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Hematopoietic stem cells under pressure

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Abstract

Purpose of review—Hematopoietic stem cells (HSCs) and progenitors are tasked with maintaining hematopoietic homeostasis in the face of numerous insults and challenges, including infection, inflammation and exsanguination. HSCs possess the remarkable ability to reconstitute the entire hematopoietic system of an organism whose own hematopoietic system has been ablated. This ability is exploited routinely in the clinic via HSC transplantation (HSCT). Here, we focus on the physiological and molecular bottlenecks overcome by HSCs during transplantation.

Recent findings—Upon transplantation, HSCs need to encounter a damaged bone marrow (BM) niche, characterized molecularly by increases in oxygen concentrations and an altered cytokine milieu. New mechanisms and pathways have been recently implicated during HSCT, including transplanted HSC-dependent secretion of conditioning molecules that facilitate engraftment and pathways that protect HSCs from perturbed organelle homeostasis.

Summary—Better understanding the molecular processes HSCs employ to withstand the stress of transplant will illuminate novel targets for further improving conditioning regimens and engraftment during HSCT.

Keywords

Hematopoietic Stem Cells; Bone Marrow transplantation; Bone Marrow reconstituting niche; oxidative stress

INTRODUCTION

HSCs are defined functionally by their ability to reconstitute hematopoiesis when transplanted into an organism whose own hematopoietic system has been ablated by irradiation, chemotherapy or disease. To preserve stemness and genome integrity, HSCs are quiescent and, in murine models, rarely divide during an individual's lifetime unless challenged by insults that stimulate them to proliferate and differentiate (*1). To reconstitute an ablated hematopoietic system, transplanted HSCs must overcome many damaging insults, such as oxidative stress and migration through a bone marrow niche physically damaged by conditioning, resulting in metabolic changes and exit from quiescence in HSCs. Despite all this, HSCT is a clinical success and is employed >50,000 times worldwide each year to treat

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Conflicts of interest.

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hematologic disease and cancer patients (2). Unfortunately, still about one-third of autologous or allogeneic transplant recipients will die for reasons other than primary disease relapse, such as infection, organ failure and Graft *vs.* Host Disease (G*v*HD) (3). A greater understanding of the molecular bottlenecks that stifle HSC function could illuminate novel therapeutic targets to improve clinical HSCT outcomes. Here, we will focus on reviewing our current understanding of the physical and molecular bottlenecks HSC must overcome during transplant to achieve stable engraftment and hematopoietic reconstitution.

A damaged niche

HSCT patients are usually pre-conditioned by chemotherapy and/or radiotherapy. The HSC BM niche is complex and multifaceted, with adipocytes, endothelial cells, megakaryocytes, heterogeneous stromal cells, macrophages, osteoblasts, and sympathetic nerves all implicated as functional contributors during homeostasis (4, 5). Transplant pre-conditioning disrupts some key components of the HSC BM niche, transforming it from a "BM homeostatic niche (h-Niche)" into what can be thought of as an acute "BM reconstituting niche (r-Niche)". For example, although osteoblasts appear minimally perturbed by conditioning (4), the sinusoidal vascular network is severely disrupted, becomes leaky, displays changes in morphology and structure, and swells. Genetic alterations of BM vascular endothelium integrity negatively impact HSC function (6**), highlighting the importance of intact vasculature for HSCs. The damaged vasculature takes weeks to recover and donor-derived hematopoietic cells, via the VEGF/VEGFR2 axis, have been implicated as participants in its recovery (4, 7). Gross changes in the levels and locations of cytokines critical to the viability and distribution of HSCs, such as SDF-1 (the Stromal Cell Derived Factor-1; also known as CXC chemokine ligand 12, CXCL12), VEGF, IGF-1, PDGF-BB, and TPO, have also been observed after conditioning (4, 5, 8). Changing CXCL12 levels following irradiation and the break-down of the sinusoidal vasculature results in redistribution of BM megakaryocytes from sinusoidal vessels to the endosteal surface; contributing to the distinct architecture of the r-Niche (4, 5, 9). Indeed, megakaryocytes facilitate BM injury recovery by producing FGF, which also stimulates HSC proliferation via the FGFR and up-regulation of NFκB and CXCR4, (the CXCL12 receptor and a master regulator of HSC trafficking and niche retention) (10). Conditioning regimens are also toxic to the nervous system, especially in children (11). During homeostasis, Schwann cell-derived TGFβ supports the integrity of the HSC pool (12, 13). Damage to BM glia by conditioning could deregulate HSC stores. Conditioning also alters the BM extracellular matrix in ways that functionally feedback on HSCs. Irradiation induces endothelial E-SELECTIN production via NFκB activation and TENASCIN-C production by stromal cells and endothelium (14, 15). Both of these molecules stimulate HSC homing, E-SELECTIN via E-SELECTIN-LIGAND-1 (ESL-1), which is expressed by HSCs, and TENASCIN C via INTEGRIN α9 (16). TENASCIN C/INTEGRIN α9 engagement can trigger HSCs to enter the cell-cycle by up-regulating *CyclinD1* and *CyclinE1* and down-regulating cyclin-dependent kinase inhibitors (*p57(Kip2), p21(Cip1), p16(Ink4a*) (14).

