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Pleiotrophin mediates hematopoietic regeneration via activation of RAS

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Hematopoietic stem cells (HSCs) are highly susceptible to ionizing radiation-mediated death via induction of ROS, DNA double-strand breaks, and apoptotic pathways. The development of therapeutics capable of mitigating ionizing radiation-induced hematopoietic toxicity could benefit both victims of acute radiation sickness and patients undergoing hematopoietic cell transplantation. Unfortunately, therapies capable of accelerating hematopoietic reconstitution following lethal radiation exposure have remained elusive. Here, we found that systemic administration of pleiotrophin (PTN), a protein that is secreted by BM-derived endothelial cells, substantially increased the survival of mice following radiation exposure and after myeloablative BM transplantation. In both models, PTN increased survival by accelerating the recovery of BM hematopoietic stem and progenitor cells in vivo. PTN treatment promoted HSC regeneration via activation of the RAS pathway in mice that expressed protein tyrosine phosphatase receptor-zeta (PTPRZ), whereas PTN treatment did not induce RAS signaling in PTPRZ-deficient mice, suggesting that PTN-mediated activation of RAS was dependent upon signaling through PTPRZ. PTN strongly inhibited HSC cycling following irradiation, whereas RAS inhibition abrogated PTN-mediated induction of HSC quiescence, blocked PTN-mediated recovery of hematopoietic stem and progenitor cells, and abolished PTN-mediated survival of irradiated mice. These studies demonstrate the therapeutic potential of PTN to improve survival after myeloablation and suggest that PTN-mediated hematopoietic regeneration occurs in a RAS-dependent manner.

Introduction

Total body irradiation (TBI) is successfully used in the conditioning of patients for hematopoietic cell transplantation (1). Radiation causes toxicity to hematopoietic stem cells (HSCs) through the generation of ROS, induction of DNA strand breaks and apoptosis, and damage to the BM microenvironment (2–4). Despite an understanding of mechanisms through which ionizing radiation causes hematopoietic toxicity, few effective mitigators of radiation-induced hematopoietic injury have been developed (5–9). The lack of effective mitigators for acute radiation sickness (ARS) has become a public health concern, as the risk of terrorism using radiological or nuclear devices has escalated (10, 11). Elucidation of novel mechanisms through which HSCs respond to radiation and the development of therapeutics targeting such mechanisms could potentially benefit not only victims of ARS but also patients receiving TBI for hematopoietic cell transplantation.

HSCs reside in specialized niches within the BM, and distinct cells within these niches regulate HSC maintenance in vivo (12–20). We and others have shown that BM-derived endothelial cells regulate the response of HSCs to genotoxic stressors such as ionizing radiation (3, 4, 16, 21). However, the precise mechanisms through which BM niche cells promote HSC regeneration after injury remain poorly understood. We recently described the hematopoietic function of pleiotrophin (PTN), a protein which is secreted by BM endothelial cells and which promotes the expansion of long-term HSCs in culture (22). Deletion of *Ptn* in the BM microenvironment significantly decreased long-term HSCs in mice (23). However, the therapeutic potential and mechanism of action of PTN remained undefined. Here, we demonstrate that systemic administration of PTN increases the survival of mice in the settings of ARS and BM transplantation. PTN mediates these effects by induction of RAS/MEK/ERK signaling in HSCs and by promotion of HSC quiescence after irradiation.

Results and Discussion

We tested whether systemic administration of PTN could mitigate hematopoietic injury and improve the survival of lethally irradiated mice. When administered beginning +24 hours following 700 cGy TBI, 80% of PTN-treated mice survived, compared with 33% of irradiated controls (Figure 1A). Irradiated PTN-treated mice displayed increased BM cellularity; increased BM cKIT⁺ SCA-1⁺LIN⁻ (KSL) cells, which are enriched for hematopoietic stem

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Conflict of interest: John P. Chute and Nelson J. Chao are cofounders of C2Regenerate, a private company that holds licenses for the development of pleiotrophin.

