Commentary

Stem Cell INVESTIGATION

The DARC-CD82 axis discloses bone marrow macrophages as guardians of long-term hematopoietic stem cells quiescence

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In all adult tissues, there is a constant supply of differentiated cells provided by the existence of stem and progenitor cells capable of dividing according to the organism's demands. One of the most paradigmatic tissues regarding the study of stemness and differentiation is the hematopoietic system. Blood homeostasis is sustained by a subset of cells known as hematopoietic stem and progenitor cells (HSPCs). This is a heterogeneous population that includes from the most immature cells, which have the ability to self-renew and give rise to cells of all blood lineages, to the so-called progenitors, whose differentiation capacity is restricted to a single lineage and display a more limited number of divisions. To date, the main way of distinguishing hematopoietic cells in different stages of maturation has been the use of surface markers based on their restricted or exclusive expression (1). Nevertheless, these markers *per se* do not show functional characteristics of the cells, since generally only their restricted expression is exploited, but not their biological activity.

The proliferative status of hematopoietic stem cells (HSCs) must be tightly regulated, since they have to replenish the pool of mature, functional cells, after injury or ablation. Consequently, they must not divide endlessly otherwise this primary source would be exhausted (2). This control is exerted by the microenvironment surrounding them, which includes both cellular and acellular factors (3). This idea was proposed in the late 70s by Schofield (4) for the hematopoietic system, who named this environment the 'niche'. Until recently, it was believed that the main cellular regulators of the cell cycle of HSCs were only the surrounding supporting cells, those non-related to the hematopoietic lineage.

According to the accepted understanding of hematopoiesis, the most immature and quiescent cell in the steady-state is the so-called long-term hematopoietic stem cell (LT-HSC). In mice, this cell divides 5 times during their lifespan (5) and can be isolated on the basis of different combinations of the expression of surface markers and/or dye-efflux activity (6,7). Common functional assays to demonstrate the stemness of HSCs purified in this manner are colony-forming unit (CFU) assays and long-term repopulation (LTR) assays (8,9). Nonetheless there is an active research in progress looking for 'functional' surface markers that may be indicative of the actual cell cycle status and that correlate with the stemness of HSCs. These new surface markers are often adhesion molecules which interact in an either homo- or heterophyllic manner with the surrounding niche. A recent example of this is the endothelial cell-selective adhesion molecule (ESAM), which has been shown to become selectively upregulated upon BM injury with 5-fluorouracil (5-FU) treatment in LSK cells, and to identify actively-cycling cells in the $CD150^{+}$ subset with an enhanced capacity of repopulation (10). Another interesting study regarding this issue has been performed by Jeannet and colleagues (11). In this work they demonstrate that activated leukocyte cell adhesion molecule (Alcam or CD166) is expressed in LT-HSCs (although not exclusively), and positively regulates both engraftment and LTR capacity. Despite the fact that Alcam is not a selective marker of HSCs, it could be used in combination with other surface antigens to simultaneously identify immunophenotypic and functional LT-HSCs. It would be very interesting to not

only discover new surface molecules, but also to find out more about the biological role of the currently employed ones in order to improve sorting methods to be used in both basic and translational research.

