runx2 causes context-specific transcriptional dysregulation

To characterize the molecular activity of RUNX2 in HSCs, we identified RUNX2 genomic binding using CUT&Tag in day-14 *Runx2*-wt CD201^{+}CD150^{+}KSL pHSCs, and profiled histone H3 lysine 3 trimethylation (H3K4me3) to annotate active gene promoters. We also performed RNA-sequencing on day-14 *Runx2*-wt, *Runx2*-het KO and *Runx2*-hom KO pHSCs to identify transcriptional changes associated with the loss of *Runx2* (**Figure 4A**).

For our transcriptomic analysis, we initially performed principal component analysis (PCA). This separated *Runx2*-wt, *Runx2*-het, and *Runx2*-hom pHSCs by across PC1, suggesting that *Runx2*-het had an intermediate transcriptional phenotype, consistent with the partial loss of *Runx2* (**Figure S4A**). We next performed differential gene expression analysis for *Runx2*-hom and *Runx2*-het samples, relative to *Runx2*-wt controls (**Figure 4B**). Overlapping differentially expressed genes (DEGs), we identified 119 common DEGs, from 564 DEGs in *Runx2*-hom pHSCs and 610 DEGs in the *Runx2*-het context (**Figure 4C**). This implied that many DEGs are *Runx2* dosage specific (**Figure 4C**). Gene Ontology analysis identified DEGs were enriched for genes associated with inflammation and interferons (**Figure 4D; Figure S4B**), which have been shown to promote HSC cell division[48].

From our CUT&Tag experiments, we identified 15,649 statistically significant RUNX2 binding sites in pHSCs, of which 58% were found gene promoters while 13% were found in distal intergenic regions, and the remaining peaks found within gene loci (**Figure 4E)**. This contrasted with its binding in osteoblasts, where only 20% of RUNX2 peaks have been found at gene promoters[49]. Motif analysis of RUNX2 peaks (filtered by genes expressed in *Runx2*-wt pHSCs) identified the RUNX motif, implying that RUNX2 directly bound these genomic sites (**Figure 4F; Figure S4C**). Suggesting potential RUNX2 binding, ETS factor and KLF factor motifs were identified along with binding motifs for KMT2A, ERF, and TYY1 (**Figure 4F; Figure S4C**).

To further understand the potential direct versus indirect regulation of the DEGs by RUNX2, we integrated our RUNX2 target genes with our DEGs (focusing on *Runx2*-hom DEGs). RUNX2 was found to be enriched in a subset of DEG promoters (**Figure 4G**). Within the *Runx2* locus, we observed RUNX2 enrichment at the *Runx2* promoter as well as several intragenic binding sites, suggesting potential autoregulation (**Figure 4H**). Interestingly, there was a strong peak at H3K4me3 for the proximal promoter in WT HSCs, and no peak at the distal promoter (**Figure 4H**), implying the proximal promoter is preferentially used in HSCs[50,51].

To further characterize the cell-type specific effects of *Runx2* loss, we performed RNA-seq on $CD201^{-}CD150^{-}$KSL pHPCs from *Runx2*-wt, *Runx2*-het KO, and *Runx2*-hom KO day-14 cultures. PCA again distinguished samples based on genotype (**Figure S4D**). Similar to the pHSCs, we identified a subset of common DEGs between the *Runx2*-hom and *Runx2*-het samples, relative to *Runx2*-wt controls; 643 of 2016 *Runx2*-het DEGs and 1095 *Runx2*-hom DEGs (**Figure S4E**). While Gene Ontology analysis also identified similar gene sets (**Figure S4F**), only 156 *Runx2*-hom DEGs were present in both HPC and HSC settings (**Figure S4G**).

We therefore conclude that RUNX2 has a context-specific and dosage-specific activity in HSPCs.

Loss of Runx2 enhances HSC self-renewal and inhibits T cell commitment

We were next interested to better understand the cellular mechanisms underlying the enhanced HSC expansion in *Runx2*-KO cultures. To investigate this, we evaluated *Runx2*-KO HSCs at the clonal level over 14-day cultures (**Figure 5A**). Overall clone size was similar between *Runx2*-wt and *Runx2*-hom KO HSCs (**Figure 5B**), and the frequency of clone survival was also similar (**Figure S5A**). However, we observed a significant increase in the number of $CD201^{+}$KSL pHSCs being generated from *Runx2*-hom KO HSC clones (**Figure 5C**). We also identified an increased proportion of HSC clones with high pHSC frequencies (>76%) from *Runx2*-hom KO HSCs (**Figure 5D**). These results suggested that loss of *Runx2* enhances HSC self-renewal divisions, rather than growth or survival. We therefore conclude that *Runx2* regulates HSC cell fate decision.

Finally, we sought to better understand the role of *Runx2* in T-cell development. As described above, *Runx2-KO* HSCs displayed reduced T-cell chimerism in transplantation assays (**Figure 2** and **Figure 3**). This could have been due to a failure of the *Runx2*-KO HSCs to generate T-cells, and/or a failure of the *Runx2-KO* T-cells to survive or proliferate. Intriguingly, several pathways related to T-cell differentiation and activity were identified in our *Runx2*-KO downregulated DEGs (**Figure S4F**), supporting the idea that loss of *Runx2* may alter T cell commitment from HSPCs.