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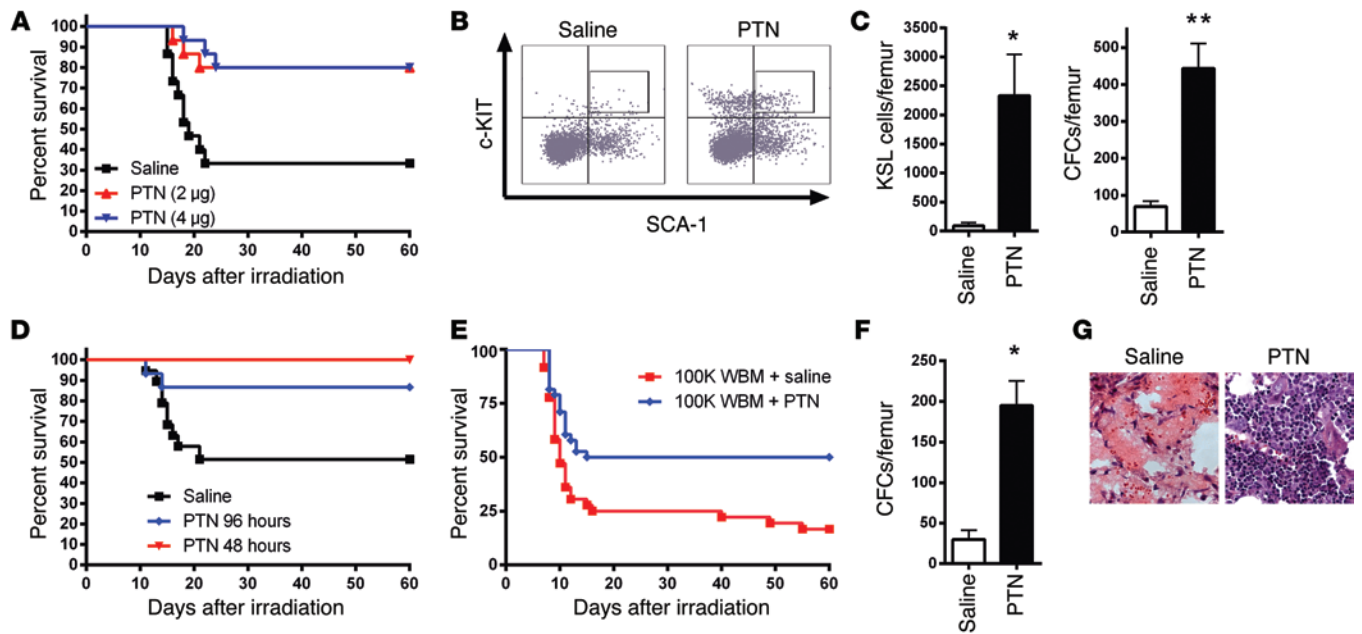


Figure 1. PTN treatment improves the survival of irradiated mice and hematopoietic cell transplant recipients. (A) Survival of 700 cGy-irradiated mice treated intraperitoneally with 2 or 4 μ g PTN or saline administered at +24 hours and every other day through day +14 (PTN-treated groups: 12 of 15 for both; saline-treated group: 5 of 15 mice; $P = 0.002$ for PTN 4 μ g vs. saline, $P = 0.004$ for PTN 2 μ g vs. saline). (B) Flow cytometric analysis of BM KSL cells from irradiated mice at day +10 treated with saline or PTN. (C) BM KSL cells and CFCs per femur ($n = 6$, $*P = 0.02$, $**P = 0.0003$). (D) Survival of irradiated mice treated subcutaneously, beginning at +48 hours and +96 hours, with PTN or saline (PTN 48 hours: 15 of 15 mice and PTN 96 hours: 13 of 15 mice; saline: 10 of 19 mice; $P = 0.002$ for PTN 48 hours vs. saline, $P = 0.04$ for PTN 96 hours vs. saline). (E) Survival of irradiated mice transplanted with BM cells and treated with PTN or saline (PTN, 19 of 38 mice, 50% vs. saline, 6 of 36 mice, 17%; $P = 0.003$). (F) CFCs per femur at day +14 following transplantation and treatment with PTN or saline ($n = 6$, $*P = 0.005$). (G) H&E images (original magnification, $\times 63$) of femurs at day +14 from transplanted mice treated with PTN or saline.

