Stem Cells, 2023, **41**, 944–957
https://doi.org/10.1093/stmcls/sxad055
Advance access publication 19 July 2023
Original Research

Stromal STAT5-Mediated Trophic Activity Regulates Hematopoietic Niche Factors

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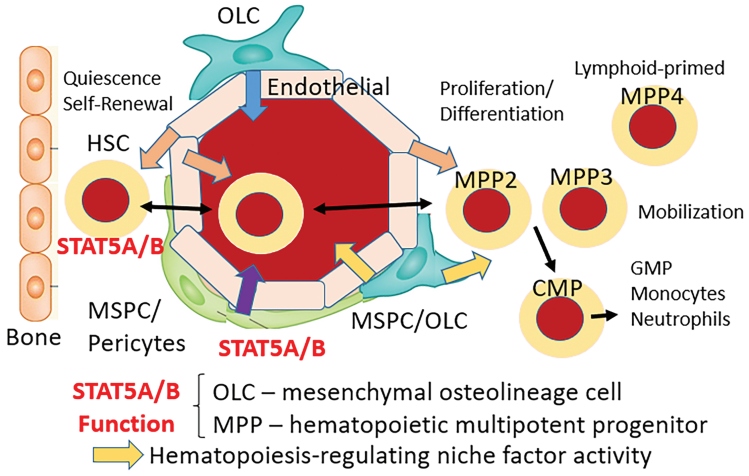
Abstract

Signal transducer and activator of transcription 5 (STAT5a and STAT5b) are intrinsically critical for normal hematopoiesis but are also expressed in stromal cells. Here, STAT5ab knockout (KO) was generated with a variety of bone marrow hematopoietic and stromal Cre transgenic mouse strains. Vav1-Cre/+STAT5ab$^{fl/fl}$, the positive control for loss of multipotent hematopoietic function, surprisingly dysregulated niche factor mRNA expression, and deleted STAT5ab in CD45^{neg} cells. Single-cell transcriptome analysis of bone marrow from Vav1-Cre/+ wild-type or Vav1-Cre/+STAT5ab$^{fl/fl}$ mice showed hematopoietic stem cell (HSC) myeloid commitment priming. Nes^{+} cells were detected in both CD45^{neg} and CD45^{+} clusters and deletion of STAT5ab with Nes-Cre caused hematopoietic repopulating defects. To follow up on these promiscuous Cre promoter deletions in CD45^{neg} and CD45^{+} bone marrow cell populations, more stroma-specific Cre strains were generated and demonstrated a reduction in multipotent hematopoietic progenitors. Functional support for niche-supporting activity was assessed using STAT5-deficient mesenchymal stem cells (MSCs). With Lepr-Cre/+STAT5ab$^{fl/fl}$, niche factor mRNAs were downregulated with validation of reduced IGF-1 and CXCL12 proteins. Furthermore, advanced computational analyses revealed a key role for STAT5ab/Cish balance with Cish strongly co-expressed in MSCs and HSCs primed for differentiation. Therefore, STAT5ab-associated gene regulation supports the bone marrow microenvironment.

Key words: signal transducer and activator of transcription-5; mesenchymal stem cell; bone marrow niche factors; hematopoiesis; cytokine signaling; single-cell RNA sequencing.

Graphical Abstract



Received: 23 March 2023; Accepted: 3 July 2023.

Graphical abstract figure, STAT5 function in hematopoiesis-supporting stromal cells. STAT5ab is expressed in both hematopoietic and stromal cells within a heterogeneous bone marrow niche that includes bone, endothelial cells, pericytes, and stromal cells. Previous studies by our lab and others showed important roles in hematopoietic stem cell and multipotent progenitor activity. However, STAT5ab expression in mesenchymal stromal progenitor cells and osteolineage progenitors (OLC) has not been explored. We show here that secreted niche factor production (arrows of different colors) supports hematopoiesis through a STAT5ab-dependent mechanism.

Introduction

The bone marrow (BM) microenvironment is comprised of niche cells that produce some cytokines in highly localized quantities requiring proximity for cells to derive a benefit. Outside of sensing these factors, hematopoietic stem cells (HSCs) are more likely to experience long-range acting cytokines and differentiate, leave the niche, or die. Although the epigenetic program[1] within each HSC ultimately dictates cell fate, the BM anatomic niche can have a profound effect on lineage commitment and self-renewal. Understanding the transcriptional regulation of HSCs and stromal cells is critical to develop new niche-based therapeutic strategies. In this study, we explored extrinsic mechanisms that support hematopoietic stem/progenitors in the BM microenvironment and identified important functions in heterotypic BM cells.

Hematopoiesis is a continuum of progressive differentiation downstream of self-renewing hematopoietic stem cells. HSCs are initially platelet-primed[2,3] and retain multipotency. With commitment, multipotent progenitors (MPPs) progressively lose CD150 (Slamf1) marker expression and myeloid potential as they progressively acquire lymphoid potential.[4-6] The regulation of this process in the BM is complex and the role of the niche in support of MPPs has not been defined, although much progress in long-term repopulating (LT)-HSC support has been gained in recent years. There is compelling data that stromal cell-derived factor-1 (SDF-1; Cxcl12) and stem cell factor (SCF; Kitl) producing cells form microniches with potentially specialized functions in hematopoietic stem/progenitor (HSC/HPC) support.[7-10] Key outstanding questions are how niche factors ensure normal hematopoiesis, whether niche factor source determines responses, and how these factors are regulated by a variety of stromal cells. Single-cell RNA sequencing studies are beginning to address heterogeneity in Vav1-Cre/+ wild-type mice on both the HSC[11] and stromal sides.[12,13] Additional hematopoietic support roles for T cells,[14,15] macrophages[16] (Mac), and megakaryocytes (Mks)[17] are described, but much remains to be learned about regulatory mechanisms controlling niche factors. Other than lymphoid-primed multipotent progenitor (LMPP; MPP4) responses to IL-7,[18] little is known about MPP2-3 beyond an important role for circadian-regulated Tnfα.[19,20]

Our prior work with signal transducer and activator of transcription-5 (STAT5) conditional knockout using Mx1-Cre showed defects in multi-lineage differentiation and survival but also in steady-state HSC homeostasis.[21] However, Mx1-Cre can also delete STAT5 in osteolineage progenitor cells resulting in reduced bone mass.[22] We have more recently used Vav1[23]-Cre[24] knockout mice to address adult hematopoiesis in the absence of an interferon response required for Mx1-Cre.[25] The Vav1-Cre mouse provides a tool for assessing effects on HSCs caused by STAT5 loss but it also has potential stromal expression.[26] Indeed, here we report efficient STAT5ab deletion in $CD45^{pos}$ and $CD45^{neg}$ cells. Since these tools can be promiscuous to some degree in non-hematopoietic cells, this led us to examine STAT5ab deletion using a suite of stroma-specific Cre transgenic strains and discovered function in hematopoiesis-supporting stromal cells.

Methods (also see Supplementary Methods)

Transgenic and Knockout Mice

All animal procedures were approved by the Emory University Institutional Animal Care and Use Committee. $STAT5ab^{flox/flox}$ (interchangeable with $STAT5ab^{fl/fl}$ as used in the text and figures) mice were originally obtained from Lothar Hennighausen (NIDDK, NIH).[27] Vav1-Cre (#035670) transgenic mice were generated and used as previously described.[25] This study used the following Cre-expressing transgenic mouse lines obtained from the Jackson Laboratory (Bar Harbor, ME) to generate new stromal knockout strains lacking expression of STAT5ab: Nes-Cre/+ (#003771), LepR-Cre/Cre (#008320), Prrx1-Cre/+ (#005584), Osx1-GFP:Cre/+ (#006361), BGLAP(OCN)-Cre/+ (#019509), and Nes-CreERT2/+ (#016261). Cdh5-CreERT2/+ mice generated by Ralf Adams were generously provided by Brian Petrich (Emory). See Supplementary Methods for detailed information on all Cre driver mice. To delete STAT5 in hematopoietic or mesenchymal lineages, various Cre mice were genetically crossed with $STAT5ab^{flox/flox}$ mice. For inducible Cre (Nes-CreERT2 or Cdh5-CreERT2/+) knockout, mice were treated with Tamoxifen (MilliporeSigma). Tamoxifen was dissolved in corn oil at a concentration of 20 mg/mL by shaking overnight at 37°C in foil paper wrapped tube to protect from light and then mice were injected with 75 mg tamoxifen/kg body weight via i.p. injection once every 24 h for 5 consecutive days. The final analysis of those mice was carried out 2 weeks after the last dose of treatment.

