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Cutting the brakes on hematopoietic regeneration by blocking TGFβ to limit chemotherapy-induced myelosuppression

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Abbreviations: Cdkn1c, cyclin-dependent kinase inhibitor 1c; CXCL12, C-X-C motif chemokine 12; GCSF, granulocyte colony-stimulating factor; Grp78, glucose-regulated protein, 78 kDa; GvHD, graft versus host disease; HLA, human leukocyte antigen; KITL, cKit-ligand; LAP, latency-associated peptide; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor kappa-light chain enhancer of activated B cells; PI3K, phosphoinositide 3-kinase; PKB, Protein kinase B; Poly(I:C), polyinosinic-polycytidylic acid; SDF-1, stromal cell-derived factor-1; Smurf2, SMAD specific E3 ubiquitin protein ligase 2; SPARC, secreted protein acidic and rich cysteine; Stat1, signal transducer and activator of transcription 1; TAK1, transforming growth factor β activated kinase 1; TPO or THPO, thrombopoietin; TRIF, TIR domain containing adapter inducing IFN-β.

Hematopoietic stressors such as infection, bleeding, or toxic injury trigger a hematopoietic adaptation that sacrifices hematopoietic stem and progenitor cell (HSPC) quiescence to meet an urgent need for new blood cell production. Once the hematopoietic demands are adequately met, homeostasis must be restored. Transforming growth factor β (TGFβ) signaling is a central mediator mandating the return of HSPCs to quiescence after stress. Blockade of TGFβ signaling after hematopoietic stress delays the return of cycling HSPCs to quiescence and in so doing promotes hematopoietic stem cell (HSC) self-renewal and accelerates hematopoietic reconstitution. These findings open the door to new therapeutics that modulate the hematopoietic adaptation to stress. In this review, we will discuss the complex context-dependent activities of TGFβ in hematopoiesis and the potential benefits and limitations of using TGFβ pathway inhibitors to promote multilineage hematopoietic reconstitution after myelosuppressive chemotherapy.

Introduction

Most hematopoietic stem cells (HSCs) are deeply quiescent but a small fraction exit G_0 to prime hematopoietic replacement of daily blood cell loss.[1,2] The signals that induce select HSCs to emerge from quiescence during homeostasis are incompletely understood and may be partly stochastic. Active HSCs contribute to hematopoiesis for variable periods and then return to dormancy.[3,4] However, things change when hematopoietic production must be urgently increased because of overwhelming infection, significant bleeding, or other causes of profound cytopenia such as myelotoxic chemotherapy or HSC transplantation. During these periods of stress, most HSCs are rapidly recruited into the cell cycle and undergo extensive self-renewal and differentiation to meet the new hematopoietic demands. Evolutionary pressures have apparently selected this "demand" hematopoietic mode as a necessary adaptation to promote survival by allowing a rapid response to acute stresses. However, unrestricted HSC cycling can lead to HSC exhaustion and hematopoietic failure.[5-8] Therefore, it is likely that evolution has also advanced mechanisms to restrict the duration of demand hematopoiesis as a means to safeguard HSCs.

A great deal is known about how hematopoietic stem and progenitor cells (HSPCs) are activated during hematopoietic stress.[9-11] But how is homeostasis restored when the stress is over? Curiously, until very recently nothing was known about how these processes wind down to allow HSCs to withdraw from the cell cycle and return to quiescence. Indeed, the *de facto* paradigm has been that homeostasis is passively re-established as stress mediators normalize. But this is a bit like driving with only a gas pedal to control velocity: fine if you want to accelerate but potentially disastrous if you need to slow down. Recently, this paradigm has been challenged. Researchers found that steady-state hematopoiesis is actively re-imposed during stress recovery and that transforming growth factor β (TGFβ) is a central mediator of this

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process.[1] Context-dependent blockade of TGFβ signaling during recovery from hematopoietic stress prolongs HSPC cycling and can augment blood count recovery from cytopenias caused by hemolysis, HSC transplantation (HSCT), or myelotoxic injury.[1,12] This finding is potentially useful because it suggests that TGFβ pathway inhibitors could be used to promote multilineage hematopoietic regeneration after myelosuppressive chemotherapy or HSCT.

Myelosuppression is among the most common life-threatening complications of cancer treatment and limits the tolerability of antineoplastic therapy. Insights from prior work defining how hematopoietic stress is activated have led to the development of a large panel of molecules that are now used to promote unilineage hematopoiesis (e.g., granulocyte colony-stimulating factor [G-CSF], erythropoietin, and thrombopoietin mimetics) and HSPC mobilization (e.g., C-X-C chemokine receptor type 4 [CXCR4] blockade with plerixafor). However, these agents have narrow activity. G-CSF is commonly used to promote granulocytic recovery after chemotherapy[13] but it does not help with dose-limiting thrombocytopenia and symptomatic anemia. The other available unilineage cytokines such as the erythroid stimulating agents (ESAs) and thrombopoietin (THPO) mimetics are less commonly used to treat myelosuppression and some risks have been identified.[14] For these reasons, blood product transfusions remain a cornerstone of supportive therapy after myelosuppressive chemotherapy or HSCT. However, transfusions are surprisingly expensive and carry the risk of severe reactions and transmission of infectious agents. New approaches are needed to promote hematopoietic regeneration after transplantation or myelotoxicity.

Only recently have we begun to understand how homeostasis is restored after hematopoietic stress. These new insights promise novel agents that promote hematopoietic regeneration by blocking the counter-regulatory signals restricting recovery rather than trying to overdrive recovery using supraphysiologic levels of unilineage cytokines. As our understanding of hematopoietic adaptation to stress improves, new approaches can be developed to promote multilineage hematopoietic regeneration without sacrificing long-term hematopoietic function.

In this review, we will discuss demand hematopoiesis with a particular focus on the context-dependent activity of TGFβ as a mediator that limits the duration of HSC activation. We also discuss the potential benefits and possible limitations of using TGFβ pathway inhibitors to promote multilineage hematopoietic reconstitution after chemotherapy-induced myelosuppression.