and progenitor cells (HSPCs) (22); and increased colony-forming cells (CFCs) compared with irradiated controls at day +10 (Figure 1, B and C, and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI76838DS1). These data suggested that PTN improved survival by mitigating radiation damage to HSPCs.

Historically, radiation mitigators have shown little efficacy when administered >24 hours after TBI (24, 25). Initiation of PTN treatment at +48 hours or +96 hours after 700 cGy significantly improved the survival of irradiated mice compared with that of irradiated controls (Figure 1D). These results suggested a unique therapeutic potential for PTN to improve survival in ARS.

We next tested whether PTN administration could accelerate hematopoietic reconstitution and improve the survival of mice receiving myeloablative (850 cGy) hematopoietic cell transplantation (14). Fifty percent of mice transplanted with 1×10^5 BM cells and treated with PTN survived, compared with 17% of transplanted control mice (Figure 1E). PTN-treated mice showed increased CFCs and BM cellularity at day +14 compared with controls (Figure 1, F and G), suggesting that PTN treatment accelerated hematopoietic reconstitution following BM transplantation.

PTN has been shown to inactivate the phosphatase domain of protein tyrosine phosphatase receptor-zeta (PTPRZ) on neural cells, thereby activating kinases, including anaplastic lymphoma kinase (ALK), which promote neurite outgrowth (26, 27). PTN treatment significantly increased p-ALK levels in HSCs from *Ptprz*^{+/+} mice but had no effect on HSCs from *Ptprz*^{-/-} mice, suggesting that PTPRZ was required for PTN-mediated activation of ALK

(Figure 2A). p-ALK can phosphorylate GRB2, which, in cooperation with son of sevenless (SOS), activates the RAS/MEK/ERK pathway (28). PTN treatment increased p-GRB2 levels and p-ERK1/2 levels in KSL cells from *Ptprz*^{+/+} mice but had no effect on p-ERK1/2 levels in KSL cells from *Ptprz*^{-/-} mice (Figure 2, B and C). p-ERK1/2 levels were also significantly decreased in KSL cells from *Ptn*^{-/-} mice compared with p-ERK1/2 levels in KSL cells from *Ptn*^{+/+} mice (Supplemental Figure 2). Expression of p-ERF, a transcriptional repressor that is regulated by ERK1/2 (29), also increased in PTN-treated KSL cells compared with that in control cells (Figure 2D). These data suggested that PTN activated the RAS/MEK/ERK pathway in HSCs in a PTPRZ-dependent manner.

Functionally, PTN treatment of BM KSL cells in cytokine cultures (thrombopoietin, SCF, and FLT-3 ligand) caused a decrease in total CFC output but increased the frequency of granulocyte-erythroid-macrophage-megakaryocyte-CFUs (CFU-GEMMs) (Figure 2E). Coupled with RAS or MEK inhibition (30), PTN-mediated maintenance of CFU-GEMMs was abolished. RAS inhibition also blocked PTN-mediated expansion of HSCs in culture, as measured with a competitive repopulation assay (Figure 2F). These results suggested that PTN-mediated expansion of HSPCs was dependent upon RAS activation.