During inflammation or vascular damage, adenosine triphosphate (ATP), uridine triphosphate (UTP) and possibly other nucleotides are released into the extracellular environment (17). Most blood cells respond to nucleotides via purinergic P2 receptors (17).

P2Y14 is highly expressed by both human and mouse HSPCs (18*, 19). *P2y14*$^{-/-}$ HSCs display hyper-radiosensitivity due to their inability to regulate ROS levels that accumulate and induce senescence through p38 hyper-activation (19). Moreover, knockdown of *P2y14* in murine HSC comprises their engraftment (18*). These data suggest that P2Y14, and potentially additional purinergic receptors, might function as critical sensors of tissue damage by detecting elevated nucleotides in the r-Niche and promoting HSC survival by controlling increasing ROS levels post-transplant.

These studies and others establish that the r-Niche is physically and molecularly distinct from the h-Niche (20). Moreover, they suggest that the recovery of the r-Niche, and successful HSC engraftment, depends, in part, on transplanted hematopoietic cells, including HSPCs.

Functional HSC must home to the reconstituting niche (r-Niche)

To effectively reconstitute hematopoiesis, transplanted HSCs must both find their way to the BM and stably establish themselves within the r-Niche. HSC BM homing following transplant is rapid (hours to 1–2 days) and requires rolling, anchorage to the BM sinusoids, trans-endothelial migration and stable interaction with niche components (8, 21). CXCL12 is critically required for HSC migration from the fetal liver to the BM during embryogenesis (22). Antibody blocking of CXCR4 (by AMD-3100) or elevated CXCL12 plasma levels can mobilize HSPCs from the BM to the periphery (13, 23). CXCL12 is critical for BM HSC homing during transplant, where it is expressed by osteoblasts and endothelium (4, 24, 25). CXCL12 levels increase in the r-Niche after conditioning, which attracts HSCs and facilitates their stable engraftment (4, 8). Since many BM proteinases, such as Matrix Metalloproteinases 2/9 (MMP2 and MMP9), can cleave CXCL12 and negatively affect its activity as a chemo-attractant, it would be of interest to study their levels in a r-Niche. Modulating the activity of these proteinases could benefit current HSCT protocols (21, 26). Other chemo-attractants have also been implicated in directing the migration and mobilization of HSCs, including CCL2, CCL5, CXCL10, IL-8, SCF, LTD4, sphingosin-1-phosphate and ceramide-1 phosphate (21, 26–29).

