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COMMENTARY

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Investigating increased hematopoietic stem cell fitness in a novel mouse model

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ABSTRACT

T-cell acute lymphoblastic leukaemia (T-ALL) is a bone marrow (BM) malignancy affecting children and adults. Typically treated with chemotherapy, leukaemia remains a major death cause in people under 20 years old. Understanding molecularly altered pathways in T-ALL may lead to new therapeutic avenues in the future. Ras pathway dysregulation is common in T-ALL. We have shown elevated expression levels of the Ras guanine nucleotide exchange factor RasGRP1 in T-ALL patients, which results in constant production of active Ras (RasGTP). When leukaemia cell lines are exposed to cytokines, RasGTP levels further increase in a RasGRP1-dependent manner. How overexpressed RasGRP1 may impact primary BM cells has remained unknown. We recently published a new *RoLoRiG* mouse model that allows for pIpC-induced overexpression of RasGRP1 in haematopoietic cells, which can be traced with an ires-EGFP cassette. This novel model revealed that RasGRP1 overexpression bestows a fitness advantage to haematopoietic stem cells (HSCs) over wild-type cells. Intriguingly, this increased fitness only manifests in native Hematopoiesis, and not in BM transplantation (BMT) assays. In this commentary, we summarize key features of our *RoLoRiG* model, elaborate on BM niche importance, and discuss differences between native Hematopoiesis and BMT in the context of stem cell metabolism.

Hematopoiesis

The blood involves multiple types of cells that carry out different functions; leukocytes are involved in immunity (both innate and acquired), erythrocytes are involved in oxygen and CO_2 transport, and platelets are responsible for clotting or wound healing. All these cell types are produced from one common source, which are haematopoietic stem cells (HSCs) that reside mainly in the BM.

Hematopoiesis initially occurs in the embryo during its development through erythroid progenitor cells in the extra-embryonic yolk sac [1,2]. These cells do not have self-renewal ability, but are important for transporting oxygen to the embryo. Later, in adult Hematopoiesis, erythroid myeloid progenitors (EMPs) are produced in the blood islands [3,4]. Eventually, adult Hematopoiesis involves multipotent HSCs that can produce all blood lineages of the adult organism. HSCs are created in the aorta-gonad-mesonephros (AGM) region of the developing embryo and subsequently migrate to the foetal liver and to the BM, where they remain in the adult organism [5]. From this moment on, HSCs are at the top of the developmental hierarchy of Hematopoiesis.

Sustaining Hematopoiesis throughout adult life needs to be accomplished by maintaining a balance between homoeostatic blood cell production, while ensuring the existence of an indefinite stem cell pool. This balance is accomplished in part through a state of dormancy (quiescence) in stem cells, but it can be quickly shifted when stem cells enter cell cycle as a response to an injury/stress or transplantation in order to regain homoeostasis [6]. HSC fate is extrinsically regulated by their microenvironment, termed as the "niche" by Schofield et al. in 1978 [7]. The 'niche' concept has evolved into an important aspect of our understanding of the haematopoietic system, as we now know it constitutes of multiple types of cells such as mesenchymal stromal cells, bone lining cells and adipocytes. Disruption of these 'niche' components often results in disease/malignancies [8].

Of mice and men

From a historic point of view, haematopoietic stem cell research was sparked by other scientific questions aimed to understand how damage to tissues from ionizing radiation (such as from atomic weapons) could be resolved. Transplantation of BM has been a treatment strategy for many leukaemia and solid tumour patients following chemotherapy, but it also serves as a therapeutic approach

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for metabolic and autoimmune diseases. The use of *in vivo* systems has been an important vessel to studying BM transplantation. In fact, understanding Hematopoiesis relied heavily on the use of such mouse bone marrow transplant (BMT) models, where BM stem cells from one mouse are transplanted into host mice after undergoing total body irradiation. A pioneering experiment performed in the early 50s showed that intravenous injection of BM cells rescued mice from radiation lethality [9] by renewing blood cell production [10]. Later, it was demonstrated that repopulation following cell engraftment occurs in a cell-number/dose-dependent fashion [11]. In the early 60s, clonal expansion of donated BM in irradiated host mice was demonstrated [12] by cytological analyses of spleen colonies which formed about 2 weeks post-transplantation. This is also how the quantitative readout for repopulating activity was discovered and named 'colony forming unit-spleen assay' [12].