PTN-treatment also significantly increased the number of CFU-GEMMs recovered from culture of irradiated BM KSL cells compared with cytokines alone, whereas RAS inhibition blocked this effect (Figure 3A). Furthermore, systemic administration of PTN promoted the recovery of BM KSL cells in irradiated mice, whereas the administration of tipifarnib, a RAS inhibitor (31),

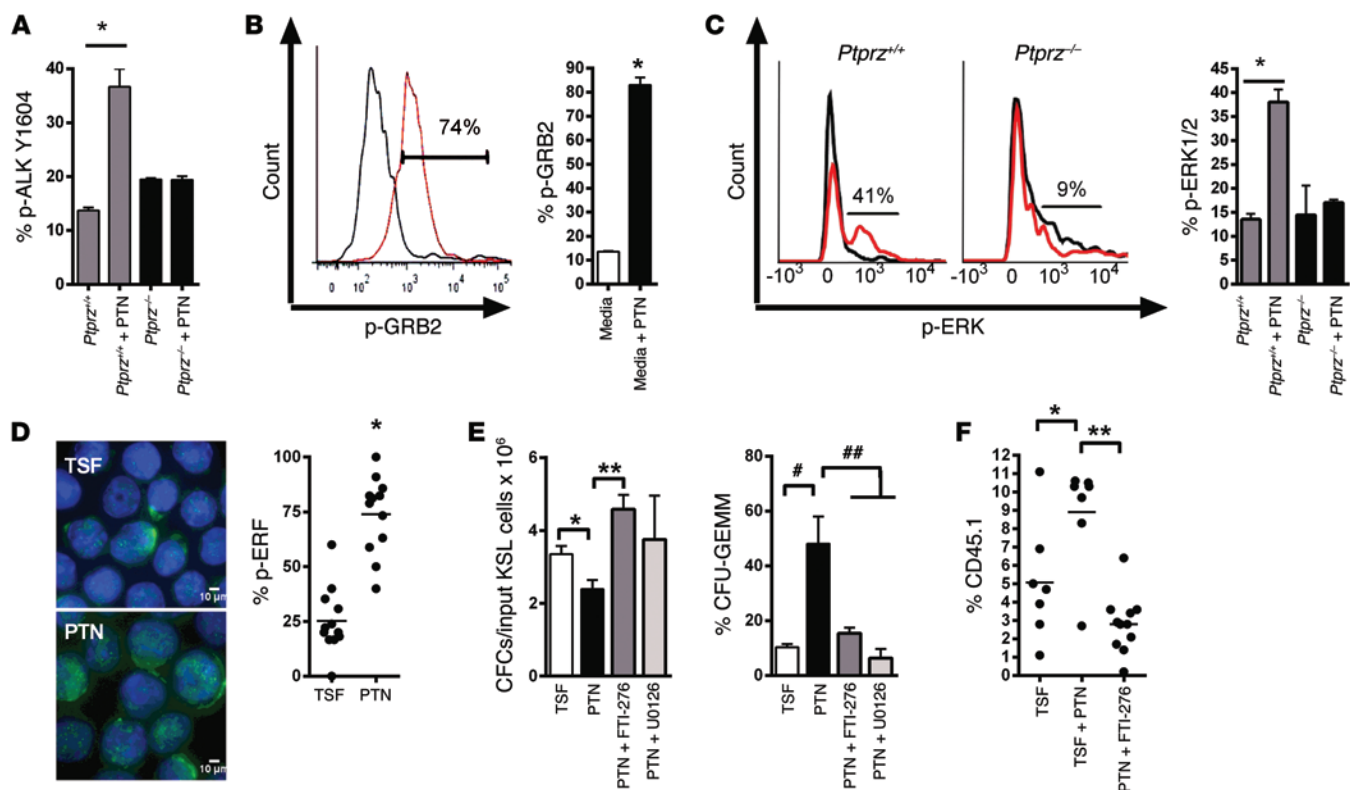


Figure 2. RAS signaling is necessary for PTN-mediated HSPC expansion. (A) p-ALK expression in BM KSL cells from the represented groups ($n = 3$, $*P = 0.003$). (B) p-GRB2 expression in BM KSL cells treated with media alone (gray curve) or PTN (red curve), with mean percentage p-GRB2 levels shown ($n = 3$, $*P < 0.0001$). (C) Representative p-ERK1/2 expression in KSL cells treated with media alone (gray curve) or PTN (red curve), with mean percentage p-ERK1/2 levels shown ($n = 5$, $*P < 0.001$). (D) p-ERF expression (green) in BM KSL cells cultured with thrombopoietin, SCF, and FLT-3 ligand (TSF), with or without PTN, and scatter plot of p-ERF levels in KSL cells (horizontal bars represent means; $n = 12$, $*P < 0.0001$). Scale bar: 10 μm . (E) CFCs per input KSL cells and percentage CFU-GEMMs at day +7 of the represented cultures ($n = 3$, $*P = 0.04$, $**P = 0.01$, $*P = 0.02$, $***P = 0.03$). (F) CD45.1⁺ donor cell engraftment at 8 weeks following competitive transplantation of the progeny of 10 CD34⁺ KSL cells cultured in the conditions shown ($n = 7$ –11 per group, $*P = 0.04$, $**P < 0.0001$).

blocked PTN-mediated regeneration of KSL cells (Figure 3, B and C). Importantly, tipifarnib treatment also blocked PTN-mediated improvement in the survival of irradiated mice (Figure 3D). Whereas 75% of PTN-treated mice (12 of 16) survived 800 cGy TBI, 28% of mice (5 of 18) treated with PTN and tipifarnib survived. These data suggested that PTN-mediated improvement in survival of irradiated mice was dependent on RAS activation.