To directly test this hypothesis, we performed *ex vivo* T-cell differentiation assays on 14-day HSPC cultures (**Figure 5E**). T-cell commitment initiates with the stepwise progression through several double negative (DN) cell stages[52], which are discriminated by CD44 and CD25 expression (**Figure 5F; Figure S5B**). We observed a significant, albeit incomplete, reduction in DN2 and DN3-stage T-cell precursors in 7-day cultures, suggesting a DN1-stage block in T-cell commitment (**Figure 5G**). Consistent with this, we also observed a significant reduction in $CD90.2^{+}CD25^{+}$ T-cell precursors (**Figure S5C**). Further discrimination of the DN1 population, using c-Kit and CD24 expression[53], identified a modest but significant build-up of $c\text{-}Kit^{+}CD24^{-}$ and $c\text{-}Kit^{+}CD24^{lo}$ DN1 cells (**Figure S5D**). These results suggest that loss of *Runx2* partially blocks T-cell commitment at an early DN1 cell stage.

To investigate this T-cell commitment in vivo, we evaluated early thymic reconstitution at 4-weeks post-transplantation (**Figure 5H**). We observed a ~2-fold increase in the frequency of *Runx2*-deficient DN1 cells as compared to *Runx2*-wt controls (**Figure 5I; Figure S5E**), in line

with the DN1 stage *ex vivo* phenotype. However, we did not observe a significant difference in the later stages, perhaps due to the different timepoint of analysis and the homeostatic expansion of these T-cell precursors. Consistent with the role of *Runx2* in pDC development, pDC frequencies were also markedly reduced in the thymus (**Figure S5F**). Within the bone marrow, we observed a significant increase in immunophenotypic multipotent progenitor cells[54] (including $Flt3^{+}CD150^{-}$KSL lymphoid-biased multipotent progenitor cells) and $CD127^{+}$KSL common lymphoid progenitor cells (**Figure 5J; Figure S5G**), suggesting that loss of *Runx2* enhances reconstitution of upstream lymphoid progenitor populations. We therefore conclude that loss of *Runx2* alters T-cell commitment but does not block lymphoid progenitor populations.

Discussion

In this study, we performed a genome-wide CRISPR screening for *ex vivo* HSPC expansion regulators. This screen identified both known and novel putative HSC regulators and may provide a useful resource for those interested in the molecular regulation of HSC activity. We have focused on validating and investigating the biology of one of its novel hits, *Runx2*. However, a number of other negative regulators remain to be investigated.

Through use of CRISPR and a hematopoiesis-specific $Runx2^{flox}$ mouse line, we have validated *Runx2* as a potent negative regulator of HSC expansion *ex vivo* and fitness *in vivo*. Notably, *Runx2*-KO HSCs engrafted at ~5-fold higher levels than controls. Clonal analysis implicated loss of *Runx2* leads to improved HSC self-renewal rather than altering rates of survival or proliferation. These results suggest that temporarily inhibiting RUNX2 function could have therapeutic potential to boost HSC expansion and engraftment in the context of stem cell transplantation. However, further studies are warranted to investigate the molecular role of *Runx2* in HSC fate decisions and human HSCs. Additionally, it would be interesting to evaluate *Runx2*-KO HSCs in a head-to-head competitive assay against wild-type HSCs.

Given the fitness advantage of HSCs following *Runx2* loss, we were surprised to find few *RUNX2* mutations in hematological malignancies (in contrast to the occurrence of *RUNX1* mutations)[55,56]. On the contrary, enforced *RUNX2* expression has been associated with the onset of AML and heterozygous loss predicted to delay leukemic onset in mice[16,18]. *RUNX2* is also highly expressed in high-risk T-acute lymphoblastic leukemia[57] and has a role in its progression[47]. From our results, we hypothesize that RUNX2 inhibitors should display a good therapeutic window in the hematopoietic system. Further evidence for this comes from patients with inherited cleidocranial dysplasia (CCD) who carry autosomal dominant mutations in *RUNX2*[58]. No significant correlations between CCD and hematological or immunological

perturbations have been reported, in line with our analysis of steady-state hematopoiesis in *Runx2*^{flox} *Vav1-iCre* mice. However, further characterization of aged *Runx2*^{flox} *Vav1-iCre* mice is warranted.

Constitutive loss of *Runx2* inhibited T-cell commitment and reduced T-cell reconstitution post-transplantation. Thus, any therapeutic potential of *Runx2* as an approach to boost HSC expansion and engraftment should rely on transient inhibition. It will be important to resolve the molecular role of RUNX2 in this process as well as its interactions with RUNX1 and RUNX3 as both factors are also regulators of lymphoid differentiation[59,60]. Given the overlapping expression and targets of Runx factors, it is interesting that loss of a single Runx factor has such strong effects in T-cell development and HSC expansion.

In summary, we used a genome-wide CRISPR KO screen approach to discover *Runx2* as a negative regulator of *ex vivo* HSC expansion and fitness. In validating this finding, we also uncovered a previously unappreciated role for *Runx2* in T-cell development. This study therefore provides important new insights into the molecular regulation of HSC fitness and T-cell commitment.

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Data availability

Sequencing datasets have been deposited on GEO (GSE288012). The datasets will be released following acceptance but are available for review using the token: adgvkawsztspbot

Authorship contributions

G.A.M and A.C.W made the figures and wrote the manuscript. G.A.M., M.N., A.S., Y.B., M.L., H.V., L.O., O.B., X.H., E.B., M.V.B., J.B., and A.C.W., performed the experiments. I.H, H.M.K, C.C, and T.M. assisted with data analysis. A.C.W conceived the study. All authors edited the manuscript.

Conflict of interest disclosures

A.C.W. is a consultant for ImmuneBridge. T.A.M. is a paid consultant for and shareholder in Dark Blue Therapeutics Ltd. JB is named as an inventor on a provisional patent application related to the LVCTM lentivirus media production system filed through Stanford University. The remaining authors declare no competing financial interests.

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