Context-Dependent Hematopoietic Adaptation to Hematologic Stress

At steady state, most HSCs are maintained in a deeply quiescent state[15,16] by paracrine factors produced by specialized bone marrow niche cells.[4,17] Yet evolution demands a rapid hematopoietic response to stressors. These triggers set off a remarkable adaptation in hematopoiesis that sacrifices HSPC quiescence to meet an urgent need for new blood cell production. The signals that awaken hibernating HSCs and activate and mobilize HSPCs during these periods of stress have been well studied.[9,11,18] Proteolytic enzymes such as matrix metallopeptidase 9 (MMP-9), cathepsin G, and elastase cleave the chemokines (e.g., CXCL12), cytokines (e.g., KITL), and adhesive interactions that retain HSCs in the niche and maintain their quiescence.[19-22] Circulating cytokine levels increase in response to cytopenias, tissue injury, and inflammation and this reinforces HSPC proliferation.[11,23]

Most mature effector cells of the innate immune system are post-mitotic and must be continually produced by bone marrow HSPCs. Severe infections consume effector cells and require emergency hematopoiesis to replenish the losses. Many signals are known to trigger increased hematopoietic output. Cytokines produced by immune cells and non-hematopoietic tissues play a central role in the stimulation of hematopoiesis during infection, often skewing differentiation toward myeloid lineages at the expense of lymphopoiesis.[21,24-26]

Inflammatory cytokines are known to act on mature effector cells and hematopoietic progenitors to support the fight against pathogens. Yet HSCs are also directly and indirectly affected by the surge of cytokines during infection. G-CSF levels increase acutely during bacterial and fungal infections to support the differentiation of mature granulocytes.[27] However, G-CSF also mobilizes HSCs from the bone marrow by triggering cleavage of membrane-bound CXCL12 (SDF1) and other factors that retain HSPCs in the niche and maintain their quiescence.[21,28] HSCs can also directly respond to inflammatory cytokines. For example, HSCs express receptors for type 1 and type 2 interferons that are induced during certain viral and chronic bacterial infections. Poly(I:C) provokes interferon-α (INF-α, Ifna) production and is known to induce HSC mobilization and recruitment into the cell cycle *via* activation of INF-α receptor (Ifnar1) and downstream Stat1/Pkb/Akt signaling in HSCs.[25,29] Interferon-γ (INF-γ, Ifng) signaling, which is induced by chronic *Mycobacterium avium* infection, also triggers HSC proliferation via its receptor, Ifngr1, and downstream Stat1-mediated signaling.[24] Successful eradication of infections requires coordinated activity of multiple cytokines.

Cytotoxic T cells (CTLs) secrete INF-γ during acute viral infections and have been recently shown to stimulate myelopoiesis by inducing non-hematopoietic bone marrow cells, possibly mesenchymal stem cells (MSCs), to release hematopoietic cytokines including interleukin 6 (IL-6).[30] In turn, IL-6 stimulates HSPCs to proliferate and differentiate with a bias toward myelopoiesis.[30] Similarly, elevated levels of M-CSF can also direct HSC differentiation toward myelopoiesis.[26] Such complex interplay between the adaptive and innate immune system is necessary to successfully eradicate infections, and the cytokine networks sculpting the immune responses act on mature effector cells, hematopoietic progenitors, and HSCs.

HSCs also express receptors that allow them to directly sense certain infections. For example, HSCs express the Toll-like receptors TLR-2 and TLR-4 to detect and respond to lipopolysaccharide (LPS), an outer membrane component of all Gram-negative bacteria.[23,31] LPS induces quiescent HSCs to enter the cell cycle *in vitro* and *in vivo*.[1,3,11,23] Expression

of TLRs is of course not restricted to HSCs, and it is recognized that TLR activation is a strong inducer of cytokine production by mature effector cells. Recently, however, researchers have found that LPS activation of TLR4 stimulates an outpouring of inflammatory cytokines by multipotent progenitors (MPPs) and short-term HSCs (ST-HSCs) that, on a per cell basis, far exceeds production by mature effector cells.[23] This regional cytokine storm is mediated by NF-κB signaling and counter-regulated by miR-146a. Of the cytokines tested, IL-6 secreted by HSPCs was again found to be the most potent inducer of myelopoiesis during endotoxin-mediated stress. These recent studies provide new insight into the inflammatory machinery that allows bone marrow HSPCs to sense and rapidly respond to acute infections.[18]

Significant cytopenias caused by acute blood loss, immunologic destruction of mature blood cells, or myelosuppressive treatments trigger demand hematopoiesis *via* mechanisms that partially overlap the adaptive responses to severe infections. Cytopenias can directly elevate the plasma level of key cytokines such as thrombopoietin (THPO) and G-CSF because they are cleared from the circulation by mature platelets and neutrophils.[32] Acute cytopenias are also commonly associated with inflammatory signals and activation of key proteases within the bone marrow.[19,33] G-CSF induces HSC proliferation not just by interfering with signaling from CXCL12 and KITL but also partly *via* activation of TLR4/TRIF signaling, thereby merging the mechanism of response to chemotherapy-induced myelosuppression with emergency myelopoiesis linked to infection.[21] Adding further complexity, signaling during demand hematopoiesis can differ from that in homeostasis. Acute hemolysis is associated with stereotyped alterations in erythropoiesis[34,35] and is also known to recruit dormant HSCs to begin active proliferation via mechanisms that have not yet been defined.[1] This makes it difficult to attribute singular functions to individual cytokines because stress hematopoiesis is a composite of many signals interacting in fluid contexts (**Fig. 1**).