Bone marrow transplantation versus native Hematopoiesis

The numerous experiments in HSC field that followed over the next 60 years used different experimental approaches and sometimes resulted in variable results with emerging complex hierarchical models of Hematopoiesis regulated by cell-intrinsic signals, as well as environmental cues. While BMT models advanced the field, their main caveat remains the irradiation needed to achieve space in the BM for transplanted cells, that when engrafting in the BM, encounter a very welcoming environment rich in cytokines and space, as opposed to the native BM environment in which cells compete in the niche. Such irradiation is perhaps relevant in T-ALL patients who undergo irradiation as part of their treatment, but not to the physiological or normal state of healthy BM. In addition, radiation not only depletes HSCs, but it damages the whole BM niche including damage to bone tissue, mesenchymal stem cells, extracellular matrix (collagen) [13], BM vasculature and particularly sinusoidal endothelial cells [14,15].

In a BMT model, BM cells are isolated from donor mice and stained for lineage markers (CD3, CD4, CD5, CD8, CD11b, B220, Ter119), c-Kit and Sca-1. The cells are then sorted by gating on a Lineage-negative (Lineage-) population that is also positive for cKit and Sca1 (cKit+Sca1+). In the field these cells are termed 'LSK cells' and with BMT approaches LSK cells are injected in the lateral tail vein of recipient mice that were lethally or sub-lethally irradiated. Therefore, BMT models are now generally perceived as 'injury' models where transplanted cells undergo stressful engraftment

and encounter a very different cytokine milieu to the one that exists under normal physiological conditions [16]. It has been demonstrated that sublethal and lethal irradiation lead to increases in pro-inflammatory cytokines such as IL-1 [17] and GM-CSF. Others have shown an increase in splenocyte-derived IL-17A following gamma-irradiation [18]. Another example following radiation is the increase in adipocytes presence in the BM, which are a rich source of stem cell factor (SCF) [19]. Therefore, stem cells that engraft in BMT models encounter an abundance of cytokines in the BM along with physical niche space and a different cellular composition of the niche.

In a landmark study, Sun J *et al.* explored new techniques with specific labelling of individual cells and their progeny *in vivo* [16]. These efforts and new approaches showed that non-transplant native Hematopoiesis is different from what had been concluded from BMT assays. BMT models supported the main dogma that multilineage Hematopoiesis is maintained by a small number of HSCs, by producing short-lived progenitor cells. The authors showed that long-lived progenitors, rather than the classical HSCs, are the main drivers of steady state Hematopoiesis [16]. These new advances in our understanding of the native (unperturbed) Hematopoiesis opened this field and inspired new discoveries.

Increased fitness in haematopoietic stem cells

The Ras guanine nucleotide releasing proteins (RasGRP) act as nucleotide exchange factors for Ras. RasGRP1, which is one of the members of this family with cell-specific expression patterns, fulfils an important role in T-cell regulation and development, but also in other cell types [20]. In our previous work we studied RasGRP1 in the context of T-cell acute lymphoblastic leukaemia (T-ALL). T-ALL is an aggressive malignancy of the BM affecting children and adults, where cure rate is less than 50% in the latter. We observed that patients with T-ALL often display abnormal levels of the Ras activator RasGRP1 [21]. Mechanistically, it has been unclear what role RasGRP1 plays in BM cells. Specifically, how overexpression of RasGRP1 may impact Hematopoiesis in the BM has never been studied.