Multi-Parameter Flow Cytometry Analysis

Bone marrow (BM) cells were stained with FITC conjugated lineage antibodies (Gr-1, Mac-1, B220, Ter119, CD3, CD4, and CD8a), APC-Cy7-c-Kit, PE-Cy7-Sca-1, eFluor 450-CD48, PerCP-eFluor 710-CD150, eFluor 660-CD34, Alexa Fluor 700-CD16/32, and PE-CF594-CD127 (IL7Rα). In some cases, BM cells were stained with Biotin-CD135 (FLK2), then PE-streptavidin along with the antibodies mentioned above. Stained samples were run on FACSymphony A5 (BD Biosciences) and analyzed by FlowJo software (FlowJo, Ashland, OR). The population of cells was defined[28] as $CD150^{+}CD48^{neg}KLS$ (HSCs), $CD150^{neg}CD48^{neg}KLS$ (previously referred to as MPP[28]), $CD150^{neg}CD48^{+}KLS$ (HPC-1), and $CD150^{+}CD48^{+}KLS$ (HPC-2). When BM cells were stained including CD34 and CD135 antibodies, stem and progenitor cells were defined as from Wilson et al[29]: LT-HSC ($CD34^{neg}CD48^{neg}CD150^{+}CD135^{neg}KLS$), MPP1 ($CD34^{+}CD48^{neg}CD150^{+}CD135^{neg}KLS$), MPP2 ($CD34^{+}CD48^{+}CD150^{+}CD135^{neg}KLS$), MPP3 ($CD34^{+}CD48^{+}CD150^{neg}CD135^{neg}KLS$), and MPP4 ($CD34^{+}CD48^{+}CD150^{neg}CD135^{+}KLS$).

Single-Cell RNA Sequencing

Tissue-derived single cells (see Supplementary Methods) were loaded onto the 10X Chromium Controller targeting 3000-6000 cells/sample. Single-cell capture, barcoding, GEM-RT, clean-up, cDNA amplification, and library construction were performed according to the manufacturers' instructions for v3 chemistry using 10x Genomics Chromium Chip B Single Cell Kit (PN 1000074) and Chromium Single Cell 3′ GEM, Library & Gel Bead Kit v3 (PN 1000092). Final gene expression

library pools were sent to Genewiz (South Plainfield, NJ) and sequenced on an Illumina Novaseq6000 instrument with a targeted sequencing depth of 150 000 reads/cell.

Single-Cell Transcriptomic Data Analysis

Raw sequencing reads were processed by Cell Ranger (10x Genomics) to generate gene expression count matrices for every sample (wild type and knockout samples for KLS and MSC, respectively). After sequence alignment to GRCm38/mm10 mouse reference genome, Unique Molecular Identifiers (UMI) counts for each gene per cell formed the gene expression count matrices. Based on UMI counts and cell barcodes, numbers of genes and cells for each sample were estimated. Clustering and UMAP visualization based on concatenation of the wild type and knockout samples did not show noticeable batch effect, which was consistent with the fact that the samples were sequenced together. Therefore, subsequent clustering and trajectory analyses were performed based on concatenation of the samples, without applying any scRNA-seq data integration workflow. Data for KLS and MSC were analyzed separately. Implemented using the Seurat package in R, the clustering workflow started with library size normalization and log transformation, followed by highly variable feature selection, principle component analysis and community detection, with clusters visualized using Uniform Manifold Approximation and Projection (UMAP). After cell clustering, differential gene expression analysis was performed using Wilcoxon rank sum test between each cell cluster against all other cells to obtain marker genes for interpreting the cell clusters. Cell clusters were annotated by a hybrid approach using marker genes as well as differentially expressed (DE) genes first computationally derived and then followed by pathway analysis. Annotation was based on these gene lists based on both marker genes and the enriched pathways according to seminal references in the field.[4,12,28,30-35]. Implemented using the Scanpy package in Python, the trajectory analysis was performed using the PAGA (partition-based graph abstraction) algorithm. The cell clusters generated in the Seurat clustering analysis served as the input of cell clusters to the PAGA algorithm, so that nodes in the graph abstraction of PAGA can be directly mapped to the cell clusters in the clustering analysis.

Statistical Analyses

All data were derived as a result of 3 or more independent experiments unless stated otherwise. Student's 2-tailed *t*-test was used to calculate *P*-values and values >.05 were considered to be significant. Throughout all figures the following nomenclature was used to indicate the level of statistical significance: $*P \le .05$; $**P \le .01$; $***P \le .001$.

Results

STAT5-Deficient KLS Cells Have Aberrant Myeloid Lineage Priming Based on Cluster-Specific Trajectory Analysis

To examine STAT5ab function in cells derived from Vav1$^+$ hematopoietic cells, STAT5 deletion with Vav1-Cre was achieved and KLS cells were sorted and analyzed by single-cell RNA seq. Sequencing data from 2841 WT cells and 7167 KO cells were obtained (KLS-WT, average of total UMIs = 10 979; KLS-WT, average number of genes = 3336; KLS-KO, average of total UMIs = 14 715; KLS-KO, and average number of genes = 3784). The subsequent data analyses yielded 13 clusters (12 hematopoietic, 1 stroma (Pdgfra$^+$, Lepr$^+$)) which is equivalent to 94% of purity for hematopoietic cells. Fig. 1 shows the results of UMAP (Fig. 1A) and trajectory analysis (Fig. 1B) as well a heat map of cluster-defining genes (Fig. 1C) with selected genes shown. Clusters were defined by manual curation of gene lists. Selected key genes for identifying HSC/HPC designations are indicated in Fig. 1C. Supplementary Fig. S1 shows some key cluster-defining genes within each cluster. In many cases, these genes are indicative of the specific hematopoietic lineage but not in all clusters. Differentially expressed gene sets among clusters were used to annotate in combination with unbiased trajectory analyses.

High levels of myeloid priming were observed with the trajectory going through myeloid-biased cells with clusters 1, 5, 7, 8, 9, 11, 12, and 13 all showing decreased Uba52, Car2, and Dntt. Myeloid-biased cluster 7 HSCs in Vav1-Cre/+STAT5 KO were characterized by downregulated of additional genes including Cd34, Mpl, Myc, and increased expression of definitive hematopoiesis (Hoxb4, Runx1, Gata2, and Mesi1) induction genes and myeloid differentiation (Hdc, Mpo, Vwf, Selp, Neo1, Hoxb5, Itg2b (CD41), Pim1, Ccnd1, Gata2, and Runx1) genes. Mpo was upregulated throughout clusters 7, 1 (also higher Ccl9, Ccl3, and Ccl4), 6 (also Dntt, Csf3r higher, and Csf1 lower), and had the largest upregulation in cluster 5. In contrast, cluster 12 was characterized by higher Gata1 expression. Within several mixed lymphoid clusters, Dntt, Flt3, Satb1, IghM, Ccl3, Ccl4, and Cd69 were highest in cluster 8 and decreased in clusters 9, 11, and 10. See Supplementary Methods for more gene lists. Peripheral blood hematology analysis of Vav1-Cre/+STAT5ab$^{fl/fl}$ mice showed decreased granulocyte-monocyte-lineage, decreased hematocrit and red blood cell count, and as expected dramatically decreased overall white blood cell count and lymphocyte count (Supplementary Table S1). Interestingly, a unique off-ramp signature was observed in cluster 2 of knockout mice where the gene signature is enriched for IL-7 response genes (not shown) which is suggestive of osteoclast-priming. Bone marrow multipotent progenitors were assessed and it was found that Vav1-Cre/+STAT5ab$^{flox/flox}$ mice had significantly reduced numbers of KLS, the most immature subsets, MPP1, and myeloid biased subsets MPP2 and MPP3 but not lymphoid-primed MPP4 cells (Fig. 1D).

Vav1-Cre is Promiscuous With STAT5ab Deletion in BM CD45neg Stromal Cells and Altered Differentiation and Niche Factor Support Gene Signatures

To correlate expression levels and Cre-mediated deletion efficiency in BM hematopoietic and stromal (cluster 4) cell types the data were analyzed further. Vav1 was expressed in a small minority of cluster 4 cells (potential stromal cell contaminants), including in 108 Pdgrfa$^+$ cells which were exclusively stromal (Fig. 2A). STAT5ab deletion was observed in Vav1$^+$, Nes$^+$, Pdgfra$^+$, and Ly6a$^+$ cells (Fig. 2B) but not in Lepr$^+$ or Acta2$^+$ cells of cluster 4 (Fig. 2C). These data show that Nes and Vav1 are co-expressed in a small percentage of hematopoietic cells and that STAT5ab was efficiently deleted in cells expressing Nes. This result suggests that reciprocally Nes-Cre should be able to delete STAT5ab in hematopoietic cells. In contrast, Vav1-Cre may be expressed more during stromal cell development leading to efficient deletion in Pdgfra$^+$ cells, despite not being highly expressed in adult stromal cell types.