Even though supporting non-hematopoietic cells comprising the niche have extensively been studied, there is a growing body of evidence that shows the influence of terminally differentiated cells on the stem cell which gave rise to them in distinct tissues (12-14). Regarding the role of mature hematopoietic cells as niche components, there are two parallel reports conducted by Frenette's and Li's teams, respectively. Both of them demonstrate the involvement of megakaryocytes (MKs) in the regulation of HSC quiescence. In the first paper, the authors state that HSCs reside in the BM close to, or in contact with, megakaryocytes, which through secretion of platelet factor 4 (PF4) would regulate the quiescence and engraftment of the undifferentiated cell. They also demonstrate that the megakaryocyte niche is spatially independent of the arteriolar niche (15). In the second one, the proximity of MKs and HSCs in the BM is also shown, together with the fact that their depletion disturbs the quiescence of the LT-HSCs, committing them to more differentiated progenitors. The maintenance of LT-HSCs in the steady state seems to be carried out through secretion of TGF-β, according to these presented results. On the other hand, after 5-FU injury, MKs are important for marrow reconstitution via FGF secretion, although TGF-β seems to be also necessary for a proper engraftment after this type of stress (16). Additionally, Nakamura-Ishizu *et al.* have described the relevance of thrombopoietin (TPO), a cytokine known for its regulatory role in the differentiation of MKs, in the maintenance of HSC quiescence (17). Interestingly, in this study it is suggested that MKs would produce and secrete TPO into the BM, and this would maintain HSC quiescence in the steady state. It makes sense that a population of cells that reside in the BM cavity and in close contact with HSCs could regulate their biology. Therefore, in line with the previous reports, it would be very interesting to explore the possibility that other committed or mature hematopoietic cells residing in the BM may have an impact on HSC function.

This question has been addressed by Hur *et al.* as described in their recently published report (18). First, they identified the widely expressed molecule of the tetraspanin family CD82 as a differential marker of LT-HSCs, as compared to more mature hematopoietic cells (ST-HSCs and MPPs). They studied the simultaneous expression of this surface antigen and the previously described selective leukocyte adhesion molecules (SLAM) markers (19) on HSCs, and found a higher expression on SLAM-HSCs with respect to more committed populations. In addition, they showed that the location of $CD82^+$ LT-HSCs is preferentially close to endosteum and arterioles in the BM, regions that are known to be important niche constituents (20-25).

Following on, the authors quantified both the immunophenotypic HSC subsets and the clonogenic ability of LT-HSCs in control and *Cd82* knockout animals, and demonstrated that only LT-HSCs, but not ST-HSCs or MPPs, were altered by the absence of this gene. Moreover, the colony-forming capacity of the LT-HSC subset was clearly diminished in KO *vs.* WT animals. Accordingly, it would seem that only symmetric divisions of LT-HSCs were compromised, as the more mature populations were not disturbed by *Cd82* deletion. However, it seems quite a strange phenomenon that one of the subsets decreases while the rest remain unchanged, given that the expected observation would have been that all populations would have been altered when one of them changed. Additionally, they provided evidence that $Cd82^{-/-}$ LT-HSCs are less quiescent than WT cells on the basis of the expression of the Ki67 antigen and some cell cycle inhibitors, together with BrdU incorporation. It would be extremely interesting to discern if these increased divisions are either symmetric or asymmetric in order to explain the constant percentages of ST-HSCs and MPPs.

To further dissect the molecular mechanism that links CD82 expression on LT-HSCs with their quiescence, Kim's team used the EML cell line, which provides larger sample amounts than Lin^- cells in order to perform the necessary experiments. They induced both overexpression and downregulation of *Cd82* in EML cells, and proved that the effect was the opposite for the two experimental approaches regarding TGF-β signaling components. Overall, their results pointed to a positive regulation of CD82 on TGF-β1 secretion and TGF-βR2 expression. TGF-β signaling, via PKCα, would lead to an increase of both total and phosphorylated forms of SMAD3, which would generate cell cycle arrest via upregulation of cell cycle inhibitors p21, p27 and p56, and dephosphorylation of Rb. This is in concordance with the work of Zhao and colleagues describing MKs as HSC quiescence guardians via TGF-β signaling (16). Thus, there seems to be consensus regarding the signaling pathways employed by mature cells to regulate stem cell activity.

Having deciphered some key signaling molecules

involved in the CD82-mediated maintenance of HSC quiescence, the next step in the work was to assess the *in vivo* repopulating capacity with and without *Cd82* expression in a wild-type background. As expected, the reduced quiescence of *Cd82*$^{-/-}$ LT-HSCs caused impaired long-term reconstitution ability after BM ablation. They observed both augmented expression of Ki67 and a reduced number of HSCs in mice transplanted with *Cd82*$^{-/-}$ cells, accompanied by a bias towards the myeloid lineage after primary and secondary transplantation.