To answer these questions, we created a new mouse model, *RoLoRiG*, allowing for inducible overexpression of RasGRP1 with an ires-GFP cassette in the targeted *Rosa26* locus, so that GFP reports on the genetic event of recombined LoxP sites as well as overexpression of RasGRP1 [22]. In this model, CRE is expressed from the IFN-α/β-inducible *Mx1* promoter by administration

of a single dose of polyinosinic-polycytidylic acid (pIpC) that leads to a genetic recombination event removing a stop codon (see Figure 1). In this work, we observed distinct outcomes for overexpression of RasGRP1 in our mice model when comparing non-transplant/native BM Hematopoiesis to BMT assays, where *RoLoRiG* BM cells are transplanted into irradiated mice. We demonstrated that through one genetic recombination event and consequent expression of RasGRP1-ires-GFP, GFP-positive cells in peripheral blood, spleen and BM increased over time. This was accompanied by splenomegaly and myeloproliferation in RoLoRiG mice spleens. GFP-positive cells outcompeted wild-type cells and dominated the peripheral blood compartment over time, and we showed that this effect originated in the BM (Figure 1).

In stark contrast, when investigated with a BMT approach, RasGRP1-ires-GFP expressing LSK stem cells did not outperform or dominate wild-type LSK cells; we observed similar percentage of LSK and other populations of stem cells in RoLoRiG and wildtype mice. Intriguingly, the increased fitness phenotype in HSCs with RasGRP1-ires-GFP was only observed in the native Hematopoiesis, and not in BMT assays into irradiated hosts.

When investigating the clonogenic capacity of progenitor cells from RoLoRiG mice, we noted a modest increase in total colony formation (CFU) and GM-CFU (Granulocyte macrophage CFU) in cytokine-rich medium. Since the BM niche is a competitive environment [23], we checked the clonogenic capacity in a medium containing limited growth factors to mimic the
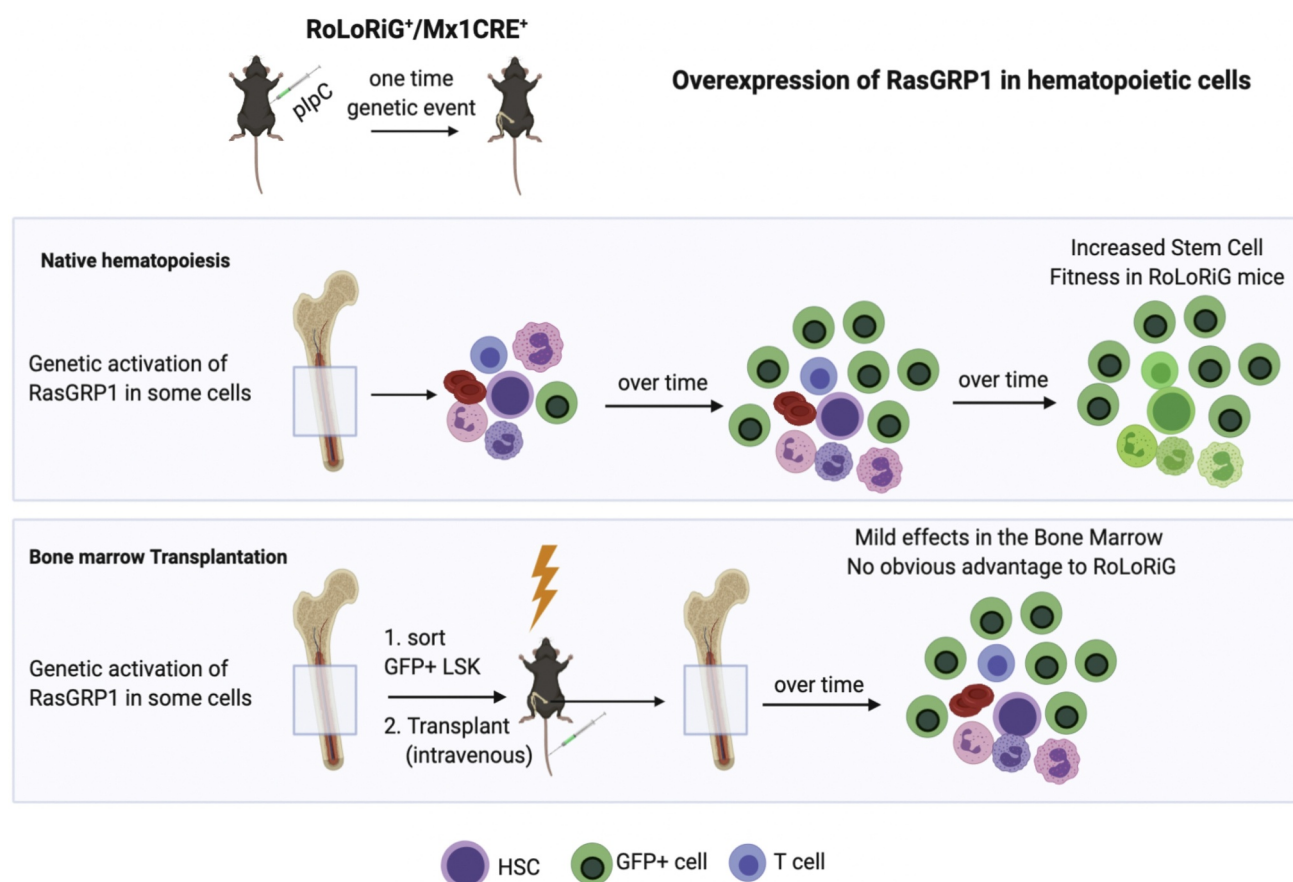

