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Dormancy regulation of hematopoietic stem cell

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Motivation and Aim: Hematopoietic stem cells (HSCs) are responsible for the production of all blood cells during life. HSCs are very heterogeneous, while most adult HSCs are maintained in a quiescent state, numerous studies have demonstrated that 20–30 % of HSCs are deeply quiescent and, therefore, called 'dormant' HSCs (dHSCs). dHSC are characterized by slow metabolism, reduced ribosomal biogenesis, and DNA replication. Dormancy protects HSCs from external stresses, accumulation of somatic mutations, prevent their exhaustion and malignant transformation. Despite their deep quiescence, dHSCs harbor the greatest long-term repopulation capacity in transplantation assays. They do not produce cells under homeostatic conditions and are activated only in response to severe stress signals such as interferons, lipopolysaccharide, and myeloablation [1]. Thus, dHSCs serve as a reserve pool of stem cells throughout life. However, excessive dormancy may prevent of hematopoietic system from an efficient response to hematological stresses. Despite the importance of dormant HSCs, their detailed characterization has been challenging due to the absence of surface markers for their ready identification and isolation. Consequently, processes involved in preserving of dHSC quiescence are poorly understood. The aim of our research was to find a surface markers for isolation dHSC and to investigate molecular mechanisms of dormancy regulations.

Methods and Algorithms: we used single-cell and bulk next generation sequencing of murine HSCs. Pseudotime analysis, dormancy score calculation. Transplantation of HSCs into lethally irradiated recipients, FACS analysis and sorting, label retaining assay in vivo, single cell tracing assay in vitro, Ca2+ flux assay, and immunohistochemistry.

Results: To capture the transition between quiescence and proliferation, HSCs from young mice were subjected to single cell RNA sequencing, and after quality control, the transcriptome profiles of 1613 individual HSCs were used for downstream analysis. To identify actively cycling cells, we calculated cell cycle and dormancy scores of individual HSCs using Seurat [2], which were based on the expression levels of cell cycle and dormancy genes [3]. We observed that cells in the S and the G2/M phases were clustered together and that, as expected, most of the HSCs were quiescent (Fig. 1). Next, we attempted to isolate cell surface markers associated with HSC dormancy and identified *Cd38* as a putative marker for dHSCs because its expression was higher in cells with high dormancy scores and corresponded with the expression of well-known long-term HSC markers.

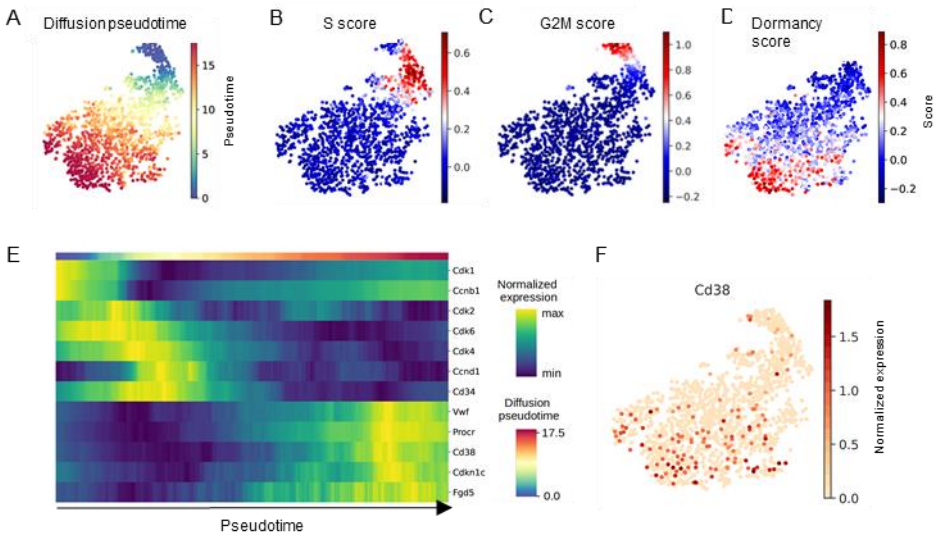

Fig. 1. Single cell transcriptome analysis of HSCs.
(A) Uniform manifold approximation projection (UMAP) representation depicting the transcriptional profiles of individual HSCs (LSK CD48^{-} CD150^{+}). (B) S-phase score along pseudotime. (C) G2/M phase score along pseudotime. (D) Dormancy score along pseudotime. For panels A–D, each dot represents a single cell. (E) Expression of selected genes along pseudotime. (F) UMAP representation showing the expression of *Cd38*. Each dot represents a single cell