To better understand the external signals through which CD82 exerts its regulation on HSCs, the authors studied the expression of the Duffy antigen receptor for cytokines (DARC), a previously described interaction partner of this membrane molecule (26), on niche supporting cells. They found that, among the populations taken from BM, only macrophages (Mφ) expressed a considerable amount of DARC. Next, they observed the physical interaction between DARC^{+} Mφ and LT-HSCs *in vivo* in Tie2-GFP mice, and *in vitro* in a co-culture of primary isolated Lin^{-} cells and F4/80^{+} macrophages. After confirming the protein interaction through immunoprecipitation assays, they determined that DARC^{+}, but not DARC^{-} Mφ, promote the quiescence of LT-HSCs in co-culture in a TGF-β1-controlled manner, as shown by means of shRNA experiments. Interestingly, CD82 expression on LT-HSCs was shown to be augmented when DARC was present on Mφ surface. However, despite that these experiments show a novel and interesting role of Mφ in the regulation of LT-HSCs quiescence, the possible influence of endothelial DARC in this process cannot be discarded, since this was the first cell type in which DARC-CD82 interaction was described (26). Given the fact that endothelial cells are well-known regulators of HSC biology, this interaction may be a component of the underlying mechanism governing this function. In fact, the authors show in their publication that upon co-culture of LT-HSCs with the endothelial cell line MS-1 overexpressing DARC the percentage of LT-HSCs in the G_0 phase was augmented. Although this was not the case for the cell line C166, it would be extremely interesting to explore this scenario in a deeper manner, and also to elucidate whether the interaction of LT-HSCs with only Mφ or both cell types (Mφ and endothelial cells) is the responsible one of the quiescence promoting effect of the DARC-CD82 interaction. In addition, although not explored in the article of Hur and colleagues, there may exist a cis regulation of the DARC-CD82 axis in Mφ or a homotypic trans CD82 interaction between two different cells of the myeloid lineage, because of the high CD82 expression reported in myeloid cells (27).

One of the most interesting sets of experiments in the report is where the authors actually show the functional relevance of the DARC-CD82 interaction on LT-HSCs cell cycle regulation upon hematopoietic ablation. 5-FU treatment caused an increase in the proliferation rate of BM cells with a concomitant drop of CD82^{+} LT-HSCs. Although they propose that this phenomenon was mediated by the depletion of DARC^{+} macrophages, their percentages did not start to be diminished simultaneously, and therefore, there must be additional mechanisms that initiate LT-HSCs proliferation at an earlier time point. The fact that upon 5-FU treatment co-localization between LT-HSCs and DARC^{+} macrophages was not observed could be a crucial point, especially if it happened before Mφ depletion. This might explain the time-course profile difference between the drop in CD82^{+} cells and DARC^{+} Mφ previously mentioned. Regardless of origin of the CD82 drop (either loss of CD82-DARC interaction or Mφ depletion) the authors clearly reveal the strong correlation between the number of DARC^{+} Mφ and the quiescence of LT-HSCs by co-culture experiments with EML cells and Raw 264.7 cells. Importantly, this quiescence was not maintained upon downregulation of DARC on the Mφ surface. Hur and colleagues went deeper into the molecular mechanism by which DARC regulates CD82 and proved, *in vitro*, that in the absence of interaction between both molecules, the latter was endocytosed and eliminated by proteasomal degradation in EML cells. Moreover, there seemed to be no need for the presence of other surface molecules to trigger the HSC quiescence effect, since treatment with recombinant DARC without Mφ present in the culture preserved the dormancy of EML cells.

Finally, they extended their findings in the mouse model to a human setting. By using UCB purified HSCs, they corroborated that CD82 was mainly expressed by quiescent cells, and that the PKC signaling initiated by DARC-CD82 interaction was conserved in humans. Similar to what occurred in the murine model, monocytes/macrophages were the main DARC-expressing cells in UCB, and co-culture of DARC^{+} monocytes/macrophages with human HSCs upregulated CD82 expression on these cells. This is clear evidence that the mechanism described for mice operates in a highly similar fashion in a human experimental setting.