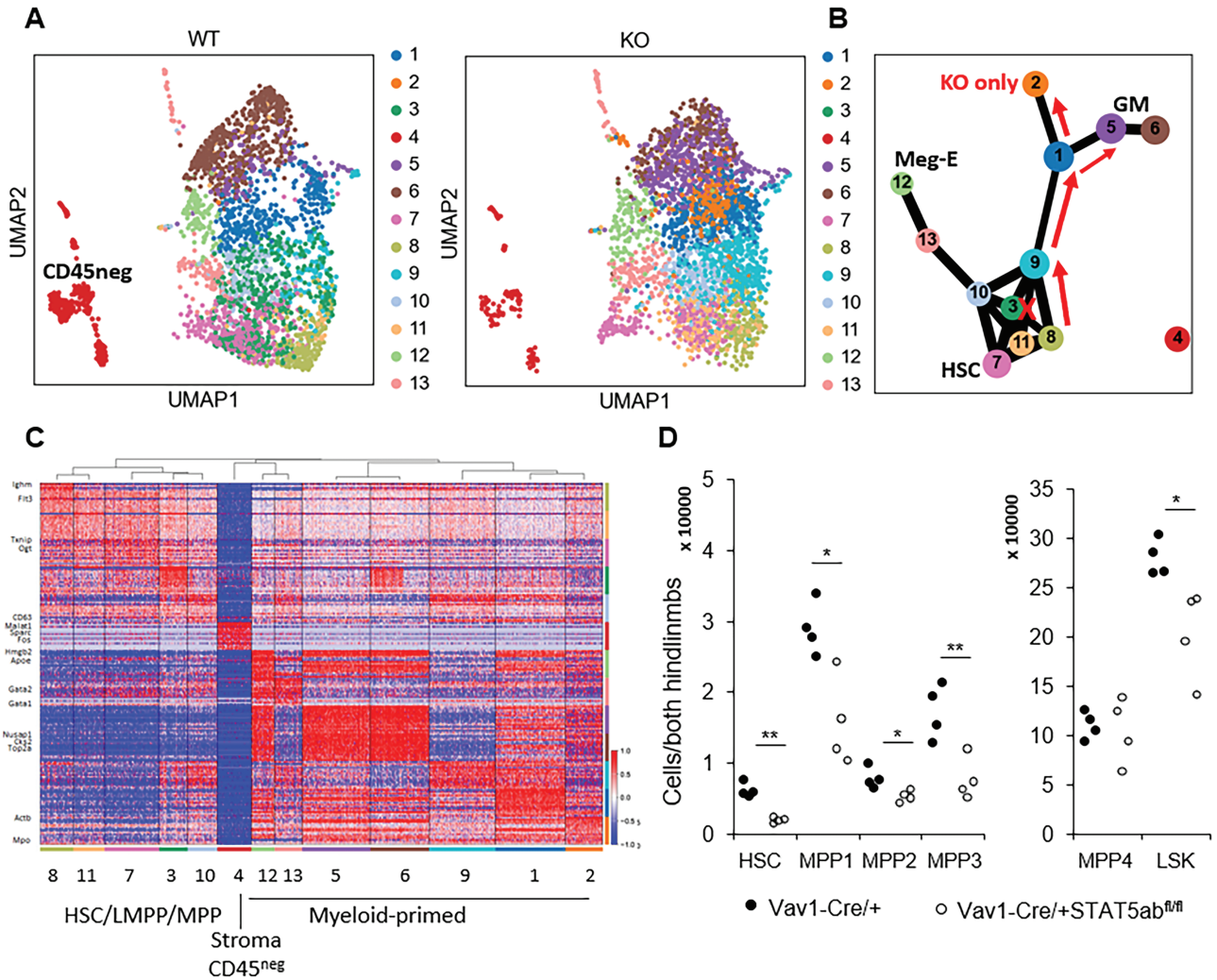


Figure 1. Single-cell RNA seq analysis reveals STAT5-deficient KLS cells are primed for myeloid lineage differentiation. (**A**) UMAP for 13 clusters from WT and STAT5 KO KLS cells. (**B**) Trajectory analysis of KLS single-cell data. (**C**) Cluster of signature genes. Expression of the top differentially expression genes (rows) across the cells (columns) in each cluster (color bar, bottom and right, as in A and B). Key genes highlighted on left. (**D**) Conditional deletion of STAT5ab with Vav1-Cre significantly altered the population of stem and progenitors in the bone marrow cells. Bone marrow cells from Vav1-Cre/+STAT5ab$^{fl/fl}$ and Vav1-Cre/+ control were assayed by multi-parameter flow cytometry to quantitate the number of stem and progenitors. BM cells were stained with antibodies against lineage markers, c-Kit, Sca-1, CD150, CD48, CD135, CD34, and IL7R. The absolute number of stem and progenitor cells in Vav1-Cre/+STAT5ab$^{fl/fl}$ and Vav1-Cre/+ control mice bone marrow samples is shown ($n = 4$ for both groups, $^{*}P \leq .05$: $^{**}P \leq .01$).

Deletion of STAT5ab was observed throughout CD45^{+} and Ly6a^{+}CD45^{neg} cells (776 cells total, including 218 WT and 558 KO). Notably, cluster 2 was unique to STAT5ab KO whereas cluster 3 was unique to WT cells. Gene expression changes were highly significant for Stat5a and Stat5b (Wilcoxon test, $P < 10^{-16}$) between Vav1-Cre/+ wild-type control cells and Vav1-Cre/+STAT5ab KO Ly6a^{+}CD45^{neg} cells (Supplementary Fig. S2). Notably, gene expression was also significantly reduced by Wilcoxon statistical analysis for the following niche factors: Cxcl12 ($P < 10^{-7}$), Igf1 ($P < 10^{-8}$), and Ntn1 ($P < 10^{-7}$) in STAT5ab KO Ly6a^{+}CD45^{neg} cells.

Stromal Cre Mouse Strains also Show Nes is Promiscuous in HSCs and STAT5ab Deletion in MSCs Decreases Hematopoietic Progenitor Numbers In Vivo

We previously showed that STAT5ab deletion reduced lymphocytes and resulted in mild anemia. Now using a series of new Cre transgenic mice we tested more direct effects of STAT5ab deletion in MSCs by comparing a variety of Cre transgenic knockouts to the Vav1-Cre knockout control. Supplementary Table S1 shows the results of peripheral blood hematology analysis from Nes and Lepr-cre, transgenic mice as drivers to knockout STAT5ab. The other transgenic mice Prrx1, Osx1, and Ocn-Cre used to delete STAT5ab are listed in Supplementary Table S2. There were some notable changes in lymphocyte, granulocyte, and red blood cell indices. These changes are visible but much milder when STAT5ab was deleted by Nes-Cre compared to Stat5ab deletion by Vav1-Cre. However, the trend of those changes included a reduced percentage of lymphocytes and an increased percentage of granulocytes when STAT5ab was knockout by Lepr-Cre and Prrx1-Cre.

To assess multi-lineage repopulating activity, functional competitive repopulation assays were performed for Vav1 control, Nes, and Lepr STAT5ab knockout mice (Fig. 3A, 3B). Only Nes-Cre resulted in significantly reduced donor