Notably, conditioning by irradiation or cyclophosphamide results in elevated cleavage of COMPLEMENT COMPONENT3 (C3) into C3a and iC3b in PB and BM (30). HSC express C3aR and CR3 (also known as CD11b/CD18 or Mac-1), which are receptors for C3 cleavage fragments (30). C3a sensitizes human and mouse HSCs to CXCL12 gradients by promoting CXCR4 incorporation into membrane lipid rafts, while iC3b deposited on damaged BM stroma increases HSC adhesion to niche components via interaction with CR3 (30). Importantly, *C3*$^{-/-}$ mice are more sensitive to G-CSF mobilization (30). Although these mice have normal steady-state hematopoiesis, they display a delay in hematopoietic recovery when subjected to sub-lethal irradiation or HSC transplantation. *C3*$^{-/-}$ HSCs function normally when transplanted into *C3*$^{+/+}$ recipients, indicating that this phenotype results from a defect in the BM niche (30). Thus, cleaved C3 is important for BM regeneration and reconstitution following conditioning and may be an essential component of the r-Niche (30).

Both mouse and human HSC have also been shown to express multiple molecules that critically regulate their ability to physically engage r-Niche cells (such as the sinusoidal endothelium). For example, blocking or genetic loss of integrins, such as α4β1, α9β1, and α4β7, inhibit robust HSC engraftment (21, 31, 32). HSC rolling on the endothelium, necessary for extravasation into the BM space, is regulated by interactions with endothelial P and L-SECTININs (33). Antibody blocking of CD44 also blocks HSC engraftment (34). More recently, CYTOHESIN-1 and JAM-B/JAM-C interactions have also been implicated in HSC niche engagement (35, 36), as have factors that likely regulate to the ability to HSCs to physically move through the vasculature and BM, such as ARHGEF5, a Rho guanine nucleotide exchange factor important for podosome formation (37). Not surprisingly, knockdown of *Arhgef5* in murine HSPCs significantly impairs their transplantation (18*). Further, conditioning regimens can impact how HSC physically engage the r-Niche. For example, BM endothelium up-regulates MAdCAM-1, an α4β1 ligand, in response to total body irradiation (31). Blocking MAdCAM-1 in this context is highly detrimental to HSC engraftment (31).

HSC-directed niche conditioning facilitates engraftment

Evidence is accumulating that transplanted HSCs can themselves act on the r-Niche in ways that promote their own engraftment. For example, knockdown of secreted factors or molecules that regulate the biogenesis of secreted factors (*e.g. Fstl1*, *Crispld1*) in mouse HSPCs reduces their transplantation (18*). Although the mechanisms here are unknown, FSTL1 is a negative regulator BMP signaling that has been implicated in vasculature remodeling and ischemic cardiomyocyte regeneration (38, 39). HSCs express putative “niche-conditioning” molecules implicated in HSC quiescence, such as RNase ANGIOGENIN, IL-8 and EMBIGEN (40**). HSCs also express ESL-1, a regulator of HSC proliferative homeostasis via repression of TGFβ production by HSCs and other hematopoietic cells. ESL-1-deficient HSCs produce more TGFβ, which conditions the niche to induce quiescence in wild type co-transplanted HSCs (41*). Degrading enzymes, such as MMPs, are important for efficient HSC homing (8). Up-regulation of MMP-2, MMP-9 and MT1-MMP facilitates HSCT and BM homing by promoting extravasation via degradation of extra-cellular matrix components, enhancing migration towards CXCL12, and by releasing soluble Kit ligand from BM stroma (42). As mentioned, transplanted hematopoietic cells promote vasculature recovery after severe irradiation (43). Conversely, transplanted hematopoietic cells can also negatively influence r-Niche recovery: donor-derived angiopoietin-1 (ANGPT-1) slows vasculature recovery after irradiation, likely by negatively regulating endothelial cell proliferation (7). Thus, a better understanding of the reciprocal interactions between incoming HSCs and the r-Niche could illuminate novel strategies for improving engraftment.

Oxidative stress and changing metabolic needs

Steady-state HSCs are largely quiescent, depend on glycolysis for energy production, and, consequently, display low levels of radical oxygen species (ROS) (44-*46). The h-Niche is irrigated with a heterogeneous network of arterioles, which carry oxygenated blood and are most abundant near the endosteum, and sinusoids, which carry less oxygenated blood and

are abundant in the central BM (20, 47–49). This creates an oxygen gradient in the BM, from ≈4% O_2 near the endosteum to ≈2% in the central marrow (50, 51). However, the distribution of HSCs throughout the BM suggests that HSC maintain a hypoxic profile regardless of their location and external O_2 tension (24, 47, 48, 50–52). Although it is formally possible that super-low O_2 levels might exist in tight regions proximal to HSCs (50).