Figure 1. Overexpression of RasGRP1 bestows an advantage to stem cells in the context of native BM but not in BMT. Injection of our novel RoLoRiG mouse model with a single dose of polyinosinicpolycytidylic acid (pIpC) leads to Mx1CRE-mediated excision of the stop codon flanked by LoxP sites and expression of a RasGRP1-ires-GFP cassette. A single pIpC injection results in RasGRP1 overexpression in a portion of the haematopoietic cells in the BM in conjunction with GFP. Under conditions of native Hematopoiesis, RasGRP1-overexpressing stem cell display an increased fitness phenotype and, over time, GFP-positive cells fill the entire BM compartment. In native Hematopoiesis, an increase in most stem cell populations is evident in RoLoRiG-derived BM. By stark contrast, when a bone marrow transplantation (BMT) approach is used, RoLoRiG-derived stem cells (LSK cells) do not have any obvious advantage over wild type-derived LSK cells, and only mild effects are detected in the BM cell population.PI3K signals play important roles in Hematopoiesis (50, 51) and aberrantly elevated PI. Figure was generated using Biorender.com

physiological competition in the niche. While colonies from normal cells were not sustained in this condition [22], RASGRP1 overexpression bestowed gain-of-function colony formation properties to BM progenitors.

Altogether, we detected differences in our RoLoRiG model when cells were in a native versus BMT context. The benefit bestowed on RoLoRiG-derived BM cells in the CFU assay pointed out that these differences could be assigned to limited nutrients, cytokines and growth factors in the physiological and competitive niche of the BM.

Nutrients in the bone marrow and metabolic mTOR pathways

As mentioned earlier, HSCs are maintained and regulated throughout life by the BM niche. Under physiological homoeostatic conditions, HSCs remain in a quiescent state (G_0) with restricted cell proliferation. This state protects HSCs from genomic instability or metabolic stress while allowing for regeneration by re-entering the cell cycle as a response to normal, physiological stimuli [24]. The switch from quiescence to activated state is regulated by changes in the BM, such as decreased availability of factors that normally maintain quiescence, or increases in growth or stem cell-mobilizing cytokines; both changes can result in proliferation and differentiation of HSCs. Many cytokines in the BM regulate the stem cell niche; G-CSF, GM-CSF and stem cell factor are among the most investigated HSCs mobilizers [25].