Interestingly, PTN treatment did not alter the percentage of apoptotic BM KSL cells early after 300 cGy irradiation in vitro (Figure 3E). However, PTN treatment significantly increased the percentage of KSL cells remaining in the G₀ phase of cell cycle after irradiation compared with that in control cells (Figure 3, F and G). Of note, RAS inhibition blocked PTN-mediated inhibition of HSC cycling following irradiation (Figure 3, F and G). These data suggest that PTN may promote HSC regeneration after irradiation via induction of HSC quiescence and that this effect occurs in a RAS-dependent manner.

Our findings have significant implications for the treatment of ARS. Recently, novel mechanisms have been described that may be targeted to mitigate radiation injury to the hematopoietic system (5, 6, 8, 32, 33). To our knowledge, PTN represents the first therapeutic demonstrated to improve survival when administered more than 24 hours after exposure. This could be advantageous in a mass casualty radiation disaster, in which medical care may be

delayed for several days. Therefore, PTN has unique therapeutic potential to improve the survival of victims of ARS. Going forward, we will generate cell-specific genetic models to discern whether the in vivo effects of PTN treatment are HSC autonomous or also reflect indirect effects on the BM microenvironment.

Our results also suggest that PTN has therapeutic potential for patients undergoing limiting dose hematopoietic cell transplantation, such as adult cord blood transplantation, which can be complicated by delayed engraftment, graft failure, and death (34). While ex vivo CB expansion is currently being tested in clinical trials to augment hematopoietic recovery (35–37), an alternative strategy would be to administer systemic therapeutics to accelerate hematopoietic reconstitution in transplant recipients. Our results suggest that systemic PTN has therapeutic potential to accelerate hematopoietic reconstitution in such a setting.