Transplant pre-conditioning, and the resulting cell damage, elevates BM O2 levels (51). Transplanted HSPCs distribute throughout this landscape of elevated oxygen (51). Under low oxygen, HIF-1α is active and transcriptionally promotes glycolysis. As O_2 levels rise, HIF-1α is targeted for degradation, promoting a shift in HSC metabolism from glycolysis to oxidative phosphorylation (OXPHOS), which further increases internal ROS levels (44, 45). Increased ROS impairs HSC self-renewal, quiescence, and promotes their mobilization from the bone marrow (53, 54). Indeed, purified ROS^{Low} HSCs display superior repopulating activity relative to ROS^{High} HSCs (44). HSCs also become exposed to supra-physiologic levels of oxygen during their isolation and handling prior to transplant (55**). Inhibition of ROS accumulation by anti-oxidants such as N-acetyl-L-cysteine (NAC) or via manipulation of signaling pathways linked to ROS accumulation (p38 MAPK, the miR-212/132 cluster (*Mirc19*) or SIRT3 overexpression) rescues HSC function and transplantation (54, 56–58). Thus, HSC must engage molecular pathways to resolve these insults in order to achieve stable engraftment and hematopoietic repopulation. Indeed, a failure to transition from glycolysis into OXPHOS blocks HSC differentiation (59, **60), indicating that is required for effective hematopoietic reconstitution. Further, constitutive mTOR activation, a master sensor of cellular metabolic needs, is detrimental to HSC quiescence and serial repopulation, which appears to be in part explained by elevated ROS (61, 62). Moreover, the PML-PPARδ pathway for fatty acid oxidation regulates asymmetric versus symmetric HSC division and disruption of this pathway leads HSC exhaustion (53).

SIRT1, a deacetylase that globally coordinates metabolic changes in response to nutrient levels, is also required for optimal HSC transplantation (63). SIRT1 functions upstream of FOXO3 in HSCs to modulate changing intracellular ROS levels (**60, 63). Indeed, FOXO factors are well known critical regulators of HSC ROS, both during homeostasis and transplantation (**60, 64). While *FoxO*-deficient bone marrow lacks long-term engraftment, *in vivo* treatment with NAC reverts this phenotype (65). Similarly, $Foxa3^{-/-}$ HSCs display high ROS post-transplant and compromised serial transplantation (18*). A SIRT1-related enzyme, SIRT3, is also required for optimal HSC transplantation, but only in aged HSC (58). SIRT3 regulates the acetylation of mitochondrial proteins (65). Thus, to effectively repopulate, transplanted HSCs must affect a balance between their shifting metabolic needs and the detrimental effects of elevated ROS on their self-renewal and differentiation.

Epigenetic regulators

Recently, several studies have illuminated single cell heterogeneity within the HSC pool and suggested a model where HSC function is transplantable and imprinted by specific epigenetic patterns, such as DNA methylation, hydroxymethylation, and histone modification (66, *67, 68). Transplantation may perturb the HSC epigenetic landscape. For

example, as HSCs switch from glycolysis to OXPHOS during transplant, increasing α-ketoglutarate and decreasing succinate may activate histone demethylases and trigger differentiation (65). The compromised differentiation potential, enhanced self-renewal, and sensitization to apoptosis of HSC deficient in *Dnmt1* or *Dnmt3a* demonstrates that DNA methylation levels can significantly impact HSC function (69, 70). Active DNA de-methylation also perturbs HSC transplantation. TET family proteins hydroxylate 5-methylcytosine to 5-hydroxymethylcytosine, which is then deaminated by AID/APOBEC proteins before processed into cytosine by BER glycosylases. *Tet2*$^{-/-}$ mice display increased HSC numbers and increased HSC repopulating activity (71). In contrast, *Aid*$^{-/-}$ mice display expansion of myeloid cells and anemia due to reduced erythroid progenitors, but display normal HSC self-renewal (72). Histone modification patterns also appear important for HSC transplantation. Loss of EED, which methylates H3K27 as part of the Polycomb Repressive Complex 2, results in HSC exhaustion (66, 73). In contrast, *Ezh2* overexpression perpetuates HSC serial transplantation (74). HSCs also display bivalent domains that contain H3K4me3 and H3K27me3 (75). To what extent the epigenetic landscape of HSC is altered by transplantation remains an open question.