These findings also show that many hematopoietic stressors lead to grossly similar consequences: recruitment of dormant

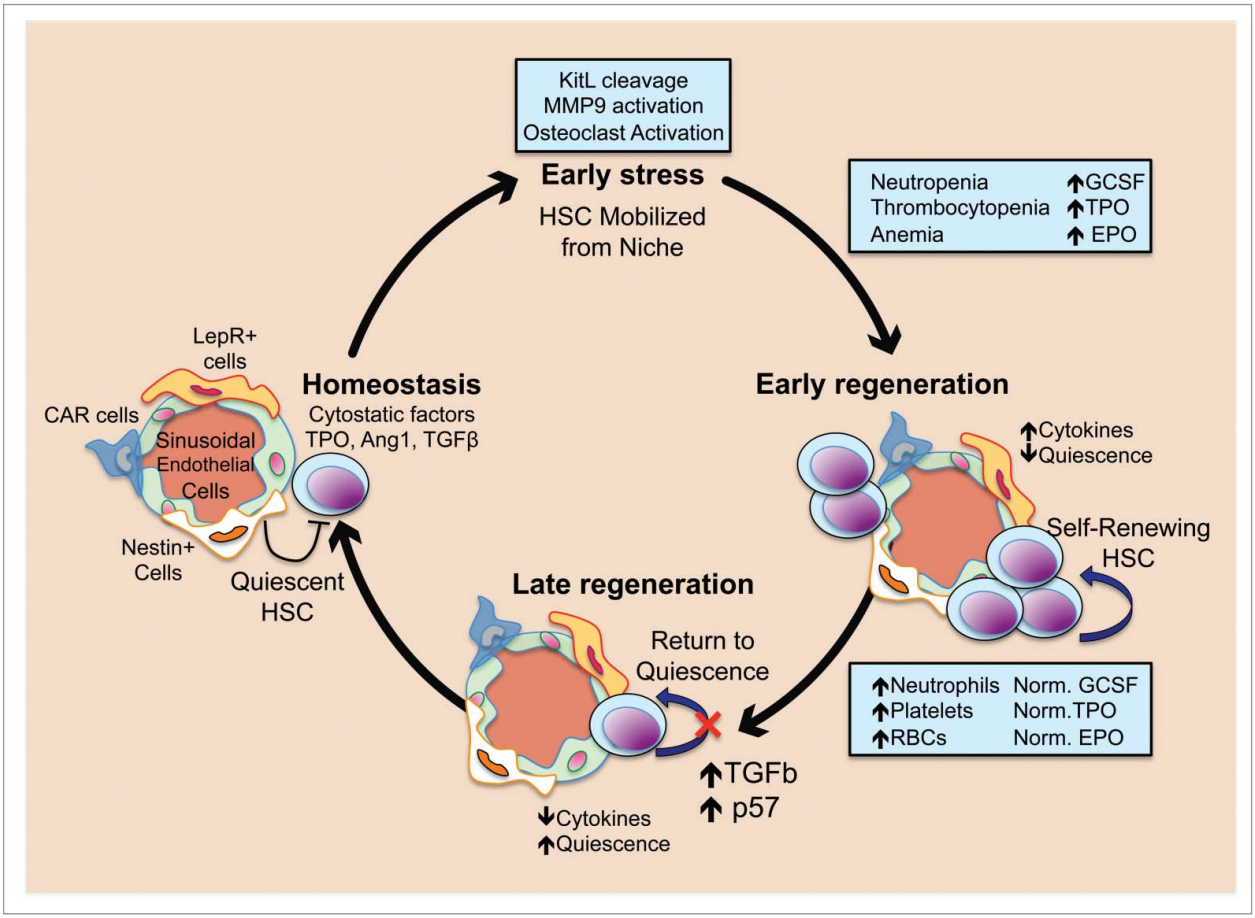

Figure 1. The lifecycle of hematopoietic stem cell quiescence. (Homeostasis) Niche factors maintain most hematopoietic stem cells (HSCs) in a quiescent state. (Early Stress) HSCs are mobilized from the niche and begin to cycle actively. (Early Regeneration) Circulating cytokine levels increase in response to cytopenia, tissue injury, and inflammation and this reinforces hematopoietic stem and progenitor cell (HSPC) proliferation. HSPCs continue actively cycling to repopulate the bone marrow. (Late Regeneration) HSPC quiescence is re-imposed and bone marrow homeostasis is restored. [CXCL2-abundant reticular (CAR) cells, leptin receptor ($Lepr^{+}$)-expressing perivascular cells, $Nestin^{+}$ mesenchymal stem cells (MSC)].

HSCs into the cell cycle; self-renewal, differentiation, and mobilization of HSPCs from bone marrow niches; and myeloid biased differentiation. Yet all of these prior studies have focused upon the initiating signals triggering demand hematopoiesis. The return to homeostasis after the major stress is over has only recently been studied.

TGFβ Signaling: A Pleomorphic System Regulated at Multiple Levels

Canonical TGFβ signaling

TGFβ is a potent growth inhibitor of epithelial, endothelial, neuronal, hematopoietic, and immune cells and performs important functions in normal tissue homeostasis.[36] The TGFβ superfamily is comprised of more than 30 closely related proteins including bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), activins, inhibins, nodal, and the 3 TGFβ isoforms that have distinct expression patterns and biologic activities.[37,38] Of these, TGFβ1 is the most highly expressed by immature hematopoietic cells and has the best-characterized activity in hematopoiesis.[39]

Canonical TGFβ signaling has been well reviewed elsewhere.[40] Active TGFβ1 binds to the high-affinity type II receptor (TβRII, Tgfbr2), inducing a heterotetrameric complex with TGFβ type I receptor (TβR1, ALK5) and leading *via* trans-phosphorylation to the recruitment and phosphorylation of Smad2