Of interest, studies have revealed an important role for metabolic pathways in the regulation of stem cells function [26–29]. Disruption of these pathways could lead to pathologies such as cancer. In fact, we now know that nutritional requirements of the stem cells vary when switching from dormancy to a cycling state. Dormant HSCs have low energy and biosynthetic requirements compared to dividing cells [30,31]. Normally, oxygen levels in the BM niche are low, leading to the activation of hypoxia inducible factor 1 (HIF1-α). HSCs mostly rely on anaerobic respiration and glycolysis [32], and HIF1-α is one of the main activators of glycolysis in dormant HSCs, as it up-regulates glycolytic genes such as pyruvate dehydrogenase kinase (PDK) [32–34]. Of note, it has been shown that fatty acid oxidation also ties into fate decisions and self-renewal [35]. Nutrient readiness in the BM regulates nutrient stress-sensing pathways, directly affecting metabolic fate of the stem cells.

mTOR (mammalian target of rapamycin) is a critical integrator of metabolic cues and receptor signals (through the activity of mTORC1 [36,37]). Nutrients, as well as growth factors, affect protein translation, mitochondrial biogenesis and autophagy through mTOR activity [38]. mTOR is serine/threonine complex which can be divided into two: mTOR complex 1 (mTORC1) which regulates the ribosomal S6 kinase (S6K1), or mTOR complex 2 (mTORC2) which regulates AKT activation [39]. It is becoming widely accepted that activation of mTOR results in increased stem cell proliferation and differentiation [40–43], and over activation of this pathway in HSCs results in leukaemogenesis [44,45]. In fact, recent studies have shown a role for mTORC1-S6K1 in regulating the self-renewal of leukaemic stem cells [46].

We questioned whether the mechanism underlying the 'fitness' observed in our RoLoRiG mice could be via an increased signalling through mTOR/PI3K. First, we noted that baseline PI3K-AKT and mTORC1-S6 signals are relatively active in haematopoietic cells, as opposed to tonic Ras-ERK signals. Furthermore, when BM cells were directly analysed ex vivo, we observed increased mTOR-S6 signals at baseline in BM from RoLoRiG mice.

Metabolic pathways in native Hematopoiesis

Baseline signalling has remained a relatively understudied area of signal transduction and thus complete mechanistic and molecular understanding of baseline signals in native Hematopoiesis is lacking [16,47]. We believe that such studies may provide insights on how altered basal signals may play a role in the recently described declining Hematopoiesis with increasing age [48]. Additionally, systematic analysis of baseline PI3K-Akt and mTOR-S6 pathways can reveal new insights into leukaemogenesis.

PI3K signals play important roles in Hematopoiesis [49,50], and aberrantly elevated PI3K signals are common in murine and human T-ALLs [21,51–54]. We reported on a large screen looking for synthetic lethality in T-ALL between PI3K inhibition and other targets in a search for effective combination therapy [55]. When PTEN, a critical negative regulator of the PI3K pathway, is deleted via MxCRE1 this leads to increased mobilization of HSCs from the BM into the spleen, accompanied by splenomegaly and a concomitant decrease in HSCs in BM of PTEN-deficient mice [56,57].

mTOR is another interesting node in Hematopoiesis and leukaemia. TSC (Tuberous sclerosis) genes are negative regulators of mTOR signalling, and downregulation of TSC2 gene expression has been reported in BM samples of Acute Leukaemia patients [58]. Raptor is a critical

component of the mTORC1 complex and its deletion results in compromised *ex vivo* colony formation with small irregular colonies in complete, cytokine-supplemented media [45]. Deletion of Raptor alone with the Mx1CRE driver does not lead to altered BM progenitor cell frequencies or leukaemia, although increased splenic LSK cells and extramedullary Hematopoiesis has been reported [45]. Intriguingly, Raptor deletion reduces the T-ALL observed in PTEN deletion, arguing that mTORC1 is a critical effector in this leukaemia with elevated PI3K signalling [45,59]. Loss of Rictor, a component of mTORC2, has little effect on normal HSC function, but similarly to Raptor deletion, deletion of Rictor normalizes the HSC proliferation in PTEN null mice and also prevents the T-ALL [59,60]. Thus, the impacts of both mTORC1 and mTORC2 in Hematopoiesis versus leukaemia appear to hinge on other signals that feed into these two kinase complexes, such as increased PI3K signals via the loss of PTEN in leukaemia [59,60].

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Disclosure statement

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