Hematopoietic Stem Cell Intracellular Levels of Ca^{2+} to the Rescue! What Next?

Hal E. Broxmeyer[1,2]

[1] Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, IN, USA

[2] Program on Hematopoiesis and Hematologic Malignancies, Indiana University Simon Cancer Center, Indiana University School of Medicine, Indianapolis, IN, USA

Calcium ions (Ca^{2+}), ubiquitous signaling and second messenger molecules, are communicators for the transmission of messages in numerous cell functions. In this issue of *Cell Stem Cell*, Luchsinger et al. (2019) provide evidence through the use of transplantation and mechanistic studies for the finding that, "Harnessing Hematopoietic Stem Cell Low Intracellular Calcium Improves Their Maintenance *In Vitro*."

A prominent role for increased intracellular [Ca^{2+}] in hematopoietic stem cell (HSC) regulation has recently gained wider appreciation. Most notably, Uemoto and colleagues recently reported that calcium levels are associated with initiation of HSC division (Umemoto et al., 2018). In a new study in this issue of *Cell Stem Cell*, Luchsinger et al. (2019) now report several new insights into how Ca^{2+} levels impact HSC maintenance.

First, mouse bone marrow (BM) HSCs manifest low intracellular levels of Ca^{2+}, which is associated with enhanced activity of "glycolysis-fueled" membrane efflux pumps. Second, low intracellular Ca^{2+} levels are associated with increased *ex vivo* HSC maintenance as denoted by HSC phenotype, functional

n-eleven translocated (TET) enzymes, which were inhibited by calpain proteases, with TET2 being required for low [Ca^{2+}] maintenance of HSCs *in vitro*.

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To assess effects of [Ca^{2+}] on maintenance of mouse BM HSCs *in vitro* for prolonged time periods, the investigators first cultured purified HSCs in calcium-free DMEM with serum-free supplement, SCF, and TPO in the presence of increasing [$CaCl_2$]. Actual numbers of phenotypically defined HSCs were 2–3 times greater in low than in high [$CaCl_2$]. Also, the cycling status of HSCs was enhanced in low [$CaCl_2$]. The functional HSC populations were tested by competitive transplantation in lethally irradiated recipients; donor cell engraftment was increased in donor cells cultured in low (0.02 mM) versus higher (2 mM) [$CaCl_2$] prior to transplantation. These results were confirmed in limiting dilution transplants. Moreover, [$CaCl_2$] had a larger effect on maintenance of the $CD150^{hi}$ HSC population, previously shown to have greater self-renewal capacity, than that on the $CD150^{lo}$ HSCs without lineage bias (similar lymphoid/myeloid ratios) in engrafted cell populations. Single-cell RNA sequencing demonstrated differences in HSCs cultured in low versus higher [$CaCl_2$].

HSC populations manifested lower intracellular [Ca^{2+}] than multipotential progenitors. To associate intracellular HSC [Ca^{2+}] with function, the authors separated HSCs based on staining with Indo-1; $Indo\text{-}1^{lo}$ HSCs were enhanced in long-term repopulation compared to $Indo\text{-}1^{hi}$ HSCs. There was low intracellular Ca^{2+} efflux pump activity in HSCs, a finding verified by active Ca^{2+} efflux in HSCs compared to progenitors. Higher levels of HSC Ca^{2+} efflux reflected glycolysis. Calpains are a family of calcium-regulated cysteine proteases. Inhibition of calpain activity was associated with low Ca^{2+} effects on HSC maintenance, which was associated with stabilization of Tet enzymes. Tet2 is required for normal functioning of HSCs. In contrast to the effects of [$CaCl_2$] on normal HSCs, cultures with low [$CaCl_2$] had no effect on the maintenance of phenotypically defined *Tet2*$^{-/-}$ HSCs, and they did not increase their competitive repopulating engraftment capability. *Tet2*$^{-/-}$ HSCs were insensitive to enhancing effects of low [Ca^{2+}]. Human cord blood HSCs had lower [Ca^{2+}] than progenitors. A reduced [Ca^{2+}] external environment or calpain inhibition promoted maintenance of human cord blood HSCs *in vitro*, as assessed by limiting dilution engraftment of human cells in sublethally irradiated NSG mice, confirming with

human HSCs some of what was found with mouse HSCs. This extensive and intriguing information opens up a number of questions (summarized in Figure 1).