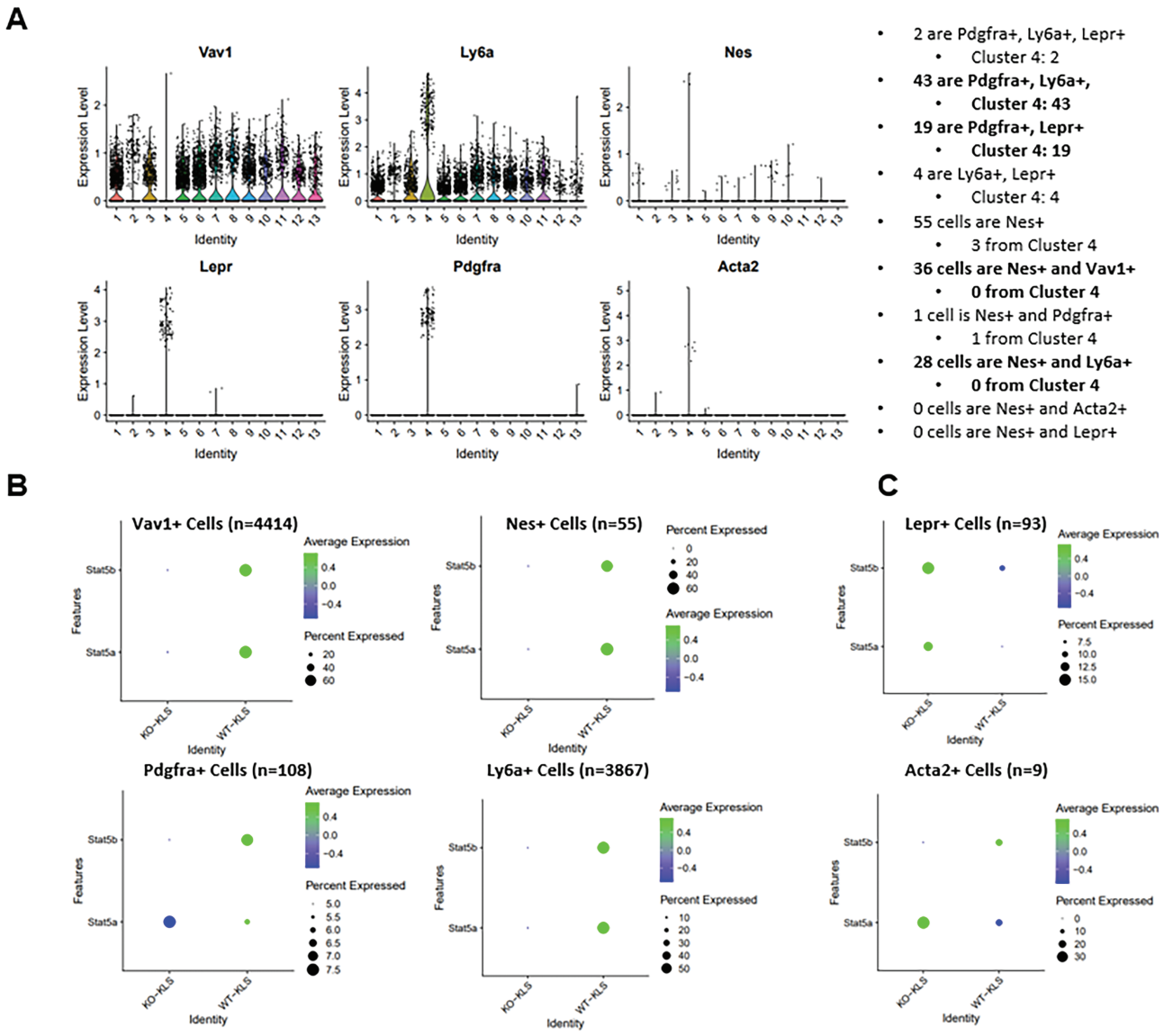


Figure 2. Vav1 is promiscuous in bone marrow CD45^{neg} stromal cells and Nes is promiscuous in bone marrow HSCs. (**A**) Violin plot shows the expression of distribution of Vav1, Ly6a, Nes, Lepr, Pdgfa, and Acta2 across 13 clusters. (**B**) Stat5a and Stat5b deletion efficiency is shown for Vav1-Cre mediated deletion within sorted KLS cell sub-populations expressing the indicated genes. The average expression and the percentage of Stat5a and Stat5b expressed between KO and WT KLS in Vav1+, Ly6a+, Nes+, and Pdgfra+ cells is shown. (**C**) The average expression and the percentage expressed of Stat5a and Stat5b between KO and WT KLS in Lepr+ or Acta2+ cells in KO KLS compared to WT KLS cells are shown.

%CD45.2 (Fig. 3A) as well as the %CD45.2$^+$ in B220$^+$, Ter119$^+$, and CD4$^+$ cells in peripheral blood (Fig. 3B). Myeloid lineage hematopoiesis was rescued in these mice presumably due to a myeloid expansion specific to that lineage. A mild increase in multi-lineage competitive repopulation was observed with Lepr-Cre mediated STAT5ab deletion. In the BM compartment, KLS cells were examined by flow cytometry and only Nes-Cre resulted in a significant reduction of donor contribution (Fig. 3C). Bone marrow competitive activity assay was also performed using STAT5ab knockout mice driven by Prrx1, Osx1 (Supplementary Fig S3A, S3B), and Ocn-Cre (Supplementary Fig S3C, S3D). All those mice had similar BM competitive activity, although Prrx1 knockout had a subtle reduction in overall %CD45.2 with no significant changes at the level of multi-lineage engraftment. To understand the role of STAT5ab in multipotent progenitors, mouse BM cells were analyzed by multi-parameter flow cytometry analysis. Bone marrow cells were first gated on c-Kit$^+$, lineage^{neg}, and Sca1$^+$ (KLS) cells. KLS cells were then subdivided into 4 populations based on the expression of CD150 and CD48: CD150$^+$CD48^{neg}KLS (HSCs) (Supplementary Fig. 4SA), CD150^{neg}CD48^{neg}KLS (MPPs) (Supplementary Fig 4SB), CD150^{neg}CD48$^+$KLS (HPC-1) (Supplementary Fig. 4SC), and CD150$^+$CD48$^+$KLS (HPC-2) (Supplementary Fig. S4D).

STAT5ab deletion with Lepr-Cre reduced restricted hematopoietic progenitor cells (HPC-2 and HPC-1[28]) with Osx1 and Prrx1-Cre on the reduction of restricted progenitors and MPPs, while only Nes-Cre knockout STAT5 had significantly reduced HSCs. Those results are consistent with a more progenitor supporting role for STAT5ab throughout the mesenchymal osteo/chondro-genic lineages and Nes expression being promiscuous in HSCs. Ocn-Cre-driven deletion of STAT5ab did not have a noticeable effect (Supplementary Fig. S5A, S5B).

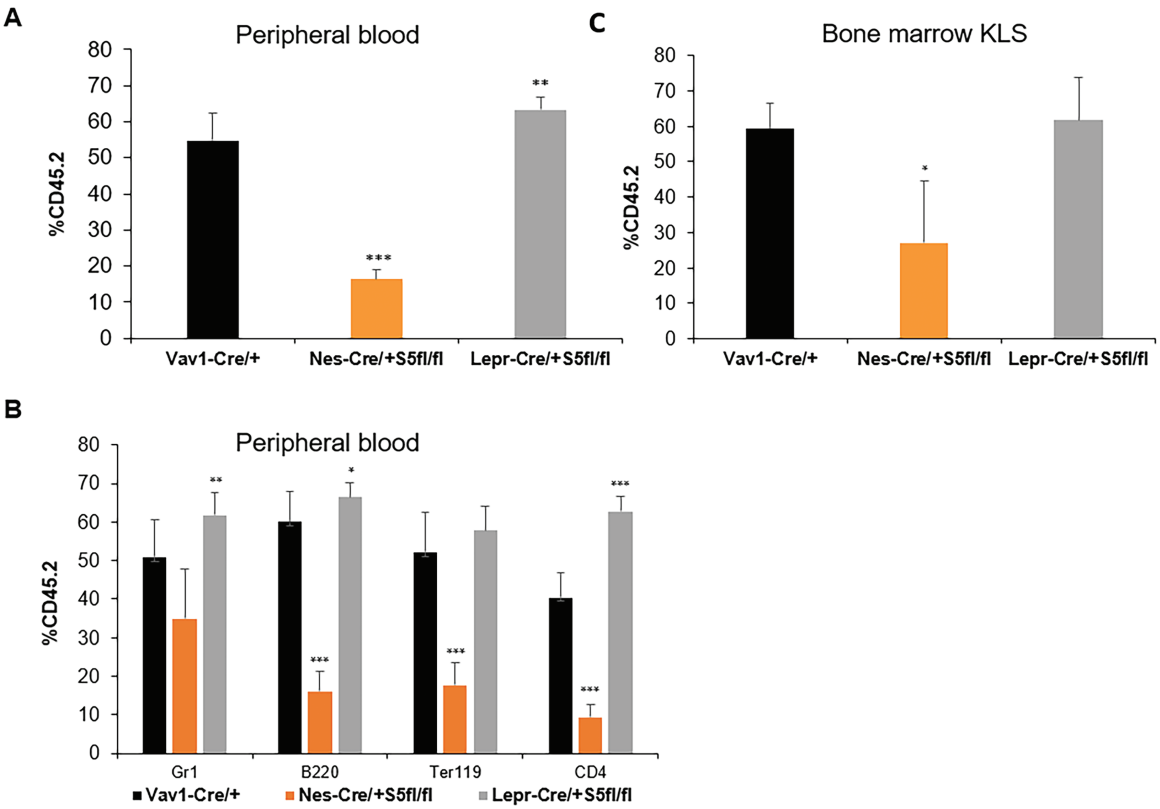


Figure 3. Conditional Nes-Cre deletion of STAT5ab leads to strong decline in mouse HSC activity while Lepr-Cre deletion has a mild reciprocal effect. Bone marrow cells from 3 to 4 donor mice were harvested from each group and mixed 1:1 with wild type Boy J competitor (CD45.1) and transplanted into lethally irradiated Boy J mice (CD45.1) for the competitive repopulation assay. Recipient mice were bled 16 weeks later for flow cytometry analysis. Shown is the average of 2 independent experiments with 5 mice per group in each experiment. The genotype STAT5ab$^{fl/fl}$ is also represented here as S5fl/fl because of space. (**A**) Overall donor percentage of CD45.2 positive in the peripheral blood by FACS analysis. (**B**) Multi-lineage analysis for the same 2 independent experiments. (**C**) Percentage of donor engraftment in the KLS fraction in the bone marrow cells.

Importantly, tamoxifen-induced Nes-CreERT2/+ to delete STAT5ab led to reduced CMP, GMP, and CD150^{neg}CD48^{neg}KLS cells but not HSCs (Supplementary Fig. S5C, S5D).

Multipotent progenitors are known to be heterogeneous. The Trumpp[29,35] and Passegue[4] groups divided the MPP population into MPP1-4 according to their immunophenotype using CD34, CD135, CD150, and CD48 along with KLS markers. Using those markers, Lepr-Cre and Nes-Cre STAT5ab knockout mice were examined more thoroughly to better understand the effects of Cre-mediated deletion of STAT5ab on the population of stem and progenitors. Nes-Cre/+STAT5ab had a consistent reduction of long-term HSCs (CD34^{neg}CD48^{neg}CD150^{+}CD135^{neg}KLS) (Fig. 4A) while STAT5ab deletion with Lepr-Cre was associated with accumulation of HSCs at the expense of MPP4/CLP lineage (Fig. 4B) as determined by flow cytometry.