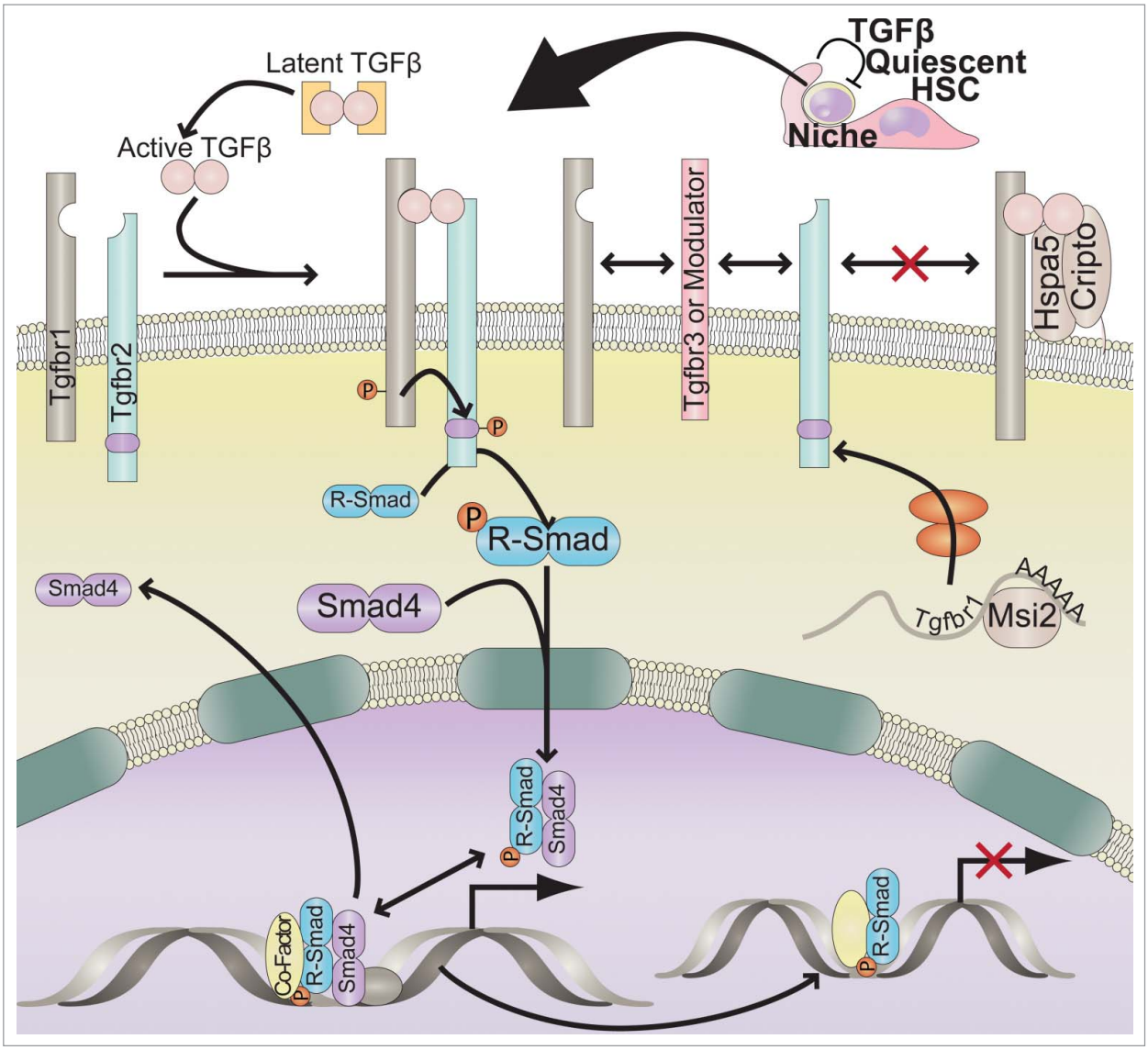

Figure 2. Schematic of TGFβ signaling. Transforming growth factor β (TGFβ) is synthesized in a latent form that is incapable of interacting with receptors. After secretion and activation, TGFβ interacts with Tgfbr2 and Tgfbr1, initiating serine phosphorylation and activation of Tgfbr1. Tgfbr1 then phosphorylates receptor activated Smads (R-Smads) such as Smad2 and Smad3. Phosphorylated R-Smads can then hetero-oligomerize with co-Smads (e.g., Smad4) and translocate to the nucleus where they interact with cofactors to induce transcription. Signaling wanes as a result of adaptation that tracks with the nuclear localization of Smad4. TGFβ receptor function can be modulated by accessory receptors such as Tgfbr3 and Endoglin and through interactions with modulators such as Cripto. The RNA binding protein Msi2 helps control Tgfbr1 mRNA and TGFβ pathway signaling.

and Smad3 in most cell types (**Fig. 2**). Smad proteins activated by phosphorylation heterodimerize with the common mediator Smad4, and the resulting complex translocates to the nucleus and recruits transcriptional cofactors to control expression of target genes.

Constraining the spatial activation of TGFβ signaling

TGFβ is secreted as a biologically inert "latent" protein incapable of signaling. Although many cell types produce TGFβ, it is secreted in non-covalent association with the latency-associated peptide (LAP) that prevents it from binding to TGFβ receptors. In turn, LAP interacts with members of the latent TGFβ-binding protein family (LTBP) that can moor the large latent complex in the extracellular matrix. LTBPs influence the release of TGFβ from LAP—a process called activation—to allow TGFβ mediated signaling *via* cell surface TGFβ receptors.[41] Latent TGFβ is activated by several mechanisms. LAP can be shed after cleavage by MMPs or plasmin, or through conformational changes induced by reactive oxygen species or adhesive interactions with thrombospondin-1 (TSP1) and integrins (e.g., αvβ6 and αvβ8).[42-46] It is instructive that all of the known mechanisms for activating latent TGFβ act locally, suggesting that TGFβ signaling likely conforms to juxtacrine or paracrine models.

Large quantities of latent TGFβ are incorporated into bone matrix and are found in the granular contents of megakaryocytes and platelets.[47] Nonetheless, few bone marrow cells show significant TGFβ signaling during steady-state hematopoiesis, suggesting that critical aspects of this signaling are regulated by the availability of active TGFβ to its cellular receptors. In addition, cells that stain for phospho-Smad2 signaling are typically adjacent to cells manifesting no TGFβ signaling, indicating that the mechanisms of TGFβ activation are spatially constrained.[1,48] These results suggest that highly specific TGFβ inhibitors could be designed if the mechanism for localized TGFβ ligand activation were known.

