Correspondence

Stem Cell INVESTIGATION

Shedding light on the DARC knight as a guardian of hematopoietic stem cell quiescence

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In-depth understanding of the crosstalk between hematopoietic stem cells (HSCs) and their niche, and identification of 'functional' surface markers of HSCs are vital in the advancement of therapies for hematological disorders (e.g., *ex vivo* expansion or transplantation of HSCs). MacNamara (1) and Pérez-Fernández *et al*. (2) respectively provided constructive comments on our results and made very interesting suggestions regarding clinical application of the CD82/DARC axis. In this Correspondence, we aim to add to the discussion (1,2) on our recent article (3).

CD82: functional marker for adult stem-progenitor cells?

Mainly known as a metastasis suppressor, CD82 is emerging as an important functional marker in adult stem cells. We recently reported that CD82 is predominantly expressed on long-term repopulating hematopoietic stem cells (LT-HSCs), and it maintains the dormancy of the most primitive HSC subset both in mice and humans (3). Few months later, Alexander *et al*. also reported that CD82 marks human myogenic cell population with higher capacity for proliferation and engraftment (4). Seemingly having tissue-dependent roles in cell cycle regulation, CD82 may be used as a functional marker for at least certain types of adult stem cells. Further studies are required for elucidating CD82 signaling in other stem-progenitor cells such as intestinal and neuronal stem cells to determine how CD82's function differs among tissue type.

In the bone marrow (BM), LT-HSCs remain largely quiescent and are anchored to surrounding niche. HSCs are kept dormant by autocrine signaling and paracrine factors [i.e., transforming growth factor β (TGFβ), stromal cell derived factor 1, thrombopoietin etc.] released from surrounding niche supporting cells (NSPs) and other niche components. In line with the previous knowledge, we reported TGFβ is downstream of CD82 and secreted from LT-HSCs [and from mesenchymal stromal cells (MSCs)], causing the stem cells to trigger quiescence program.

Besides, CD82 may be involved in physical interaction with HSC niche, which is also critical in cell cycle regulation. In leukemic cells, CD82 promotes N-cadherin cluster formation in the plasma membrane thereby facilitating their homing to the BM (5). CD82 also upregulates the expression level and the formation of integrin α4β1 cluster causing increased adhesion to BM microenvironment (6). Based on these studies, as Pérez-Fernández *et al*. suggested (2), it is conceivable that CD82 may put HSCs to sleep not only by TGFβ-SMAD3 pathway

but also by promoting spatial rearrangement of adhesion molecules which favors HSC-to-niche interaction and concomitant cell cycle exit.

An interesting observation in our study is that although LT-HSC number was decreased by *Cd82* deletion, $Lin^-Sca\text{-}1^+cKit^+$ (LSK) population [namely, hematopoietic stem-progenitor cells (HSPCs)] was not affected. One possibility is that *Cd82*$^{-/-}$ or $CD82^{low/neg}$ LT-HSCs may have a tendency to undergo "myeloid bypass", that is, give rise to myeloid restricted repopulating progenitor cells via asymmetric division (7). It is also possible that short-term repopulating HSCs and multipotent progenitors may not be affected by *Cd82* loss since they minimally express CD82. According to our RNA-seq results, *Cd82* expression was positively related to mRNA level of negative regulators of myeloid leukocyte differentiation. Consistent with the RNA-seq data, *Cd82*$^{-/-}$ BM cells showed a higher percentage of myeloid population; similar phenomena were observed after primary and secondary BM transplantation (3). CD41 (integrin alpha-IIb) and, more importantly, tetraspanin CD9 are associated with myeloid and megakaryocyte commitment (8,9). Therefore, it is interesting to speculate tetraspanin CD82 may determine lineage preference.

NSPs and dormancy-inducing factors in the BM

In addition to non-hematopoietic NSPs such as osteoblasts, MSCs, endothelial cells (ECs), growing evidence shows that hematopoietic cells also contribute to HSC dormancy (3,10,11). Therefore, we can postulate that progeny cells that are derived from HSPCs become NSPs that contribute to HSC quiescence. Exploring possible crosstalk between megakaryocytes, DARC-expressing macrophages and non-hematopoietic NSPs (e.g., MSCs) will deepen our understanding of HSC niche biology. Other than autocrine TGFβ signaling of $CD82^+$ LT-HSCs, NSPs may provide LT-HSCs with TGFβ in a CD82-independent manner. Interestingly, one-third of *Cd82*$^{-/-}$ LT-HSCs are in G_0 phase, implying CD82-independent quiescence signaling exists. Various HSC regulating factors including growth factors, ECM components, and adhesion factors may account for the observation. Also, given that TGFβ is secreted as an inactive form, elucidating the mechanism that leads to TGFβ activation is of particular interest.