Single-Cell RNA Seq of CD45^{neg} Stromal Cells Using Lepr-Cre Deletion of STAT5ab Reveals Overlap Between Several MSC Markers

Having defined the impact of STAT5ab deletion on the numbers of hematopoietic stem/progenitors, we next explored molecular mechanisms that might be responsible. To examine STAT5ab function in cells derived from Lepr^{+} stromal cells, STAT5ab deletion with Lepr-Cre was used and CD45^{neg}CD31^{neg}Ter119$^{neg/low}$CD71^{neg}CD44^{neg} living cells were sorted and analyzed by single cell RNA seq. Sequencing data from 565 WT and 349 KO cells were obtained (MSC-WT, average of total UMIs = 2789; MSC-WT, average number of genes = 1131; MSC-KO, average of total UMIs = 2990; MSC-KO, average number of genes = 1100). The subsequent data analyses yielded 6 clusters (1 early erythroid with some lymphoid genes as well, 5 stromal) and genes for defining clusters are listed in Supplementary Fig. S7. This result is consistent with a report that BM CD45^{neg} contains erythroid and lymphoid progenitors.[36] The yield of captured single MSCs was lower than obtained for the KLS during the process of single-cell RNA seq and thus resulted in fewer clusters.

Clusters were manually annotated using a panel of partially overlapping markers including genes such as Lepr, Pdgfra, Nes, Acta2, Sca1 (Ly6a), and Bglap. The UMAP (Fig. 5A) and lineage trajectories (Fig. 5B) are shown. Notably, Pdgfra was expressed in clusters 2, 3, 4, like that of Lepr (Fig. 5C). Interestingly, Lepr^{+} cells mostly co-expressed STAT5b but not STAT5a and Lepr-Cre caused some deletion of STAT5b mRNA levels (Fig. 5D). In contrast, Nes was expressed rarely (only ~1% of MSC sequenced) in adult stroma and appeared to mark a potential transient "activated" cell intermediate based on trajectory analysis and cluster 5. Ly6a was also expressed in these 3 clusters with an expression pattern that was reciprocal to Lepr. Vav1 was expressed in cluster 5 but not enough to explain deletion resulting in adult mice. This suggests that Vav1 may be expressed in a transient early pre-MSC that results in the deletion observed in the KLS cluster 4. Lepr-Cre deleted very well in defined Lepr^{+} cells, although these cells were a minority of the cluster that we defined as the Lepr^{+} cluster

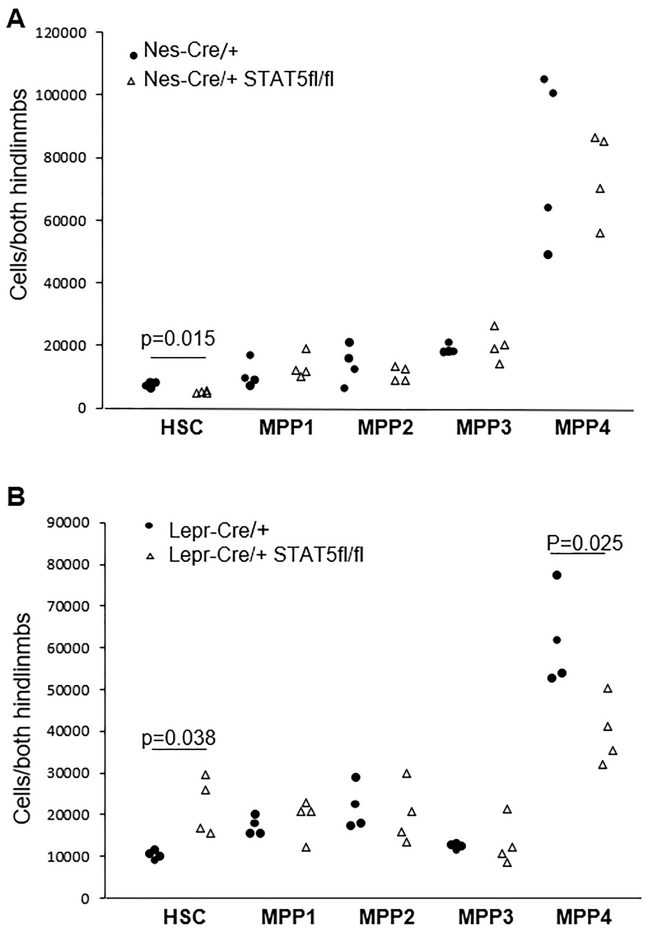


Figure 4. Conditional deletion of STAT5ab with Nes-Cre and Lepr-Cre has reciprocal effects on the number of HSCs in the bone marrow. Bone marrow cells from Nes-Cre/+STAT5ab$^{fl/fl}$ and Nes-Cre/+ or Lepr-Cre/+STAT5ab$^{fl/fl}$ and Lepr-Cre/+ control were assayed by multiparameter flow cytometry to quantitate the number of stem and progenitors. BM cells were stained with antibodies against lineage markers, c-Kit, Sca-1(Ly6a), CD150, CD48, CD135 (FLK2), CD34, and IL7R. The genotype STAT5ab$^{fl/fl}$ is also represented here as STAT5fl/fl because of space. (**A**) Absolute number of stem and progenitor cells in Nes-Cre/STAT5ab$^{fl/fl}$ and Nes-Cre/+ control. (**B**) Absolute number of stem and progenitor cells in LepRCre/STAT5ab$^{fl/fl}$ and Lepr-Cre/+ control (n = 4 for each group).

because of the highest Lepr expression levels. Notably, of the 6 clusters identified, deletion of STAT5ab was observed mainly when looking at the Lepr$^+$ cells but not by looking at the cluster designations despite cluster 4 being the most enriched for Lepr$^+$ cells. Additionally, Lepr-Cre deleted Stat5a/b efficiently in Pdgfra$^+$ cells but not in Nes$^+$, Ly6a$^+$, or Acta2$^+$ cells (Fig. 5D).

Combined STAT5ab/Cish Co-expression Algorithms and Knockout Differentially Expressed Genes Predict Differentiation Priming in Both MSCs and HSCs

To address Stat5a and Stat5b separately a computational approach was taken to develop a new algorithm for co-expression analysis with genes in scRNAseq data sets. First, MSC datasets published by Scadden[12] were validated using maximum mean discrepancy (MMD) analysis in comparison with 2 other co-expression algorithms (Iacono[37] and MAGIC[38]) (Supplementary Fig. S8, S9). Cish, a known direct target and negative feedback inhibitor of Stat5ab was included for co-expression analysis in combination with Stat5a and Stat5b. The combination of Stat5ab/Cish proved to be a powerful combination for identifying gene signatures in BM stromal cell types associated with glucocorticoid signaling and osteogenic/chondrogenic differentiation of mesenchymal stromal cells (Supplementary Fig. S10, S11).

In hematopoietic stem/progenitor cells using the scRNAseq dataset published by Gottgens,[11] Stat5ab/Cish unique and overlapping signatures were associated with distinct quiescence/self-renewal vs. cell cycle activation gene signatures (Supplementary Fig. S12). MMD comparison of 3 different publicly available data sets (MSC) or 2 different publicly available data sets and our own (HSC) showed overlap among gene sets within the top 5000 most co-expressed genes from independent studies of MSCs or HSCs (Supplementary Fig. S13). The Scadden dataset was used to interrogate Lepr-MSCs (Supplementary Fig. S14A) and our dataset was used to interrogate HSCs (Supplementary Fig. S14B), respectively. Comparisons were made with both versus our wild-type and STAT5ab knockout MSC and HSC clusters for maximum validation. Combined STAT5ab knockout and wild-type co-expression analyses identified common Stat5ab/Cish regulation of MSC/HSC differentiation priming (Supplementary Fig. S14C) with no distinct differences between STAT5a or STAT5b. Overall, these computational analyses suggest that the STAT5ab-mediated gas/brakes driving MSC/HSC differentiation priming are conserved in 2 heterotypic BM cell types which cross-talk to regulate normal hematopoiesis.

Functional Defects in Niche Factor Expression and Support Activity With STAT5ab Deletion

Analysis of the scRNAseq data set for Lepr-Cre mediated STAT5 deletion in stroma showed strong sets of cluster-defining genes, including common hematopoietic niche factors (Fig. 6A–C). Since decreased local production of Igf1 in the BM microenvironment causes many hallmarks of LT-HSC aging,[39] including cell cycling, this may explain some of the LT-HSC accumulation observed with Lepr-Cre. Reciprocally, Grem1 was the 6th most induced gene in the MSCs which is a known marker for loss of multipotency (Fig. 6B). Cluster 5 was not as changed as other clusters and this correlated with weaker STAT5ab expression levels in WT. It did trend toward more osteoblast differentiation genes and myeloid cell differentiation genes with loss of negative regulation of MAPK, p38 MAPK, and ERK1/2 pathways. Other gene sets increased included regulators of osteoblast differentiation, BMP response, fat cell differentiation, as well as early response genes (fosb, jun, junb, fos, ebf1, and dusp1). Overall, the gene signature in cluster 4 as well as in cluster 2 suggests more priming toward osteo/chondro-genic differentiation (Fig. 6B) very similar to what was observed with the KLS cluster 4 (the only CD45^{neg} cluster). In contrast, clusters 3 and 6 became very chondrocyte-primed (Fig. 6C). Lepr-Cre deleted very well in MSCs (n = 914; 349 KO, 565 WT) and correlated with loss of mRNAs for key niche factors Cxcl12 (P = .006) and Igf-1 (P = .0006) (Fig. 6D). See Supplementary Methods for more MSC differentially-expressed (KO vs. WT) gene lists.