Constraining the temporality of TGFβ signaling

Negative feedback mechanisms limit the duration of TGFβ signaling by restricting receptor expression, transmembrane signaling, nuclear transit of mediators, and their transcriptional activity. At the cell surface, TGFβ occupancy of receptors initiates their internalization with consequent recycling or ubiquitin-mediated degradation.[49-51] The TGFβ target Smad7 feeds back to block activation of Smad2/3 *via* the type I receptor (Tgfbr1) and pairs with Smurf2 to trigger degradation of this receptor.[51,52] Other TGFβ target genes such as Ski and SnoN disrupt the transcriptional activity of intranuclear Smad2/3/4.[53] As a result of these feedback mechanisms, or others that have not yet been defined, TGFβ signaling is temporally constrained and appears to be most responsive to changes in TGFβ ligand availability rather than the total amount of active TGFβ.[54]

Modulating TGFβ signaling

By pre-receptor/receptor signaling

TGFβ receptor function can also be modulated by accessory receptors such as endoglin (Eng). Endoglin is expressed in a subset of HSCs and is upregulated during hematopoietic stress[55] but it is not known how endoglin affects TGFβ signaling in adult HSCs. In endothelial cells, endoglin directs TβRII to signal *via* ALK1 rather than ALK5.[56] By re-partnering TGFβ receptors, endoglin abrogates the cytostatic response of TGFβ mediated by ALK5-phosphorylated Smad2/3, and activates a more proliferative/invasive program mediated by ALK1-activated Smad1/5. Endoglin is not the only TGFβ modulator that is differentially expressed in HSPCs. Among partially understood signaling modulators, Grp78 (Hspa5), the receptor for the TGFβ pathway modifier CRIPTO (Tdgf1), distinguishes deeply quiescent HSCs during homeostasis[57,58] whereas the membrane protein GARP (glycoprotein A repetitions predominant, Lrrc32) serves as a membrane reservoir of latent TGFβ.[59]

Non-canonical TGFβ signaling

TGFβ signaling independent of Smad activation *via* TAK1/MAPK, Rho-like GTPase, and PI3K/AKT pathways is well described.[60-62] This non-canonical intracellular TGFβ signaling can oppose the cytostatic activity of TGFβ to promote motility, invasion, metastasis, and epithelial to mesenchymal transition (EMT) and is particularly well described in malignant cells.[63] Much of the non-canonical signaling is mediated by Tgfbr2 and can occur independent of the type I receptor. Very little is known about how these alternative signaling pathways affect the outcome of TGFβ signaling in HSCs and it is possible that TGFβ ligand traps and inhibitors specific for the type I or type II receptor could have different effects.

TGFβ Signaling Triggers the Return to Homeostasis

Hematopoietic stress sacrifices HSPC quiescence to meet increased hematopoietic demands. Once these demands have been adequately met, homeostasis must be restored. It has long been assumed that this is a passive process with homeostasis returning as stress mediators normalize, but researchers have recently found that this assumption is incorrect.

The first evidence that homeostasis could be actively reimposed emerged from timed studies of HSC cell cycle during recovery from myelosuppressive chemotherapy with 5-fluorouracil (5FU). Most HSCs rapidly emerge from quiescence after 5FU treatment and extensively proliferate for almost 2 weeks (**Fig. 3**) but then abruptly return to quiescence just as bone marrow cellularity has recovered and the blood counts normalize.[1] A similarly rapid return to quiescence was also seen during recovery from acute hemolysis (modeled using phenylhydrazine) or sepsis (modeled by LPS). It was later found that TGFβ signaling is a central mediator mandating the return of HSPCs to quiescence after stress. During late hematopoietic regeneration, as homeostasis is restored, the level of active TGFβ spikes in whole bone marrow and downstream signaling (as reported by Smad2 phosphorylation) increases in hematopoietic stem and progenitor cells (HSPCs), thus limiting HSC self-renewal.

TGFβ blockade using a neutralizing antibody (Genzyme, 1D11) or a small molecule inhibitor of TβRI (Lily, LY2157299)