Transplant challenges organelle homeostasis

Recent data suggests that transplanted HSCs must cope with perturbations in organelle homeostasis. For example, the essential autophagy gene, *Atg7*, is required for HSC repopulation (76). Further, as transplanted HSC exit quiescence and increase oxidative phosphorylation, mitochondria numbers increase (44, 55**, 77). This activates mitochondrial pathways regulating oxidative stress, such as SIRT1, a deacetylase that targets FOXO transcription factors in HSCs (**60). Loss of SIRT7, a regulator of the mitochondrial unfolded protein response, result in reduced repopulating activity, reflecting the dependence of transplanted HSCs on this pathway (**78).

Oxidative stress, accumulating mis-folded proteins, or calcium disequilibrium can induce endoplasmic reticulum (ER) stress (79). Glucose-regulated protein 78 (GPR78), an ER chaperone, regulates and inactivates multiple ER stress sensors (80). *Gpr78*-deficient mice show a loss in HSCs (81). The ER unfolded protein response (UPR) is resolved by inhibition of translation, activation of ubiquitin-dependent degradation of mis-folded proteins or by increased ER biogenesis (79). Indeed, overexpression of ERDJ4 (a canonical UPR chaperone) in human HSCs enhances their repopulating activity, indicating that ERDJ4 protects against transplant-induced ER stress (**82). Further, human HSPCs display higher expression of PERK pathway members and decreased expression of IRE1, suggesting that HSCs depend on this ER stress response pathway (**82).

Conclusion

Classic pre-transplant conditioning triggers a plethora of cellular responses that cumulatively select for the small population of bone marrow cells we know as transplantable HSCs. These cellular responses likely exist to counteract environmental insults to the hematopoietic system (*e.g.* infection, exsanguination, starvation, etc…) by allowing HSC division and activation while safeguarding genome integrity and stemness. Although the molecular

mechanisms behind many of these responses have been recently illuminated, we are still far from fully understanding HSC engraftment. Indeed, numerous novel alternative conditioning methods reveal that HSCT does not depend on complete bone marrow ablation (*e.g.* dietary Valine depletion, anti-c-Kit or CD45 antibodies, CD45-saporin, inhibition of stromal cell heparan sulfate synthesis and E-SELECTIN antagonists (**83,84). These new methods hold the promise of alleviating the non-hematopoietic toxicity associated with classic conditioning regimens, which will be especially important for children and young adults. Further, each likely imposes unique molecular pressures on transplanted HSCs. Further study of these alternative conditioning regimens will yield new insight into additional molecular bottlenecks that stifle transplanted HSC.

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KEY POINTS

- Transplant conditioning regimens induce dramatic changes in the HSC bone marrow niche.
- Transplanted HSCs are subject to significant metabolic changes, perturbed organelle homeostasis, and elevated ROS levels.
- HSCs autonomously condition their new home to facilitate engraftment.