To investigate whether these changes are observed in cultured MSCs in vitro, MSCs were analyzed by qRT-PCR for reduced mRNA and protein for STAT5a and STAT5b separately (Fig. 7A, 7B). The key gene from the global transcriptome analysis that was increased was Grem1 and

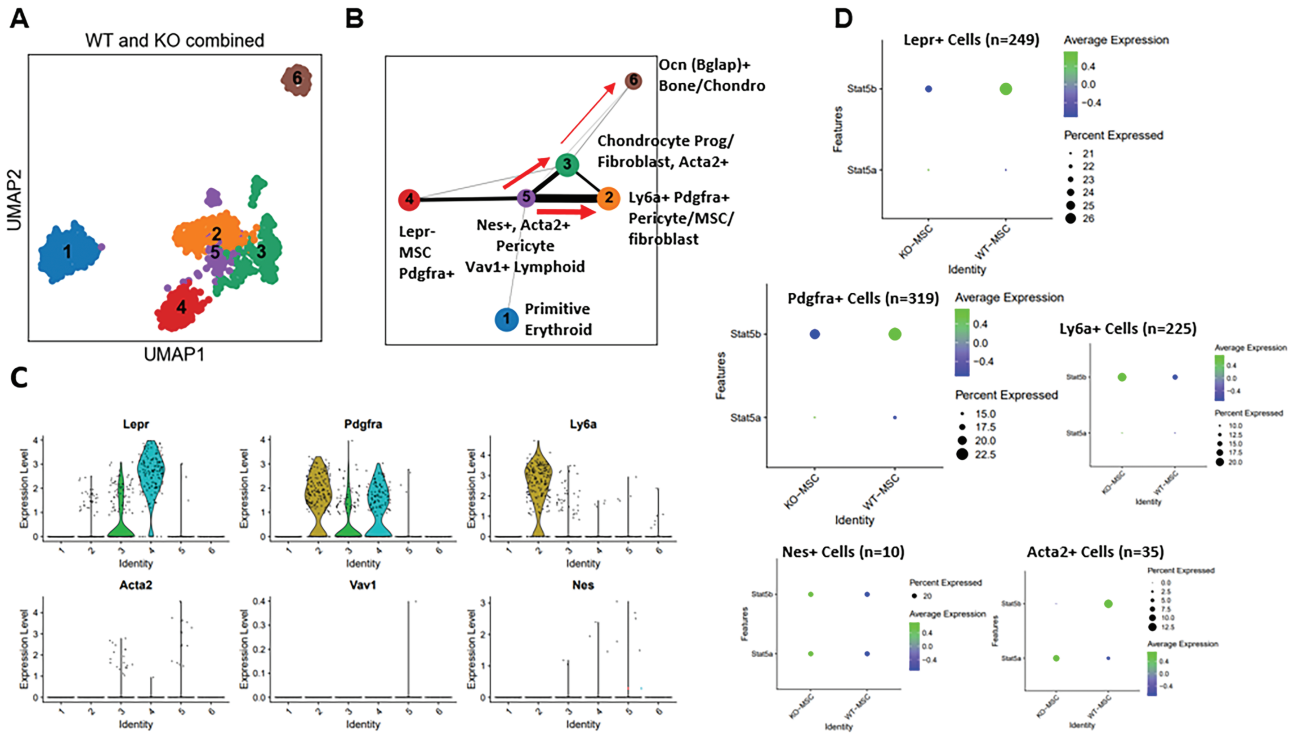


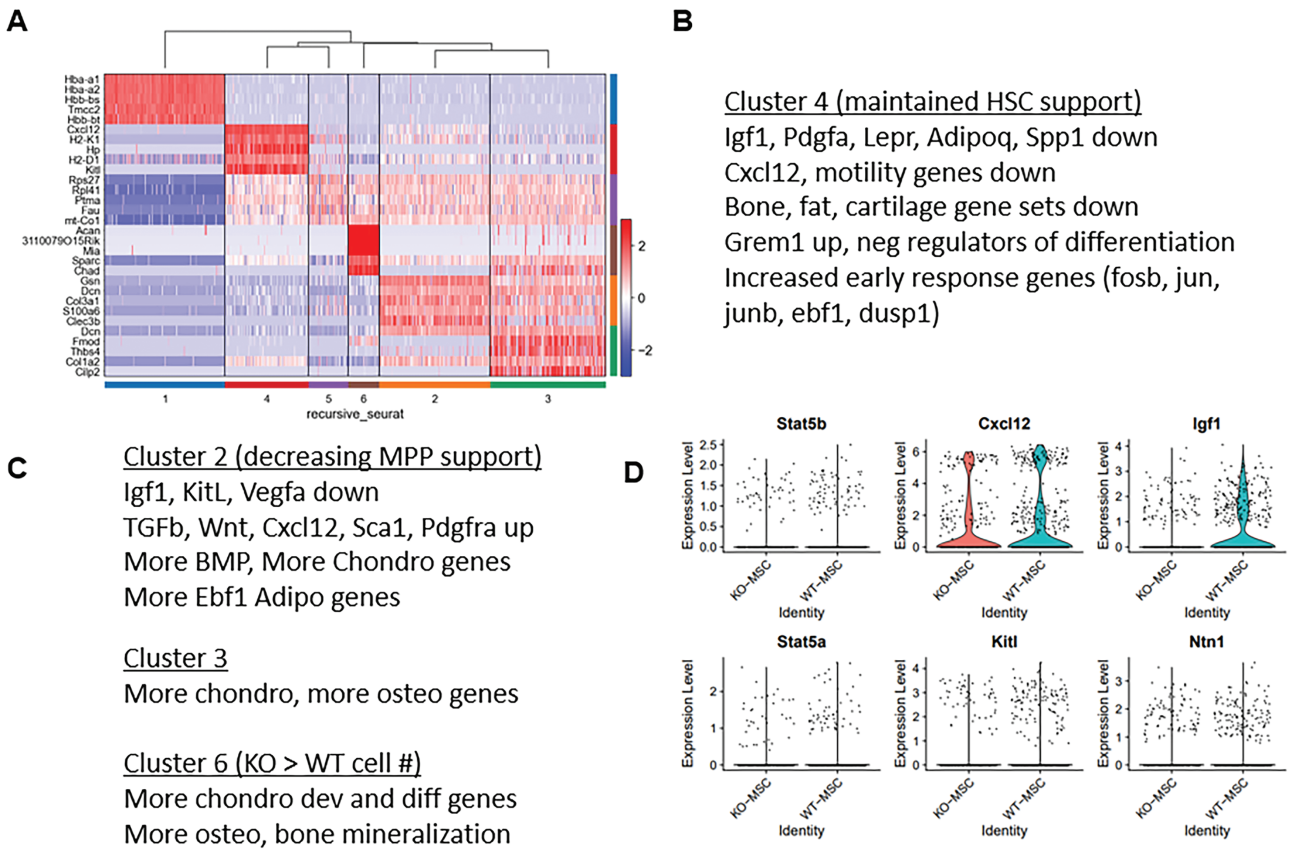
Figure 5. Single-cell RNA seq of CD45^{neg} stromal cells using Lepr-Cre deletion of STAT5ab reveals overlap between several MSC markers. Single-cell RNA seq analysis was performed on CD45^{neg}CD31^{neg}Ter119^{neg}CD71^{neg}CD44^{neg} BM cells from Lepr-Cre/+Stat5ab$^{fl/fl}$ and its control. (**A**) UMAP for 6 clusters of stromal cells single-cell RNA seq data. (**B**) Trajectory analysis of single stromal cells by RNA seq data. (**C**) Distribution of expression level for selected marker genes across 6 clusters with violin plot representation. (**D**) Stat5a and Stat5b average expression and percent expressed among selected marker gene-positive cells between Lepr-Cre/+ STAT5ab$^{fl/fl}$ (KO-MSC) and its control (WT-MSC).

likewise it was sharply increased in cultured MSCs (Fig. 7C). In contrast, Cxcl12, Angpt1, Igfbp4, Igf1, and Il34 were all decreased like the global scRNAseq data (Fig. 7D). Also, similarly increases in Kitl were observed. Functional analysis of STAT5ab-deficient MSCs were precluded by difficulties in culturing them following deletion for prolonged time. Therefore, to test trophic function in vitro, mouse embryonic fibroblasts lacking STAT5ab were used (Fig. 7E, 7F). Support function for the SCF-dependent cell line EML was tested by co-culture on STAT5ab-deficient MEFs. Reductions in support were observed compared to wild-type MEFs, consistent with a functional role for STAT5ab in providing necessary trophic support to hematopoietic progenitors. To check the production of the secretory niche factors, wild-type or STAT5ab-deficient MEFs or MSCs were cultured. The same number of cells (0.1 million) were seeded in a 24-well plate. The second day, the fresh medium with reduced FBS (2%) was changed and the supernatant was harvested 24 h later for IGF-1 and SDF-1α ELISA assay. MEFs had a relatively lower level of basal IGF-1 and SDF-1α compared with MSCs but both STAT5-deficient MEFs (Fig. 7G) and MSCs (Fig. 7H) had significantly reduced secretory niche factors IGF-1 and SDF-1α.

