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RESEARCH HIGHLIGHT

Niche-less maintenance of HSCs by 2i

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Maintenance of hematopoietic stem cells (HSCs) *in vitro* has been believed to be difficult due to a lack of complete understanding of HSC quiescence maintained by the niche. Recent evidence suggests that *in vitro* maintenance of human and mouse long-term HSCs (LT-HSCs) is possible through dual inhibition (2i) of both GSK-3 and mTOR in the absence of cytokines, serum, or feeder cells.

Hematopoietic stem cells (HSCs) are generally quiescent, and have the ability to self-renew or to differentiate into mature blood cells. Despite recent advances, it has not been possible to maintain functional long-term HSCs (LT-HSCs) outside the hematopoietic niche, because mechanisms by which HSC quiescence is maintained by the niche [1] have not been fully understood. There have been many attempts to expand HSCs and hematopoietic progenitor cells *in vitro* using hematopoietic cytokines combined with factors, including Wnt activators [2-4], glycogen synthase kinase 3 (GSK-3) inhibitors [5], Notch ligand, HoxB4, prostaglandin E2, aryl hydrocarbon receptor antagonists, angiopoietin-like proteins, or pleiotrophin [6, 7]. However, all studies have required hematopoietic cytokines, which may promote lineage commitment at the expense of LT-HSC maintenance.

Huang *et al*. [8] previously reported that disruption of GSK-3 in hematopoietic cells in mice leads to an increase in the number of HSCs through Wnt activation, and that the subsequent depletion of LT-HSCs occurs because inhibition of GSK-3 also activates mammalian target of rapamycin (mTOR) (Figure 1A). The mTOR pathway is recognized as an established nutrient sensor, and nutrient-sensing systems are associated with HSC homeostasis. Indeed, HSCs reside in a low-perfusion environment in the bone marrow with low oxygen and low nutrition. Activation of mTOR has been shown to increase the proliferation of committed progenitors at the cost of HSC maintenance (Figure 1A), indicating that low nutrient availability is an essential characteristic of the niche. Thus, Huang *et al*. hypothesized that low nutrient availability might contribute to HSC maintenance.

Recently, Huang *et al*. [9] clearly demonstrate that human and mouse LT-HSCs can be maintained *in vitro* by inhibiting both GSK-3 and mTOR, in the absence of cytokines, serum, or feeder cells (Figure 1B). Moreover, the combination of two clinically approved inhibitors, lithium (GSK-3) and rapamycin (mTOR) (Figure 1C), increases the number of functional LT-HSCs in mice. First, Huang *et al*. [9] determined whether dual inhibition (2i) of GSK-3 and mTOR would be sufficient for maintaining HSCs *in vitro*. They cultured mouse c-Kit$^+$ or Lin$^-$ Sca1$^+$c-Kit$^+$ (LSK) cells in X-VIVO 15 (Lonza) (which is chemically defined, serum-free, hematopoietic cell medium) supplemented with inhibitors of GSK-3 (CHIR99021 or lithium) and mTOR (rapamycin) for 7 days in the absence of cytokines, serum, or feeder cells. They subsequently assessed the hematopoietic potential of the cultured HSCs by competitive repopulation assay. It was confirmed that HSCs cultured with 2i maintained long-term reconstitution potential, and that the frequency of HSCs was similar to that in uncultured c-Kit$^+$ cells. Similarly, they confirmed that the effects of 2i on LT-HSCs were recapitulated in human HSCs that are present in umbilical cord blood CD34$^+$ cells. To explore the mechanism by which 2i preserves HSCs, they also investigated cell cycle status in mouse LSK cells. They found an increased percentage of quiescent cells by 2i, suggesting that the maintenance of LSK cells by 2i is the result of increased dormancy *in vitro*. Finally, they demonstrated that GSK-3 and mTOR inhibition increases mouse LT-HSCs *in vivo*. They treated mice with lithium and rapamycin for 2 weeks, and found that both the overall bone marrow cellularity and the absolute number of LT-HSCs increased in the treatment group. In a competitive repopulation assay, the absolute number of competitive rescue units was increased by 2-fold in bone marrow of treated mice.