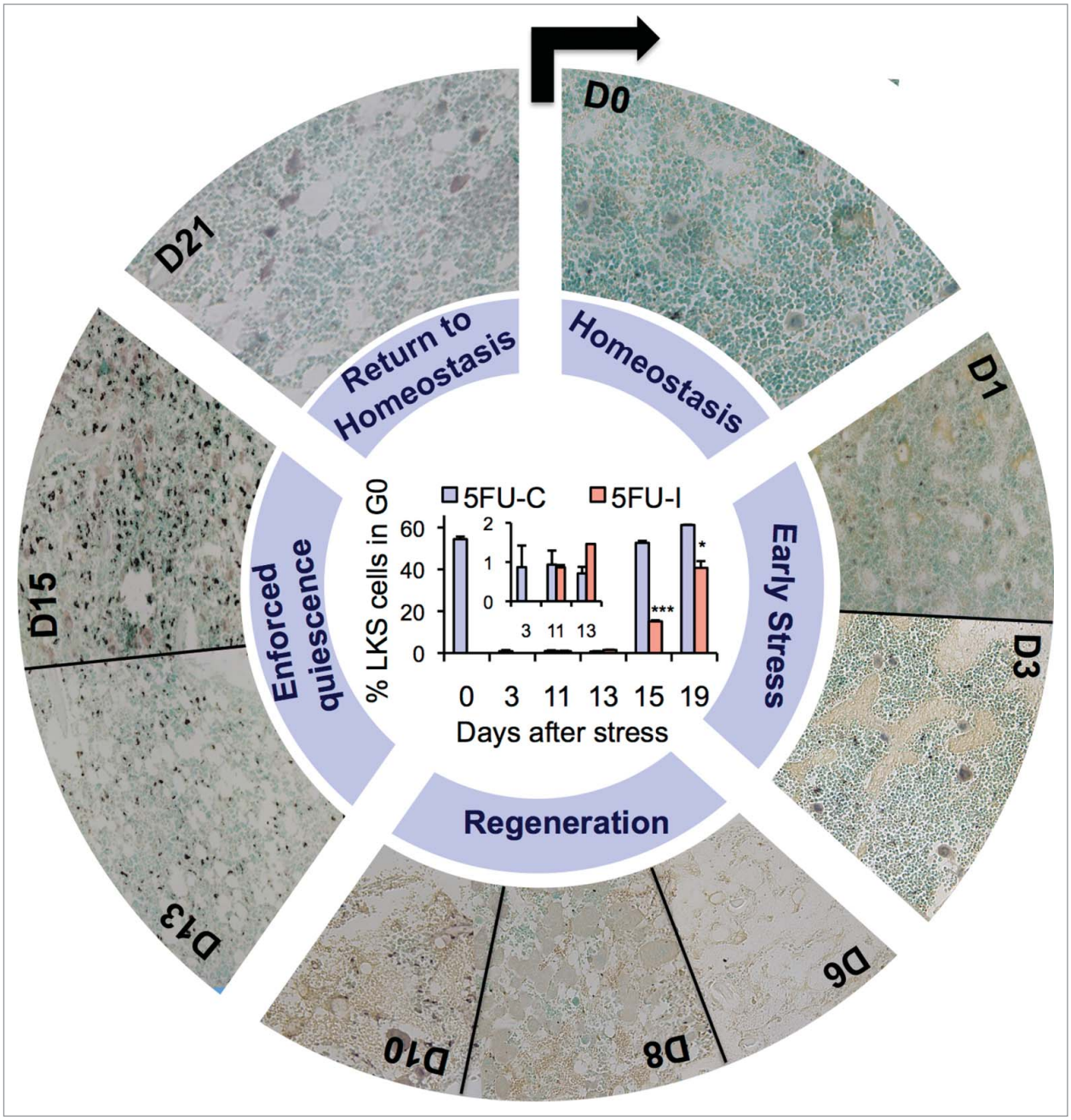

Figure 3. Quiescence is actively re-imposed by activation of TGFβ signaling after myelotoxic stress. Mice (WT, C57BL/6) were treated with a single dose of 5-fluorouracil (5FU, 250 mg/kg) on day 0 and immunohistochemical (IHC) staining for pSmad2 (brown) was performed on bone marrow sections collected before and after chemotherapy. The lifecycle of hematopoietic stem and progenitor cells (HSPC) after stress is represented: Homeostasis (D0), early stress (D1-3), regeneration (D4-10), enforced quiescence (D13-15), return to quiescence by D21. Sections were counterstained with the nuclear stain methyl green to assess cellularity. The percentage of quiescent LKS^+ (Lin-$cKit^+Sca1^+$) cells in G_0 phase is shown before treatment (D0) and at various times after treatment with 5FU and the TGFβ neutralizing antibody 1D11 (5FU-I, red bars) or the control antibody 13C4 (5FU-C, blue bars). (Adapted from Brenet et al., 2013).

after chemotherapy delayed the return of HSCs to quiescence and promoted HSC self-renewal and hematopoietic regeneration. Similarly, TGFβ blockade during recovery from other stressors—phenylhydrazine (PHZ)-induced hemolysis, LPS-modeled sepsis, or syngenic HSCT using lethal radiation as conditioning—hastened blood count recovery, prolonged HSC cycling, and expanded *bona fide* long-term engraftable HSCs. The duration of HSC cycling and hematopoietic stress differs significantly from 5FU, LPS, and PHZ stresses and, unsurprisingly, different schedules of TGFβ blockade were required to modulate HSC quiescence in these diverse settings. Whereas TGFβ levels spike approximately 10 days after 5FU treatment, TGFβ1 expression

is strongly induced in MPPs/ST-HSCs within 1 or 2 days of LPS challenge.[1,12,23] Nonetheless, the return of HSCs to quiescence after LPS, 5FU, or PHZ-hemolysis tracks with intracellular Smad2 phosphorylation in HSCs and is modulated by TGFβ blockade.[1] Although the clinical significance of these vastly dissimilar models is not known, these studies demonstrate that spatiotemporally constrained activation of TGFβ signaling during bone marrow recovery from stress mandates the return of HSCs to quiescence.

Although TGFβ has pleiotropic activities and is known to affect and be affected by many other signaling pathways involved in demand hematopoiesis, it also modulates the bone marrow microenvironment. Recently, genetic deletion of SPARC produced by non-hematopoietic cells was found to hasten the return of HSC quiescence and limit the hematopoietic toxicity of 5FU, but no linkage to TGFβ signaling could be made.[12] CD81 expression modestly promotes the return of HSCs to quiescence after 5FU but CD81 is not a target of TGFβ in HSCs and unlike TGFβ, which is known to block receptor clustering into lipid rafts, CD81 appears to require clustering to induce its effects on HSCs.[64-66] These results suggest that other signaling pathways likely help mediate the return to quiescence in some contexts.

Confusion from Murine Genetic Studies

TGFβ is one of few negative regulators of hematopoiesis [66,67] and is known to be a potent inhibitor of cytokine-driven HSC proliferation *in vitro*,[66,68-71] but its role in hematopoiesis has been hard to establish *in vivo*.[72-76] Constitutive knockout of signaling components causes embryonic lethality or a lethal inflammatory disorder that precludes routine analysis of steady-state adult hematopoiesis.[74,77-79] Genetic deletion of TβRI (ALK5) does not appear to affect HSC quiescence or exhaustion[75] and ALK5 may not even be expressed in homeostatic HSCs.[65] In contrast, HSC self-renewal during stress is strongly influenced by knockout of TβRII and by manipulation of the downstream effectors Smad4 and Smad7.[48,80,81] As a result, available studies provide a confusing picture: on the one hand canonical TGFβ signaling from Tgfbr2 is critical for control of HSC quiescence and self-renewal[48,80,81] while on the other hand Tgfbr1 (ALK5), Eng, and TGFβ1 have no effect on these same processes.[74-76,82]

The reasons for the conflicting results of murine genetic studies have not been defined. It is plausible that experimental details play a significant role in determining the TGFβ phenotypes assessed. For example, the consequences of TGFβ signaling appear to be at least partially dependent on dose, duration, and context. TGFβ signaling cues HSCs to return to quiescence during recovery from hematologic stress, but the role of TGFβ in homeostasis, when many niche signals are available, may be redundant and possibly dispensable.[1,83,84] Experimental systems that use chemotherapy, retroviral transduction, transplantation, or poly(I:C) for Mx1-Cre induction or surgical alteration of the bone marrow are all necessarily influenced by hematopoietic stress; this may accentuate or mask the phenotypes observed. HSPCs also appear to be sensitive to the concentration of available TGFβ, with high concentrations being inhibitory and low concentrations augmenting cytokine-driven proliferation, possibly via non-canonical signaling.[85,86] Although TGFβ has been known to affect HSPCs for 15 years, the receptors mediating these effects and the downstream targets remain largely undefined. TGFβ signaling is adaptive and can be much more sensitive to changes in TGFβ ligand concentrations than to steady-state ligand availability.[54] Indeed, such adaptive signaling is expected to enhance the responsiveness of HSPCs to the spike in bone marrow TGFβ levels during recovery from hematologic stress.[1] The outcome of TGFβ signaling and the regulation of HSPCs in the bone marrow microenvironment is more complex than previously appreciated but a fuller understanding of the spatiotemporal context of signaling during bone marrow regeneration promises new classes of therapy to treat myelosuppression.