We consider this report to be a very elegant study, not only because of the technical complexity of the

experimental design and the detailed view it presents regarding the molecular mechanism that governs the HSC-niche interaction, but also because of its scientific relevance and the interesting focus from which it was performed. On one hand, Hur *et al.* have contributed to reformulate CD82 as a surface antigen defining functional LT-HSCs by showing its differential expression and biological role in this subset. CD82 has been previously described as a regulator of cell adhesion through control of alpha 4 integrin (28). We and others have demonstrated the importance of cell adhesion molecules in the regulation of HSC quiescence (10,11,23,29,30). Therefore, we would not discard the possibility that, in addition to TGF-β signaling, cell adhesion might have a role in CD82-mediated control of the cell cycle. As indicated above, defining functional markers is very relevant to improve cell sorting protocols and studies regarding the repopulating capacity of HSC. On the other hand, despite that Mφ have previously been proposed as niche components (31), Hur *et al.* describe for the first time their role as gatekeepers of the quiescence and the long term repopulation ability of HSCs through direct contact. These studies, in conjunction with the above pointed reports involving MKs in HSC regulation in the BM cavity (15-17), depict a new paradigm of the HSC niche in which its cellular composition is not restricted to supporting, non-hematopoietic cells, i.e., the traditional vascular and osteoblastic niches. Perhaps, in light of these results, we should start to consider some kind of "single-cell niche", taking into consideration every cell surrounding a particular stem cell/progenitor, even another HSPC, given the evidence showing autocrine regulation of HSCs in terms of stemness (32). In the medium/long term it will be extremely interesting to consider other hematopoietic subsets occupying the BM cavity, either progenitors or more committed cells, as crucial regulators of the cell cycle and differentiation of HSPCs, in order to unravel the complex network of interactions that presumably govern these critical processes. As the authors propose in their paper, their study not only sheds light on the regulation of LT-HSCs quiescence by $DARC^+$ Mφ, but also opens new therapeutic possibilities, with respect to the use of rhDARC and TGF-β in the clinic, to improve *ex vivo* expansion protocols for HSCs, which is one of the most currently pursued goals in the field of hematopoiesis.

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Footnote

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References

1. Doulatov S, Notta F, Laurenti E, et al. Hematopoiesis: a human perspective. Cell Stem Cell 2012;10:120-36.
2. Orford KW, Scadden DT. Deconstructing stem cell self-renewal: genetic insights into cell-cycle regulation. Nat Rev Genet 2008;9:115-28.
3. Wang LD, Wagers AJ. Dynamic niches in the origination and differentiation of haematopoietic stem cells. Nat Rev Mol Cell Biol 2011;12:643-55.
4. Schofield R. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. Blood Cells 1978;4:7-25.
5. Wilson A, Laurenti E, Oser G, et al. Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. Cell 2008;135:1118-29.
6. Wilson A, Oser GM, Jaworski M, et al. Dormant and self-renewing hematopoietic stem cells and their niches. Ann N Y Acad Sci 2007;1106:64-75.
7. Nakamura-Ishizu A, Takizawa H, Suda T. The analysis, roles and regulation of quiescence in hematopoietic stem cells. Development 2014;141:4656-66.
8. Jones RJ, Sharkis SJ, Celano P, et al. Progenitor cell assays predict hematopoietic reconstitution after syngeneic transplantation in mice. Blood 1987;70:1186-92.