Discussion

Signal transducer and activator of transcription (STAT) family of 7 transcription factors are activated by a diverse set of receptors and Janus kinases (JAKs). Our lab[21,25,40-49] and others[50-52] have shown that conditional deletion of STAT5 with Mx1-Cre caused loss of quiescence and progressive loss of CD150 expression, along with impaired lymphocyte[53] and leukemia development.[54,55] Within KLS cells, deletion of STAT5ab increased LMPPs[25] at the expense of megakaryocyte/erythroid (Mk-E) priming, much like the Thpo receptor (Mpl) knockout.[56] A recent study highlighted Cxcr4 conditional knockout using Flk2-Cre in hematopoietic cells and the impact due to inability to sense Cxcl12 which resulted in greater responses to another key niche factor KitL.[57] We feel that the similar phenotypes of STAT5ab knockout with Lepr-Cre are most likely modestly expanding HSCs through the same mechanism, whereby more Kitl and less Cxcl12 produced by the KO stromal cells themselves cause dysregulated signaling. While the competitive repopulation advantage was multilineage, this could promote quick passage through the MPP4 stage toward mature lymphocytes to account for the enhanced lymphoid recovery (more B220 and CD4) observed in Fig. 3.

Molecular investigations of the phenotypic HSC compartment have also defined a metabolically activated compartment with limited self-renewal, termed MPP1, which may represent an initial differentiation step. Interestingly, among the top cluster defining genes in this study there was a high representation of translation-associated gene expression in STAT5ab knockout KLS cells. These RPS/RPL genes were ranked as high, med, or low within the top cluster-defining genes. The early clusters 7, 8, 10, 11, 3 all had more cluster-defining RPS/RPL genes whereas the more Mk/E and GM clusters did not have any (Supplementary Fig. S1). This likely indicates that STAT5 deficiency causes cellular activation and abnormal myeloid lineage priming in early MPPs. In the MSC clusters, only cluster 5 is primed with RPS/RPL gene, which represent an "activated MSC" state intermediate between



Figures 6. Analysis of the scRNAseq data set for Lepr-Cre mediated STAT5 deletion in stroma shows dysregulated osteo-chondrogenic differentiation and niche factor gene expression. (**A**) Cluster of signature genes that define each of the MSC clusters. Expression of the top differentially expression genes (rows) across the cells (columns) in each cluster (color bar, bottom and right, as in A and B). Key genes highlighted on left. (**B**) The gene signature in cluster 4 maintained HSC and MPP support. (**C**) The gene signature in the other stroma clusters. (**D**) Deletion of Stat5a and Stat5b as well as reduced expression of Cxcl12 and Igf1 in the aggregate total throughout all 6 clusters of MSCs. All gene sets were identified from Panther GSEA as significantly changed and $P << .05$.

quiescent Lepr-MSC and the osteo/chondro progenitors. That fits the GSEA and lineage trajectory analyses.

Expression of transcription factors, including Foxc1 and Ebf3 are essential for HSC niche formation and/or maintenance[58,59] in CAR/Lepr$^+$ cells. Indeed, we found that Foxc1 is increased (ranked 54/13 968 genes) in cluster 4. Ebf3 was also increased (ranked 72/13 968 genes) in cluster 4. Therefore, these are highly induced in KO vs. WT. Intrinsic metabolic reprogramming of MSCs has also been described to control their cell fate. Naïve MSCs are ROS^{low} and favor glycolysis in the hypoxic niche but switch to oxidative phosphorylation (oxphos) and become ROS^{high} with differentiation.[60] STAT5ab co-expressed genes were associated with priming for translation and STAT5 may play a role to ready MSCs for lineage commitment with Cish co-expression. In contrast to reduction of pTyr dosage which may impair terminal differentiation, STAT5 protein deletion may permit more osteolineage commitment at the expense of adipocyte priming due to loss of as yet undefined non-pTyrSTAT5 regulatory functions. pTyrSTAT5 seems likely for full lineage differentiation, however. Lepr-MSC1-4 differentiation continuum genes include glucocorticoid response genes, supporting the known ability of dexamethasone to induce MSC commitment to differentiation.[61] Non-genomic roles for non/low pTyrSTAT5a and to a lesser extent STAT5b in golgi have also been reported[62–64] with knock-down in vitro resulting in golgi fragmentation. MSCs have been extensively studied in vitro and in vivo under steady-state and stress conditions (high GC) and STAT5b transcriptionally promotes osteolineage (OLC) differentiation,[65] yet understanding of STAT5-mediated hematopoiesis-support is unknown. Innervated Nes$^+$ pericyte MSCs respond to the sympathetic nervous system in a circadian manner to regulate the secretion of niche factors such as Cxcl12[66] and nerve injury impairs hematopoiesis.[67]

Extrinsic activation of Stat5ab/Cish as a key signaling cascade can also be part of the BM microenvironment to regulate MSC function. A single skeletal stem cell produces chondrocytes, osteoblasts, adipocytes, and fibroblasts. STAT5 primes multipotency and KO increases differentiation potential but cannot dictate terminal differentiation. The circadian response is a major regulator of hormone[68,69] and cytokine[70] trafficking/secretion and it is significant to better understand whether STAT5 isoforms have evolutionarily conserved roles in the BM microenvironment to support niche factor production.[71,72] Since the 1990s, roles for STAT5a (mammary gland-prolactin-milk proteins) and STAT5b (liver-growth hormone (GH)-insulin-like growth factor 1 (Igf1)) have been shown to be distinct[73] despite 95% homology. During sleep, expression of GH or prolactin precedes the glucocorticoid (GC) pulse, permitting STAT5 to cooperate with the glucocorticoid receptor (GR) in MSCs. A conserved role for STAT5 in regulating hematopoiesis through BM niche factors was