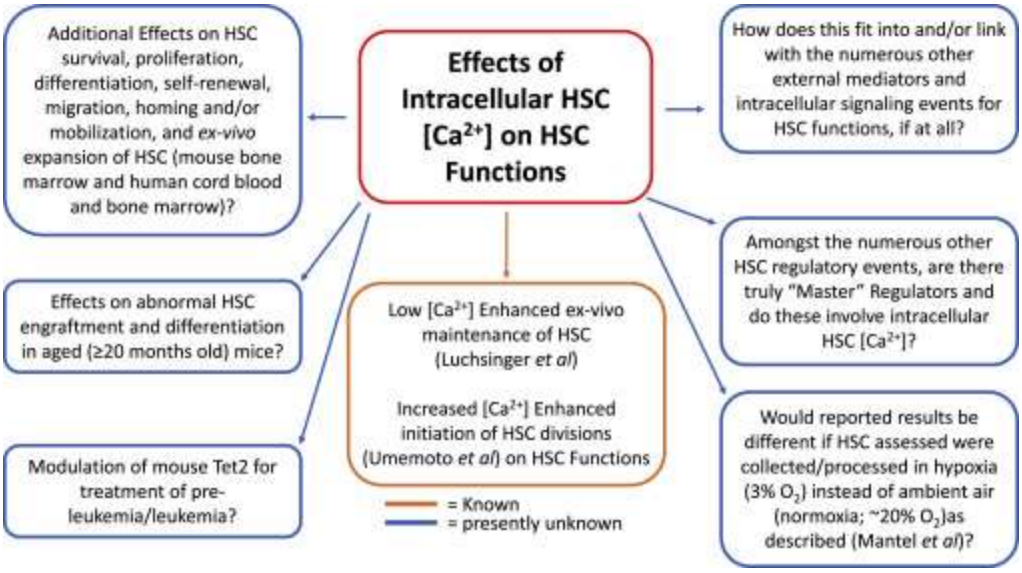

Figure 1. Known and As Yet Unknown Role(s) of Hematopoietic Stem Cell Intracellular Levels of [Ca^{2+}] and Ca^{2+} Efflux in HSCs

HSCs in old mice (e.g., ≥20 months old) are "defective" compared to those in young mouse HSCs. HSC aging is characterized by increased numbers of phenotypically defined HSCs but greatly decreased engrafting capability in lethally irradiated mice, with altered myeloid-lymphoid ratios of engrafted cells. These findings beg the question as to whether there are abnormalities associated with changes in intracellular HSC [Ca^{2+}] or plasma membrane Ca^{2+} efflux pump efficiency in old mice as compared to young mice.

TET2 is implicated in the regulation of normal HSCs and leukemia stem/initiating cells (Cai et al., 2018). *Tet2*$^{-/-}$ HSCs have pre-leukemia and/or leukemia characteristics. Luchsinger et al. tantalizingly suggest that low [Ca^{2+}] had some suppressive effects on *in vitro* maintenance of *Tet2*$^{-/-}$ HSCs. Repopulating *Tet2*$^{-/-}$ cells might be less sensitive to enhancing effects of low intracellular [Ca^{2+}]. Can modulation of intracellular [Ca^{2+}] serve as an intervention treatment for pre-leukemia and/or leukemia?