Our study showed DARC-expressing macrophage, a hematopoietic cell type, governs LT-HSC dormancy through direct contact. Interestingly, in BM ablation model, percent of $CD82^+$ LT-HSCs dropped although percent of DARC-expressing macrophages remained high. Immunofluorescence analysis of the BM revealed the loss of direct interaction between LT-HSCs and macrophages. Additionally, the number of DARC-expressing macrophages was reduced (unpublished data). While these data indicate the physical contact with DARC is crucial in maintaining surface CD82 level *in vivo*, we cannot exclude the possibility in which other factors (e.g., cytokines) lowered CD82 expression in earlier time point.

Although we only included $CD11b^+$ fraction of macrophages ($CD11b^+Gr1^{low}F4/80^+SSC^{low}DARC^+$) in the report, $CD11b^-$ macrophages also slightly express DARC (unpublished data). Considering rhDARC treatment or co-culture with *Darc*-overexpressing ECs could maintain/increase G_0 portion of LT-HSCs, it is conceivable that regardless of cell type, surface DARC molecule may be involved in HSC dormancy. Furthermore, considering that the arteriolar niche in murine BM is critical in maintaining HSC quiescence (12) and human endothelial DARC expression is tissue-dependent (13), it remains to be determined whether human vascular niche impacts LT-HSC dormancy via the CD82/DARC axis.

In clodronate-induced *in vivo* macrophage depletion model, we observed that both total and $CD82^+$ LT-HSCs plummeted in number, which seems to contradict a previous paper (14). However, it should be noted that the experimental setting of ours differs from that of McCabe *et al*. in many aspects including LT-HSC definition ($CD34^-Flt3^-LSK$ *vs*. $LK^+CD150^+CD48^-$) and injection route of clodronate [intraperitoneal (IP) *vs*. intravenous (IV)]. As MacNamara pointed out (1), IP injection may have caused significant inflammation in the stomach, leading to increased "demand-adapted hematopoiesis". Chow *et al*. reported HSC mobilization into peripheral blood on day 1 post-clodronate injection (15). IP injected clodronate, unlike IV injected one, is delivered to lymph nodes before they reach the BM. Therefore, IP injection may induce additional removal of macrophages, necessitating HSC differentiation to replenish the cells. Finally, McCabe *et al*. saw an increase in HSC number on day 2 (14). Therefore, it is possible that BM HSC number time-dependently changes in response to clodronate stimuli.

It is an interesting hypothesis that interferon-gamma, which maintains/increases the pool of BM macrophages, may lower their DARC expression, inducing cell cycle and consequently reducing the number of HSCs. Investigation into a mechanism regulating DARC expression, such as inflammation, may provide a strategy to manipulate HSC

function *in vivo*.

Clinical relevance of the CD82/DARC signaling

Though understanding of the CD82/DARC axis is in its earliest stage, taking advantage of these two molecules seems to be of particular clinical importance. The most-pursued goals in the field of hematology include improving the efficiency of large-scale *ex vivo* expansion and engraftment of functional HSCs. Because quiescent HSCs show better engraftment and repopulating capacity, a procedure that consists of purification of dormant HSCs using CD82, *ex vivo* expansion, followed by re-induction of quiescence using recombinant DARC protein is conceivable.

Successful elimination of leukemic stem cells (LSCs) by either targeting LSC-specific antigens (16) or bringing dormant LSCs into cell cycle and treating with traditional chemotherapy (17) suggests the CD82/DARC axis may also be used to develop a relapse-prevention strategy. Previous studies indicate that the CD82 may be exploited by LSCs. CD82 is highly expressed on and therefore is regarded as a marker for chemoresistant LSCs (reportedly enriched in $CD34^+CD38^-$ subpopulation). CD82 elevates anti-apoptotic protein BCL2L12 (18), downregulates tumor suppressor gene expression (19), promotes ECM adhesion (20) of $CD34^+CD38^-$ subset. In contrast to its dormancy-enforcing role in normal LT-HSCs, CD82 seems to favor LSC proliferation. In order to clarify CD82's function in LSCs, establishing a precise molecular definition for authentic LSCs by long-term repopulation assay is necessary. In addition, considering posttranslational modification (PTM) of CD82 affects its function, we hypothesize that leukemic CD82 may differ in PTM status or carry mutation at the genetic level. Further investigation is warranted to unveil whether CD82/DARC signaling is altered in leukemia patients. On the contrary, if the quiescence-promoting function of these molecules is conserved in LSC niche, this may confer LSCs chemoresistance. Hence, it is possible that blocking the CD82/DARC axis will awaken LSCs and make them sensitive to chemotherapy. Thorough understanding of LSC niche will enable the establishment of relapse-free leukemia treatment.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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