TGFβ Blockade as a Double-Edged Sword to Fight Cancer

Blockade of TGFβ signaling after myelosuppressive chemotherapy delays the return of cycling HSPCs to quiescence.[1,12] Unlike current approaches using cytokines (e.g., G-CSF) with their regenerative activity restricted to a single lineage, TGFβ blockade after chemotherapy promotes recovery of all *in vivo* lineages because it acts on HSCs and early multilineage progenitors.[1] This suggests that TGFβ blockade could be an effective way to promote multilineage bone marrow regeneration after injury or hematopoietic stem cell transplantation. Nonetheless, successful translation of this research to clinical care will require a more complete understanding of the mechanisms of TGFβ activation within the bone marrow, and the safety and feasibility of this approach has to be fully evaluated.

Basic and clinical research has shown that transient blockade of TGFβ signaling does not cause the toxicities (e.g., autoimmune organ damage) in mice and humans that have been observed with genetic deletion of TGFβ signaling components in engineered mouse models. Thus, it is evident that the consequences of prolonged and short-term TGFβ inhibition are important determinants of potential toxicity. Because TGFβ blockade after chemotherapy prolongs HSC self-renewal, a potential concern is that this approach could lead to HSC exhaustion. Preliminary studies begin to alleviate this concern because HSCs obtained from mice treated with 5FU and then a TGFβ neutralizing antibody outcompete HSCs obtained from mice treated with the same chemotherapy and a control antibody.[1] Similarly HSCs from mice deficient in the critical downstream TGFβ target gene *Cdkn1c/p57* have a competitive advantage over wild-type HSCs after chemotherapy.[1,66] Importantly, cycling HSCs return to quiescence after TGFβ blockade or when p57 is deleted, suggesting that, rather than permanently disrupting homeostasis, these approaches simply reschedule

homeostasis for a later time and in so doing promote hematopoietic regeneration.

The timing of TGFβ blockade during demand hematopoiesis is important. For instance, administering a TGFβ neutralizing antibody on days 5, 7, and 9 after 5FU chemotherapy improved blood count recovery and delayed HSC quiescence to a greater degree than the same antibody doses administered before or after this time (Brenet & Scandura, unpublished). Similarly, the schedule of TGFβ blockade after LPS challenge (day 1), PHZ (day 3), or after lethal radiation and HSCT (second week) needed to be tailored to the type of hematopoietic stress. The schedule of TGFβ blockade is also potentially important because delayed HSPC quiescence could sensitize hematopoiesis to repeated chemotherapy cycles. However, preliminary testing suggests that this need not be the case. Cyclic chemotherapy actually caused less toxicity in mice deficient in the TGFβ target gene p57.[1] The TGFβ blocking agent used and its dose schedule will likely influence the risk of chemosensitization from closely spaced repetitive cycles of S-phase active chemotherapeutics. For example, the pharmacology of TGFβ inhibition using the 1D11 neutralizing antibody differs significantly from blockade of Tgfbr1 using the small molecule inhibitor LY2157299. Murine IgG_1 such as 1D11 has a terminal half-life of 3–5 days whereas the half-life of LY2157299 is just a few hours.[87] Although both agents promote hematopoietic recovery after chemotherapy, the long half-life of antibodies in the circulation makes it infeasible to rapidly "turn off" TGFβ blockade using 1D11. In principle, a short-acting inhibitor such as LY2157299 allows for tighter control over the timing of TGFβ signaling blockade (albeit somewhat at the expense of efficacy). Further work is necessary to determine how finely the return to quiescence can be modulated by TGFβ pathway inhibitors after chemotherapy.

The context of TGFβ inhibition is also important. Interestingly, it was only during recovery from demand hematopoiesis that TGFβ blockade using a pan-TGFβ neutralizing antibody (1D11) prolonged HSPC proliferation and augmented blood count recovery. In homeostatic mice, this same inhibitor failed to induce quiescent HSCs to enter the cell cycle and did not increase blood counts or bone marrow cellularity. The context-dependent activity is important because TGFβ signaling seems to be dispensable for the maintenance of quiescence during homeostasis whereas it is a central mediator mandating the return of HSPCs to quiescence after stress.[1] This finding potentially conflicts with recent work demonstrating a role for glial fibrillary acidic protein $(GFAP)^+$ Schwann cells as a source of bone marrow TGFβ that can control the dormancy of HSCs.[48] This discrepancy can be explained by either exclusion of 1D11 from homeostatic HCS niches or by the use of experimental methods that could deviate from homeostasis. For instance, the functional role of $GFAP^+$ cells was demonstrated by unilateral mobilization of HSPCs after unilaterally transecting postganglionic sympathetic nerves in the lumbar trunk. However, surgical trauma or the resulting unilateral bone marrow inflammation due to degenerating neurons could have mobilized HSCs from the marrow instead of the loss of homeostatic TGFβ signaling. Genetic studies using GFAP-Cre deleter strains may help resolve this question.

TGFβ blockade also has potential application in the setting of HSCT. The availability of suitably HLA-matched adult donors remains a major obstacle that prevents many patients from receiving a curative allogeneic HSCT.[88] As a cryopreserved product, publicly banked umbilical cord blood (CB) is a readily available source of HSPCs for transplantation of patients lacking a suitable donor. CB has many appealing features[88] and greatly extends HSCT access, but the low number of HSPCs available in CB leads to prolonged cytopenia following HSCT. Delayed engraftment is a major problem because it is associated with prolonged hospitalization, increases transplant-related mortality, and increases the cost of CB-HSCT. Resolving this issue by blocking TGFβ to enhance HSPC engraftment after CB-HSCT would fulfill a significant unmet need and expand the pool of suitable donors for HSCT. However, new research must first determine the extent to which interfering with TGFβ signaling promotes a graft versus leukemia effect or graft versus host disease (GvHD).