The above findings by Huang *et al*. [9] are outstanding, but many questions need to be answered in future studies. i) The authors examined 2i cultures for 7 days *in vitro* (and 2 weeks *in vivo*), and it would be interesting to examine how long it is possible to maintain LT-HSCs in vitro under 2i condition. However, as the authors mentioned [9], prolonged activation of Wnt signaling might be associated with transformation *in vitro*, and might have the risk of inducing colorectal cancers and leukemias when GSK-3 inhibitors are administered *in vivo* [10]. Nevertheless, lithium (GSK-3 inhibitor) has been used to treat bipolar disorder for over 50 years and is not

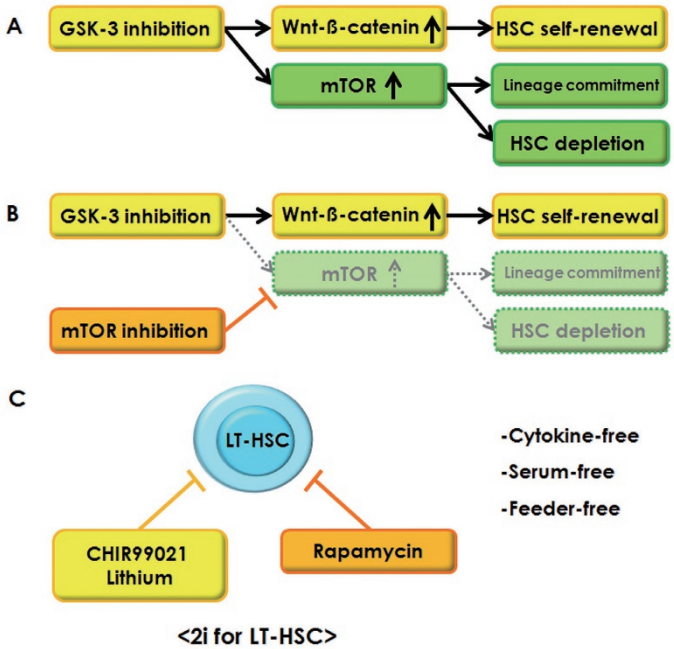

Figure 1 Schematic diagrams of maintenance of LT-HSCs. **(A)** Disruption of GSK-3 results in HSC self-renewal through Wnt activation in a β-catenin-dependent manner. However, in assays of LT-HSC function, disruption of GSK-3 leads to depletion of HSCs through activation of mTOR. GSK-3 regulates both Wnt and mTOR signalings in HSCs [8]. **(B)** GSK-3 inhibition-induced mTOR activation is attributable to HSC depletion, which can be prevented by mTOR inhibition [9]. **(C)** *In vitro* maintenance of LT-HSCs is possible by dual inhibition (2i) of GSK-3 and mTOR under cytokine-free, serum-free, feeder-free conditions [9]. CHIR99021 and lithium are GSK-3 inhibitors, and rapamycin is mTOR inhibitor.

associated with an increased risk of malignancies [11], as the authors pointed out [9]. ii) In cytokine-free medium, is there cytokine production by HSCs or progenitor cells? It may be possible that cytokine production would contribute to the maintenance of LT-HSCs in an autocrine or paracrine manner. iii) Although feeder cells and/or serum are not defined factors for culture, it would be of interest to investigate whether 2i culture in the presence of supporting cells would further improve the maintenance of LT-HSCs. Some extrinsic regulators for HSC quiescence [12], such as N-cadherins, could contribute to LT-HSC maintenance in cooperation with 2i. iv) In addition to iii), hypoxic environment is known to be an extrinsic regulator for HSC quiescence [12], as the bone marrow niche is a low-perfusion environment. Hypoxic culture might synergize with 2i. v) When human ESCs/iPSCs are induced to differentiate into HSCs, it is difficult to capture true human LT-HSCs *in vitro*. If this is due to inability to maintain human LT-HSCs *in vitro*, it would be interesting to examine whether 2i culture would enable *in vitro* induction and maintenance of transplantable LT-HSCs derived from human ESCs/iPSCs.

In summary, dual inhibition (2i) of GSK-3 and mTOR allows for the maintenance of human and mouse LT-HSCs *in vitro* (Figure 1C), and this may resolve the difficulty in culturing HSCs, which in turn, may improve basic research of HSCs (e.g., gene editing *in vitro*) and human HSC transplantation outcomes. Furthermore, although the effect of 2i on expansion of HSCs is relatively small, a combination of 2i drugs may increase human clinical trials [1, 6, 7] that use 2i *in vivo* for the aim of increasing the number of LT-HSCs, since 2i drugs are known as clinically tolerated medications. Insights gained from the discovery of 2i for HSC maintenance may lead to great benefits for patients with hematologic disorders, hopefully in the near future.

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