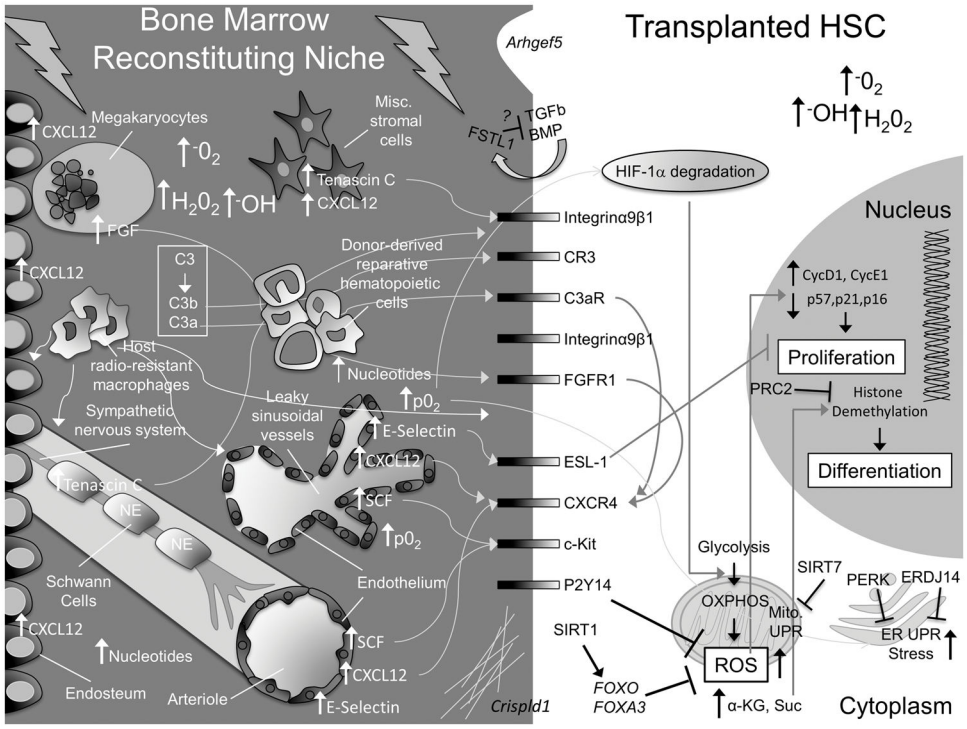

Figure 1. Summary of molecular alterations driven by classical pre-transplant conditioning regimens in HSCs and the bone marrow niche

Here, we present a schematic to highlight some of the gross physical and molecular changes that occur in the bone marrow niche and within HSCs. For simplicity, not every known cellular component of the niche is pictured. In the niche, C3 is cleaved to C3b and C3a, which interact with HSC CR3 and C3aR receptors and stimulate homing by increasing, among other things, CXCR4. Megakaryocytes, which are attracted to the endosteum from sinusoidal vessels by increasing endosteal-CXCL12, also upregulate CXCR4 on HSCs via increased secretion of FGF. Schwann cells and stromal cells release TENASCIN C, which stimulates HSC migration and adhesion. Endothelial cells upregulate E-SELECTIN, CXCL12 and SCF. Sinusoidal vessels are damaged and leaky, resulting in an increase in O_2 partial pressure (pO_2) and BM ROS levels. This contributes to H1F-1α degradation in HSCs, promoting their transition from glycolysis to oxidative respiration (OXPHOS), which further increases intracellular ROS levels. FOXOs, FOXA3, and signaling downstream of P2Y14 help HSCs cope with rising ROS levels. SIRT1 activates FOXOs. SIRT7 inhibits the increase in the mitochondrial unfolded protein response (UPR). Increased ROS stimulates HSC division and an ER-UPR. PERK and ERDJ14 counteract this effect in transplanted HSCs. Free nucleotide levels rise in the BM and are sensed by purinergic receptors, like P2Y14, which regulates ROS. Increasing intracellular α-Ketoglutarate (α-KG) promotes HSC differentiation via Histone demethylation. PRC2 complex counteracts this effect by promoting Histone methylation. Transplanted HSCs condition the reconstituting niche by secreting FSTL1 and extracellular matrix components (via *Crispld1*) and (very likely) additional factors (*e.g.* IL-8). Transplanted hematopoietic cells facilitate recovery of the conditioned niche. Figure Key: the bone marrow space is depicted on a dark gray background, the HSC intracellular space is light gray, and the HSC nucleus is dark gray.

Major cell types are labeled in white font, major changes in the bone marrow space are labeled in white font, and major changes in the HSC are labeled in black font.