Mechanistically, our data suggest that PTN-mediated expansion of HSPCs, in steady state or following irradiation, is dependent upon RAS activation. While overexpression of oncogenic RAS in hematopoietic cells causes a myeloproliferative disorder (38), the effects of physiologic RAS activation in HSCs are less well understood. Overexpression of oncogenic H-RAS in human HSCs, coupled with pharmacologic Ras inhibition, was previously suggested to promote HSC expansion (39). We postulate that PTN

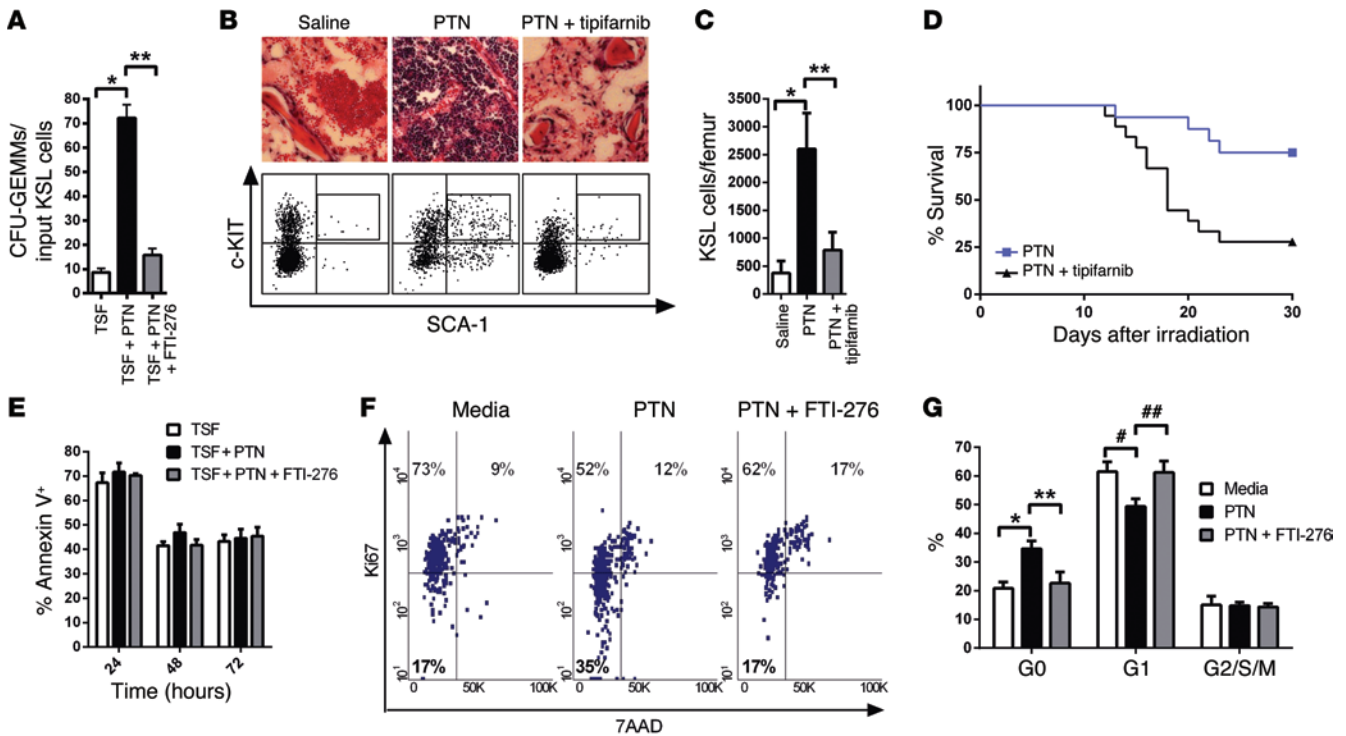


Figure 3. PTN promotes hematopoietic regeneration and survival following irradiation in a RAS-dependent manner. (A) CFU-GEMMs at day +3 of culture of KSL cells following 300 cGy ($n = 6$, $*P < 0.001$, $**P < 0.001$). (B) H&E-stained femurs (original magnification, $\times 63$) and flow cytometric analysis of KSL cells from mice at day +10 following 700 cGy and the treatments shown. (C) KSL cells per femur at day +10 following 700 cGy and the treatments shown ($n = 3$, $*P = 0.004$, $**P = 0.01$). (D) Survival curves of 800 cGy-irradiated mice treated with 4 μ g PTN every other day, with and without 1 mg tipifarnib, through day +21 ($P = 0.004$, log-rank test). (E) Percentage annexin-positive KSL cells following 300 cGy ($n = 5$). (F) Representative percentages of KSL cells in G_0 (Ki67 $^-$ 7AAD $^-$), G_1 (Ki67 $^+$ 7AAD $^-$), and G_2 /SM phase (Ki67 $^+$ 7AAD $^+$) at 6 hours following 300 cGy and cultured as shown. (G) Mean G_0 , G_1 , and G_2 /SM levels for each group ($n = 5$, $*P = 0.004$, $**P = 0.04$, $\#P = 0.02$, $##P = 0.01$).

promotes HSC expansion via activation of physiologic RAS signaling. Our data also suggest that PTN activates MEK/ERK signaling downstream of RAS in HSCs. In a prior study, we observed that PI3K inhibition abrogated PTN-mediated expansion of KSL cells in culture (22). Since RAS proteins can activate both PI3K/AKT and MEK/ERK signaling, it is possible that PTN activates both arms of RAS signaling. Going forward, we will use *Mx1-Cre Erk1 $^{-/-}$ Erk2 $^{fl/fl}$* mice (40) to determine whether PTN-mediated HSC regeneration is dependent upon RAS/MEK/ERK signaling.