9. Visser JW, de Vries P, Hogeweg-Platenburg MG, et al. Culture of hematopoietic stem cells purified from murine bone marrow. Semin Hematol 1991;28:117-25.
10. Sudo T, Yokota T, Oritani K, et al. The endothelial antigen ESAM monitors hematopoietic stem cell status between quiescence and self-renewal. J Immunol 2012;189:200-10.
11. Jeannet R, Cai Q, Liu H, et al. Alcam regulates long-term hematopoietic stem cell engraftment and self-renewal. Stem Cells 2013;31:560-71.
12. Sato T, van Es JH, Snippert HJ, et al. Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. Nature 2011;469:415-8.
13. Tong CK, Chen J, Cebrián-Silla A, et al. Axonal control of the adult neural stem cell niche. Cell Stem Cell 2014;14:500-11.
14. Hosoda T, Zheng H, Cabral-da-Silva M, et al. Human cardiac stem cell differentiation is regulated by a mircrine mechanism. Circulation 2011;123:1287-96.
15. Bruns I, Lucas D, Pinho S, et al. Megakaryocytes regulate hematopoietic stem cell quiescence through CXCL4 secretion. Nat Med 2014;20:1315-20.
16. Zhao M, Perry JM, Marshall H, et al. Megakaryocytes maintain homeostatic quiescence and promote post-injury regeneration of hematopoietic stem cells. Nat Med 2014;20:1321-6.
17. Nakamura-Ishizu A, Takubo K, Fujioka M, et al. Megakaryocytes are essential for HSC quiescence through the production of thrombopoietin. Biochem Biophys Res Commun 2014;454:353-7.
18. Hur J, Choi JI, Lee H, et al. CD82/KAI1 maintains the dormancy of long-term hematopoietic stem cells through interaction with DARC-expressing macrophages. Cell Stem Cell 2016;18:508-21.
19. Oguro H, Ding L, Morrison SJ. SLAM family markers resolve functionally distinct subpopulations of hematopoietic stem cells and multipotent progenitors. Cell Stem Cell 2013;13:102-16.
20. Zhang J, Niu C, Ye L, et al. Identification of the haematopoietic stem cell niche and control of the niche size. Nature 2003;425:836-41.
21. Calvi LM, Adams GB, Weibrecht KW, et al. Osteoblastic cells regulate the haematopoietic stem cell niche. Nature 2003;425:841-6.
22. Sugiyama T, Kohara H, Noda M, et al. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. Immunity 2006;25:977-88.
23. Winkler IG, Barbier V, Nowlan B, et al. Vascular niche E-selectin regulates hematopoietic stem cell dormancy, self renewal and chemoresistance. Nat Med 2012;18:1651-7.
24. De Grandis M, Lhoumeau AC, Mancini SJ, et al. Adhesion receptors involved in HSC and early-B cell interactions with bone marrow microenvironment. Cell Mol Life Sci 2016;73:687-703.
25. Lilly AJ, Johnson WE, Bunce CM. The haematopoietic stem cell niche: new insights into the mechanisms regulating haematopoietic stem cell behaviour. Stem Cells Int 2011;2011:274564.
26. Iiizumi M, Bandyopadhyay S, Watabe K. Interaction of Duffy antigen receptor for chemokines and KAI1: a critical step in metastasis suppression. Cancer Res 2007;67:1411-4.
27. Lebel-Binay S, Gil ML, Lagaudriere C, et al. Further characterization of CD82/IA4 antigen (type III surface protein): an activation/differentiation marker of mononuclear cells. Cell Immunol 1994;154:468-83.
28. Termini CM, Cotter ML, Marjon KD, et al. The membrane scaffold CD82 regulates cell adhesion by altering α4 integrin stability and molecular density. Mol Biol Cell 2014;25:1560-73.
29. López-Ruano G, Prieto-Bermejo R, Ramos TL, et al. PTPN13 and β-catenin regulate the quiescence of hematopoietic stem cells and their interaction with the bone marrow niche. Stem Cell Reports 2015;5:516-31.
30. Umemoto T, Yamato M, Ishihara J, et al. Integrin-αvβ3 regulates thrombopoietin-mediated maintenance of hematopoietic stem cells. Blood 2012;119:83-94.
31. Ludin A, Itkin T, Gur-Cohen S, et al. Monocytes-macrophages that express α-smooth muscle actin preserve primitive hematopoietic cells in the bone marrow. Nat Immunol 2012;13:1072-82.
32. Rehn M, Olsson A, Reckzeh K, et al. Hypoxic induction of vascular endothelial growth factor regulates murine hematopoietic stem cell function in the low-oxygenic niche. Blood 2011;118:1534-43.

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