The biology of TGFβ is complex; its cytostatic properties have led to its categorization as a tumor suppressor gene, but TGFβ signaling also has well-recognized effects on the microenvironment, cell motility, and immune surveillance. It is now evident that malignant cells can selectively shed the growth suppressive functions of TGFβ while retaining signaling that promotes local tumor growth and metastasis by driving invasion and migration within the microenvironment and allowing the tumor cells to evade the immune system. The effects of oncogenic TGFβ signaling are the best studied and can be summarized as an epithelial to mesenchymal transition (EMT) in breast cancer, but similar themes have been described in other tumor types. Several classes of TGFβ inhibitors under development loosely fall into 4 major classes: TGFβ ligand trap; peptide aptamers; antisense oligonucleotides [5]; and small molecule receptor kinase inhibitors.[89] Details of these agents and the early clinical and preclinical studies are beyond the context of this review but these agents have shown promise in many solid tumors.[90-93] This suggests that TGFβ blockade after chemotherapy could provide a double-edged sword to attack cancer by blocking aggressive tumor phenotypes while limiting chemotherapy-induced myelotoxicity.

Major Gaps in our Understanding of TGFβ Signaling in Hematopoiesis

More research is needed before we can realize the full potential of modulating the return to steady-state hematopoiesis for therapeutic purposes. Because TGFβ exerts its activity in tightly constrained spatiotemporal contexts it should be possible to design highly specific inhibitors capable of regional context-dependent activities. For instance, pre-receptor inhibitors could be designed if the mechanism for regional activation of latent TGFβ were known. Similarly, a more complete understanding of the activation of TGFβ signaling during bone marrow regeneration could yield new approaches to target the important receptor/accessory protein complexes and kinase activity, promote internalization or degradation of receptors, or interfere with downstream TGFβ

target genes. Major gaps in our current understanding of how this pathway is activated during regeneration indicate that this potential is not yet mature.

It is likely that counter-regulatory TGFβ signaling will be mechanistically linked to other signaling pathways involved in demand hematopoiesis but the connections have not been established. The surge in TGFβ during recovery from hematopoietic stress could limit signaling from CTLs because TGFβ suppresses the production of inflammatory mediators such as INF-γ, potentially curtailing HSC proliferation driven by IFN-γ during certain chronic infections.[24,94] Indeed, HSCs appear to be resistant to long-term interferon signaling, possibly as a result of induction of interferon regulatory factor 2 (Irf2) in HSCs.[95,96] The mechanism by which HSCs become refractory to repeated interferon dosing is not well defined, but TGFβ is implicated because acute INF-α modulates the expression of mediators of TGFβ signaling in HSCs.[96] Similarly, TGFβ could safeguard HSCs during recovery from stress by antagonizing the production of granzyme B (Gzmb), which has recently been found to mediate HSC apoptosis after LPS challenge *via* TLR4/TRIF/NF-kB signaling.[97] TGFβ and thrombopoietin (Thpo) are the only 2 factors known to induce p57 (Cdkn1c), a critical regulator of quiescence, in HSCs.[1,65,66,84,98] Although no clear linkage between these signaling pathways has been established during homeostasis or stress, it is possible that Thpo and TGFβ regulate p57 in different contexts, with Thpo maintaining HSC quiescence during homeostasis[7,84] and TGFβ driving p57 expression and the return to quiescence during stress conditions, when high Thpo levels appear to be incapable of restricting HSC cycling and may even augment it.[84] It will be critical to understand how divergent initiators of hematologic stress each trigger TGFβ pathway activation during recovery.

Although many cell types produce latent TGFβ it cannot bind TGFβ receptors until LAP is shed. Diverse mechanisms of TGFβ activation likely underlie the context-dependent, downstream biological effects of TGFβ, but little is known about how they function in hematopoiesis. This gap in our understanding of pre-receptor spatiotemporal activation of TGFβ in bone marrow severely limits the study of TGFβ in hematopoiesis. Although it is generally accepted that TGFβ signaling is initiated by the availability of ligand, this has never been definitively shown and alternative hypotheses have not been tested. While the canonical TGFβ signaling pathway is well appreciated, it is not clear how this critical pathway is interpreted by HSCs during homeostasis nor it is known how the signaling mechanism is altered by stress. For instance, altered expression of TGFβ receptors, receptor modulators, or downstream target genes during homeostasis and recovery from hematopoietic stress could lead to different signaling outcomes. Indeed, the role of non-canonical TGFβ signaling in HSCs has not been fully explored. Without understanding these mechanisms, it will be difficult to modulate TGFβ signaling to selectively regulate HSC quiescence in particular physiologic contexts. This is important because disruption of homeostatic HSC quiescence can lead to exhaustion of HSCs[65] whereas blockade of this pathway during hematopoietic regeneration can mitigate the effects of bone marrow injury.[1] Once these gaps in our understanding are filled, locally active, context-dependent inhibitors of TGFβ signaling will be possible because the cellular source of TGFβ and its mechanism of activation will be known in homeostasis and during recovery from hematopoietic stress. Ultimately, this should permit tight pharmacologic control over HSPC quiescence and promote hematopoietic regeneration after myelosuppressive chemotherapy while minimizing potential toxicities.

Summary and Closing Statement

The *de facto* paradigm that homeostasis is passively re-established as stress mediators normalize is incorrect: rather than being a passive process, steady-state hematopoiesis is actively re-imposed. TGFβ pathway activation marks the return of regenerating HSPCs to quiescence and this context-depending signaling helps re-establish homeostasis during recovery from chemotherapy. Therefore, myelosuppression does not drive hematopoiesis using only a cytokine-fueled gas pedal but also taps an active braking mechanism once sufficient recovery has been attained. TGFβ pathway inhibitors could promote multilineage hematopoietic reconstitution. However, the lack of mechanistic details and poor understanding of the context-dependent activities of TGFβ has confounded prior attempts to unravel TGFβ signaling in HSCs. Nonetheless, efforts to understand the spatiotemporal aspects of TGFβ signal transduction hold the promise that modulation of TGFβ signaling could permit tight control of HSC quiescence and hematopoietic function during recovery from myelosuppression, massive infection, and hemolysis/hemorrhage.

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No potential conflicts of interest were disclosed.

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