Activation of the RAS pathway has not been previously shown to radioprotect HSCs. Here, we observed that PTN treatment promoted HSC quiescence after injury in a RAS-dependent manner. The effects of RAS/MEK/ERK signaling on cell cycle status are context dependent and may be related to the effects of cooperating oncogenes or inactivation of tumor suppressor genes (41–43). Going forward, we will perform RNA sequencing analysis of PTN-treated KSL cells to identify downstream effectors that may be responsible for RAS-dependent inhibition of HSC cycling. One candidate is p21, since prolonged activation of RAF/MEK/ERK signaling in fibroblasts induced p21-mediated cell cycle arrest (41, 44). The cyclin D binding myb-like transcription factor 1 (DMTF1) is also responsive to the RAS/MEK/ERK pathway and has been shown to promote HSC quiescence (45).

Broadly, our results suggest that the pharmacologic induction of HSC quiescence after irradiation can mitigate radiation injury

to the hematopoietic system. Several studies have shown the lack of efficacy of cell cycle-inducing cytokines in promoting survival when administered to mice after irradiation (24, 25). However, when administered prior to TBI, these same cytokines can radioprotect, perhaps by promoting the synchronized entry of HSCs into late S phase, a radioresistant phase of the cell cycle (24, 46). Conversely, administration of a CDK4/6 inhibitor within the first +20 hours after TBI improved the survival of lethally irradiated mice (7). Our results are most consistent with these findings and those of Cheng et al. (47), who showed that cycling HSCs from *p21 $^{-/-}$* mice displayed increased sensitivity to 5-FU chemotherapy and poor serial transplant capability compared with more quiescent *p21 $^{+/+}$* HSCs. Our studies suggest that PTN, a BM niche-derived protein, promotes HSC quiescence early after irradiation and powerfully mitigates radiation injury to the hematopoietic system.

Methods

For more detailed information, see the Supplemental Methods.

Mice. We used PTN-deficient (*Ptn $^{-/-}$*) mice and PTPRZ-deficient (*Ptprz $^{-/-}$*) mice as previously described (23). RAS and MEK inhibitors were provided by Christopher Counter and Donita Brady (Duke University).

Statistics. Survival analyses were performed using the log-rank test. Data are presented as mean \pm SEM throughout, and the Student's 2-tailed *t* test was used for comparisons. $P < 0.05$ was considered significant.

Study approval. Animal procedures followed protocols approved by the Duke University and UCLA animal care committees.

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