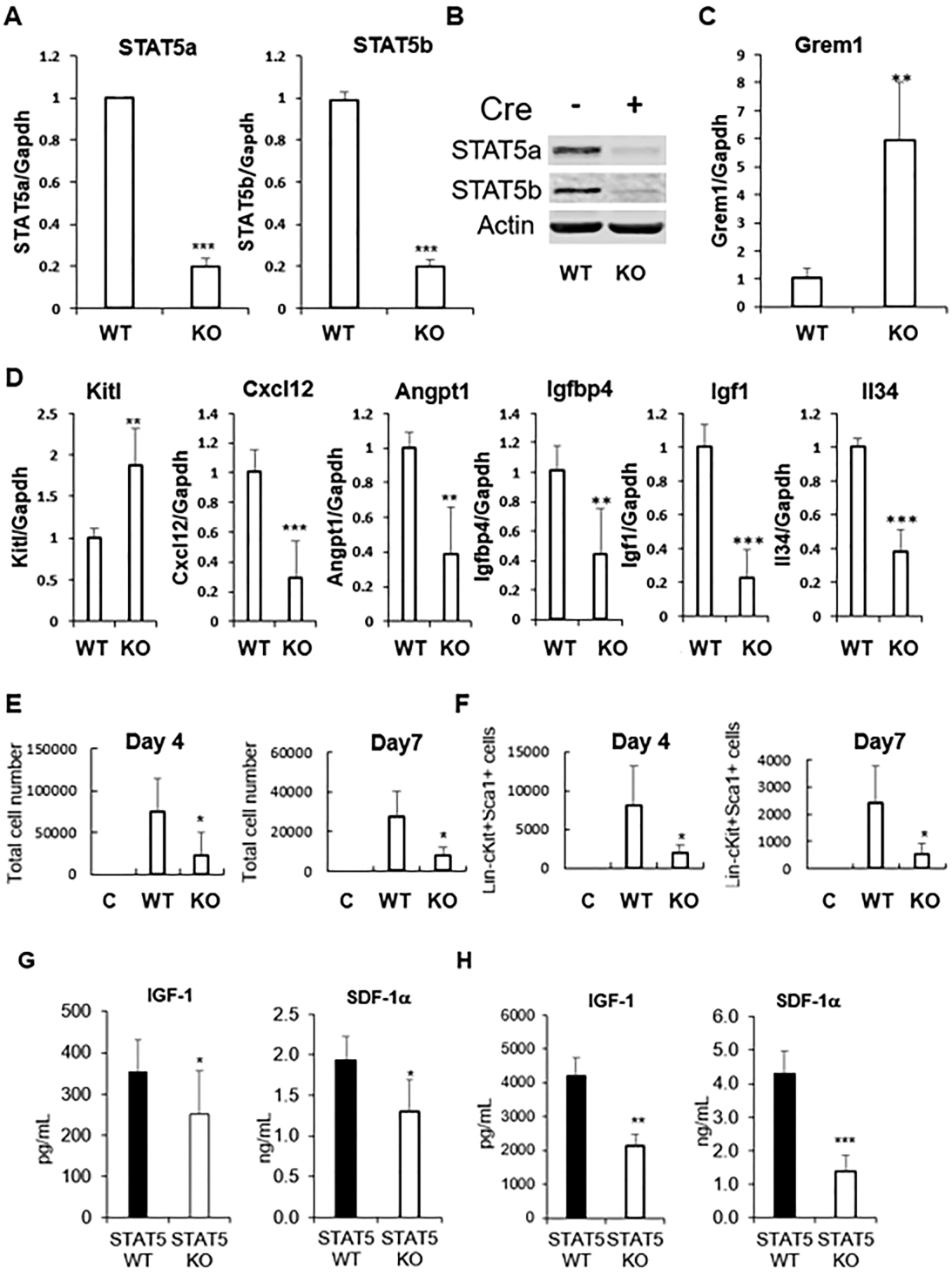


Figure 7. Deletion of STAT5ab with Lepr-Cre leads to alteration of bone marrow niche factor expression. (**A**) Real-time PCR showed the deletion of Stat5a and Stat5b in MSCs from Lepr-Cre/+STAT5ab$^{fl/fl}$ compared to the MSCs from control mice. GAPDH was used as the internal control for real-time PCR. (**B**) Western blot showed the deletion of STAT5a and STAT5b protein. Actin was used for the loading control. (**C, D**) Expression of bone marrow niche factor including Grem1, KitL, Cxcl12, Angpt1, Igfbp4, Ifg1, and Il34 by real-time PCR compared WT MSCs with STAT5ab deleted MSCs. (E, F) STAT5ab$^{null/null}$ MEF cells have reduced hematopoietic supporting activity for EML C1 cells. The total number of EML C1 that were co-cultured for 4 days ($n = 4$, $P = .04$) or 7 days ($n = 4$, $P = .03$), and for Lin^{neg}Kit^{+}Sca-1^{+} cells co-cultured for 4 days ($n = 4$, $P = .05$) or 7 days ($n = 4$, $P = .04$) were compared to those co-cultured with WT MEF cells or without MEF (C: control). Niche factor SDF-1α and IGF-1 are significantly reduced when STAT5ab is deleted in MEF and MSCs. (G) Conditioned medium from wild type and STAT5ab$^{null/null}$ MEFs was assayed for SDF-1α and IGF-1 by ELISA assay ($n = 5$, $P = .02$ for SDF1α; $n = 11$, $P = .01$ for IGF-1). (H) Conditioned medium from wild type and STAT5ab deleted MSCs was assayed for SDF-1α and IGF-1 by ELISA assay ($n = 5$, $P < .001$ for SDF1α, $P = .002$ for IGF-1).

still surprising. Screening for highly ranked Cish co-expressed receptors highlighted the following potential inflammatory genes (Supplementary Fig. S11) that might be dysregulated in Lepr-MSCs: IL-4ra (rank 605), IL-13ra (2037), IL-17ra (67), IL-17rd (475), IL1r2 (2760), and Tnfrsf1a (3248) out of 14 446 total number of genes. Ramping down negative feedback by inhibiting these receptors could have clinical utility in myelosuppression recovery and anti-inflammatory feedback/injury repair mechanisms involving IL-4/13/17 induced Cish co-expressed Tnfaip6 (Tsg6; rank 6), Ccl2 (402), Traf6 (949), and Stc1 (988). Modulation of STAT5b function downstream of SNS-regulated hormones and cortisol in the BM niche is potentially targetable to modulate IL-4/IL-13/IL-17 licensing of BM stromal immunomodulatory activity and will be examined in future studies.

STAT5 roles in MSC adipocyte[74–76] and osteoclast differentiation[22,77] have been described to come together in regulating bone development.[78] However, there has not been a previous in-depth examination of STAT5ab in MSCs at the single-cell level as shown in this study. Gremlin1 (Grem1) is an antagonist for bone morphogenic protein (BMP) and importantly was the main differentiation marker increased in STAT5ab knockout MSCs. Osteochondroreticular (OCR) stem cells have been recently described as a population of Nes^{neg} mesenchymal precursors that can differentiate into osteoblasts, chondrocytes, and reticular marrow stromal cells, but not adipocytes in vivo. Grem1-Cre cells can be found concentrated within the metaphysis of long bones, but not in the perisinusoidal space where the main hematopoiesis support activity occurs.[79] There are a number of markers found in the perivascular BM niche including PDGFRβ, PDGFRα, CD146, Nestin, LepR, and Cxcl12, while arterial vasculature is associated with cells expressing PDGFRβ, PDGFRα, Sca1, and LepR.[8,34,80–82]

An important finding from these studies was promiscuous expression by Vav1 and Nes in stromal and hematopoietic compartments respectively. Nes^{+} cells were discovered within hematopoietic KLS clusters and showed STAT5ab deletion with Vav1-Cre. Therefore partial overlap among Nes-Cre and Vav1-Cre may explain HSC-intrinsic repopulating defects observed with Nes-Cre. Out of 55 Nes^{+} cells (out of 10 008 total), 43 are $CD45^{+}$ (78%) and 12 are $CD45^{neg}$ (12%) and were found throughout all of our KLS scRNAseq clusters. Of 19 $Vav1^{neg}Nes^{+}$ cells, 13 are $CD45^{+}$ and 6 are $CD45^{neg}$. Notably, Vav1-Cre deleted STAT5ab in $CD45^{neg}$ cells. The majority of the $CD45^{neg}CD31^{neg}Ter119^{neg}$ BM cell fraction is of hematopoietic origin and contains erythroid and lymphoid progenitors.[36] However, a sub-population of stromal BM cells are $CD45^{neg}Nes^{+}$ and differentiate into multiple lineages, including osteoblasts, adipocytes, and chondrocytes. Importantly, Nes-CreERT2 which is stroma-specific did not confer a reduced HSC number like that observed from Nes-Cre. Likewise, Vav1 was not only promiscuous in some mesenchymal progenitor cells but Vav1-Cre deleted Stat5a and Stat5b in $CD45^{neg}$ cells which correlated with reduced Cxcl12 gene expression. Vav1 is normally abundant throughout most of the $CD45^{+}$ KLS cell clusters but was surprisingly expressed within $CD45^{neg}$ cells. Future studies will determine the cell-type identity and altered biology of these $Vav1^{+}CD45^{neg}$ cells.

Conclusion

In summary, this study shows for the first time that STAT5ab is active in heterotypic BM stem/progenitor cells to regulate hematopoiesis. Further expansion of the predictive co-expression algorithm has prognostic potential for using STAT5ab/Cish regulated biological processes as a biomarker.

Acknowledgments

Technical support was provided by Emory Integrated Genomics core for single-cell RNAseq analysis.

Funding

This work was funded by the I3 Rapid RFP Emory SOM/Georgia Tech Computational and Data Analysis to Advance Single Cell Biology Research Award (K.B. and P.Q.); Aflac Cancer and Blood Disorders Center Pilot Grant (K.B.), NIHR01DK059380 (K.B.), Aflac Cancer & Blood Disorders Center of Children's Healthcare of Atlanta and Emory University School of Medicine (K.B.) We acknowledge the generous support of the Emory + Children's Pediatric Research Center and the Emory/Pediatrics Winship Flow Cytometry Core.

Conflict of Interest

Dr. Bunting owns a consulting company in the State of Georgia called Valhalla Scientific Editing Service, LLC. The other authors declared no potential conflicts of interest.

Data Availability

The newly generated mouse models described here will not all be sustained long-term in the lab. However, all mouse strains used are commercially available, except the Cdh5-Cre mice. All strain numbers from JAX have been provided. Antibodies used are listed in Supplementary Table S3 and DNA oligonucleotide sequences used for qRT-PCR are listed in Supplementary Table S4. EML c1 cells are commercially available. STAT5ab knockout MEFs were generated in the Bunting lab and will be provided as needed. Newly generated scRNAseq data has been submitted to GEO with accession # GSE214857. The MMD co-expression code used in this manuscript was submitted to the GitHub repository. Publicly available scRNAseq data was obtained from the following sources: NCBI Gene Expression Omnibus, GSE128423, GSE108892, GSE132151, GSE100428, and GSE81682.

Author Contributions

Z.W., G.E., H.S.L., W.Z., A.K., E.G. P.Q., and K.B. collected, analyzed data and prepared the figures. P.Q., Z.W., and K.B. conceived and designed the work and wrote the manuscript. All authors had final approval.

Supplementary Material

Supplementary material is available at *Stem Cells* online.

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