We found that about 50 % of HSCs expressed CD38 on the surface at a high level. Using lable retaining assay together with cell cycle analysis of HSCs in vivo in steady state condition and in response to several hematopoietic stresses, we have confirmed that CD38+ HSCs represent the most quiescent population of stem cells. Moreover, CD38+ HSCs have higher repopulation capacity compared to their CD38- counterparts in serial transplantation assay. Therefore, our results indicate that CD38^{+} HSCs reside at the top of the hematopoietic hierarchy. Hence, CD38 represents a marker that will help circumvent the limitations of the long-term label-retaining assays or even negate the necessity of reporter mice for studying the mechanisms underlying HSC dormancy.
CD38 is a multifaceted nicotinamide adenine dinucleotide (NAD) catabolic ecto-enzyme that metabolizes NAD and its precursors (nicotinamide mononucleotide-NMN and nicotinamide riboside-NR) into adenosine diphosphate ribose (ADPR) and cyclic-ADPR (cADPR). We have found that inhibition of CD38 ecto-enzymatic activity led to the cell cycle activation of dHSCs. Moreover, we found that cADPR, the product of NAD conversion by CD38, activates Ca^{2+} release from endoplasmic reticulum in CD38+ dHSCs.
To clarify how the CD38/cADPR/Ca^{2+} axis regulates HSC dormancy, we performed a bulk transcriptome RNA sequencing of CD38+ and CD38- HSCs and found that while 205 genes were significantly down-regulated in CD38+ HSCs, 225 were up-regulated. Gene sets controlling the response to calcium ions, extracellular matrix interaction, and TGF-β1 response were up-regulated in CD38+ HSCs. HSC-related genes, such as *Hoxb9*, *H19*, *VwF*, *Clu*, and *Selp*, as well as genes associated with HSC dormancy, namely, *Gprc5c*, *Meis2*, and *Neo1*, were up-regulated in CD38+ HSCs. We did not find significant differences in *Cdk2*, *Cdk4*, *Cdk6*, and *CyclinD1* expression, but CD38+ HSCs

expressed the cell cycle inhibitors *Cdkn1a* and *Cdkn1c* at higher levels than CD38- cells. Intriguingly, the transcription factor *Fos*, whose expression was previously correlated to cell cycle activation, was one of the most significantly up-regulated genes in CD38+ HSCs. This observation was further corroborated by the fact that AP-1 complex responsive genes were enriched in CD38+ HSCs compared to CD38- counterparts. Moreover, $CD38^{+}$ HSCs displayed higher levels of transcriptionally active phosphorylated c-Fos (at Threonine 232, p-c-Fos) than CD38- cells. Using in vitro and in vivo assays, we found that inhibition of c-Fos transcription activity activated cell cycle entrance of dormant CD38+ but not CD38- HSCs. We identified that c-Fos regulates HSC dormancy via up-regulation of *Cdkn1c* expression ($p57^{kip2}$-cell cycle inhibitor). Importantly, we found that CD38 enzymatic activity on neighboring cells regulated the proliferation of CD38-negative human HSCs. In human bone marrow, several cell types express CD38: multipotent and restricted hematopoietic progenitors, plasma cells, activated T and B-lymphocytes, and NK cells. Therefore, some of these CD38+ cells can be the neighbors for human HSCs. Several hematological malignancies (chronic myeloid leukemia, acute myeloid leukemia, acute lymphoblastic leukemia, and multiple myeloma) express CD38 at a high level. We hypothesize that tumour microenvironment enriched in the products of CD38 ecto-enzymatic activity may keep healthy HSCs in the quiescent state leading to cancer-related pancytopenia as well as it may preserve the dormancy of cancer stem cells leading to disease persistence. Therefore, a better understanding of the mechanisms controlling human HSC dormancy is required to support healthy hematopoiesis in patients with hematologic malignancies and develop more powerful strategies for cancer eradication.

Conclusion: Here, we identify CD38 as a novel and broadly applicable surface marker for the enrichment of murine dHSCs. We reveal that the CD38/cADPR/Ca^{2+}/c-Fos/$p57^{kip2}$ axis regulates HSC dormancy. Mechanistically, we demonstrate that CD38 itself regulates stem cell dormancy by shuttling intracellular Ca^{2+} in a CD38/cADPR-dependent manner, which results in a consequent increase in c-Fos and the expression of the cell cycle inhibitor $p57^{kip2}$. Manipulation of this axis can potentially stimulate dHSC expansion and their efficient response to hematopoietic stress.

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