Studies compared human cord blood to mouse bone marrow HSCs. While more studies are warranted with human HSCs, would there be differences in human cord blood versus human bone marrow HSCs? How might modulation of intracellular Ca^{2+} levels additionally influence cell survival, proliferation, self-renewal, differentiation, migration, homing, and/or mobilization of HSCs? Luchsinger et al. provide significant insight into HSC maintenance. Will this phenomenon have therapeutic benefit? The efficacy of hematopoietic cell transplantation (HCT) is sometimes limited by low donor cell numbers (e.g., cord blood). *Ex vivo* HSC expansion is being considered to address this concern as reported in recent papers (Li et al., 2018, Wilkinson et al., 2019), but only a few such previous *ex vivo* procedures have been clinically evaluated, and not yet confirmed by others for efficacy. Can modulation of intracellular [Ca^{2+}] synergize with various cytokines and effectors? Enhanced HSC maintenance *ex vivo* should link to *ex vivo* increased functional HSC numbers. Is Ca^{2+} involved in the recently reported massive 236- to 899-fold *ex vivo* expansion of HSCs (Wilkinson et al., 2019)?

Most intriguing to me is the plethora of publications offering mechanistic insight into HSC regulation, papers seemingly being reported endlessly. How do these regulatory molecules and interactions fit together, and potentially so in the context of intracellular HSC [Ca^{2+}]? Regulators include, but are not limited to, cytokines, chemokines, and growth modulating proteins including non-chemokine heterochromatin remodeling nuclear protein DEK that when released from the cell functions as a cytokine acting through the CXCR2 chemokine receptor (Capitano et al., 2019), prostaglandin E, ascorbate (Agathocleous et al., 2017), and valine (Taya et al., 2016), with numerous intracellular signaling molecules being implicated. Interactions of this vast number of regulatory molecules and elements are not fully understood in the context of overall regulation of HSC biology. How are these numerous regulators linked? Are there truly "Master" regulators for different HSC functions, and how might they be related to [Ca^{2+}], if at all? We tend to study specific regulators to achieve insight into HSC regulation, but we typically ignore how these fit into an overall schema of HSC function or functions. Clinical efforts, even "successful ones," are usually associated with side-effects, which are sometimes worse than the disease

itself. To better understand potential treatment outcomes, putting together an inclusive or close to inclusive "map" of all regulators and regulation would be extremely helpful. This may seem overwhelming, but a start in this direction, sooner rather than later, could entail meetings of investigators invested in this area of research think-tanking this massive undertaking. Linking or un-linking various regulatory molecules with external and internal influences could lead to more rational treatments and improved health benefits.

Does the present work by Luchsinger et al. on Ca^{2+}, as well as most other regulatory studies reported, accurately mimic activities of HSCs when in their *in vivo* hypoxic microenvironment? Mitochondria are linked to HSC biology (Filippi and Ghaffari, 2019). Roles for mitochondria in HSC function in the context of cell collections and processing in hypoxia (3% O_2) versus ambient air (normoxic, ~20% O_2) have been reported (Mantel et al., 2015). While low O_2 effects on the growth of HSCs and progenitors have been documented since the 1970s, such cells assessed under low O_2 cultures had been first removed in ambient air, which was found to induce rapid differentiation of HSCs to progenitors, a phenomenon termed Extra Physiologic Shock/Stress (EPHOSS). HSC and progenitor numbers and functions in multiple knockout mouse studies were grossly different with cells collected and processed at 3% O_2 (never exposed to ambient air) compared to those collected and processed in ambient air (Mantel et al., 2015). Would intracellular Ca^{2+} levels and their effects on HSC function be different if cells were collected and processed in hypoxia?

There is clearly much more that needs to be done regarding elucidating roles for Ca^{2+} and other HSC regulatory events. Hence, there exists a continued need to investigate HSC biology and optimize HSC modulation for clinical advantage.

Declaration of Interests

H.E.B. has just become a member of the Scientific Advisory Board of Elixell.

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