Epidermal growth factor regulates hematopoietic regeneration after radiation injury

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The mechanisms that regulate hematopoietic stem cell (HSC) regeneration after myelosuppressive injury are not well understood. We identified epidermal growth factor (EGF) to be highly enriched in the bone marrow serum of mice bearing deletion of Bak and Bax in TIE2-expressing cells in Tie2Cre; Bak1−/−; Baxlox/− mice. These mice showed radioprotection of the HSC pool and 100% survival after a lethal dose of total-body irradiation (TBI). Bone marrow HSCs from wild-type mice expressed functional EGF receptor (EGFR), and systemic administration of EGF promoted the recovery of the HSC pool in vivo and improved the survival of mice after TBI. Conversely, administration of erlotinib, an EGFR antagonist, decreased both HSC regeneration and the survival of mice after TBI. Mice with EGFR deficiency in VAV-expressing hematopoietic cells also had delayed recovery of bone marrow stem and progenitor cells after TBI. Mechanistically, EGF reduced radiation-induced apoptosis of HSCs and mediated this effect through repression of the proapoptotic protein PUMA. Our findings show that EGFR signaling regulates HSC regeneration after myelosuppressive injury.

HSCs can be found in proximity to bone marrow sinusoidal vessels1, and bone marrow endothelial cells regulate HSC homeostasis and regeneration2–8. It has been previously reported that maintenance of the HSC pool in mice is dependent on the expression of stem cell factor by bone marrow endothelial or perivascular cells, demonstrating the important role of endothelial and perivascular cells in maintaining the HSC pool during homeostasis8. We have shown that adult sources of endothelial cells produce soluble growth factors that promote the expansion of human HSCs in vitro2 and support the regeneration of mouse and human HSCs in vitro after radiation exposure2,3,9–11. We have also demonstrated that systemic infusion of autologous or allogeneic endothelial cells accelerates bone marrow HSC reconstitution and hematologic recovery in mice after radiation-induced myelosuppression4,12. A previous study demonstrated a requirement for vascular endothelial growth factor receptor 2 (VEGFR2)-expressing sinusoidal endothelial cells for normal hematologic recovery after TBI6. Similarly, systemic delivery of an antibody to vascular endothelial cadherin (VE-cadherin), which inhibits bone marrow vasculogenesis, substantially delays hematologic recovery after myelosuppression4,5. However, the precise mechanisms through which bone marrow endothelial cells regulate hematopoietic regeneration remain unknown.

Here, using a screen for cytokines in the bone marrow serum from Tie2Cre; Bak1−/−; Baxlox/− mice, which lack Bak and BAX in TIE2-expressing endothelial cells13 and show a strong radioprotective phenotype, we identify EGF as a candidate endothelial cell–derived mediator of radioprotection of the hematopoietic system and show that EGFR signaling regulates hematopoietic regeneration in vivo. Notably, systemic administration of EGF accelerated the recovery of long-term HSCs and improved the survival of mice after radiation-induced myelosuppression, whereas pharmacologic inhibition or genetic deficiency of EGFR antagonized hematopoietic regeneration in vivo.

RESULTS

EGF mediates HSC regeneration in vitro

We previously developed a genetic model to delete the genes encoding BAK and BAX, which regulate the intrinsic pathway of apoptosis13, in TIE2-expressing endothelial cells as a means to protect bone marrow endothelial cells from radiation-induced injury14. After high-dose TBI, Tie2Cre; Bak1−/−; Baxlox/− mice showed protection of the bone marrow vascular and HSC compartments as well as marked improvement in survival of the mice compared to Tie2Cre; Bak1−/−; Baxlox/+ mice, which retain one allele of Bax, or compared to wild-type mice14. To identify secreted factors produced by TIE2-expressing bone marrow endothelial cells that might contribute to the radioprotection observed in Tie2Cre; Bak1−/−; Baxlox/− mice, we generated primary bone marrow endothelial cell lines (CD45−, von Willebrand factor (VWF)+, lectin+ and acetylated low-density lipoprotein (AcLDL)+) from Tie2Cre; Bak1−/−; Baxlox/− mice (FL−/− endothelial cells) and Tie2Cre; Bak1−/−; Baxlox/+ mice (FL/+ endothelial cells), as previously described2,3,9,15. When we irradiated wild-type bone marrow c-Kit+Sca-1+Lin− (KSL) progenitor cells with 300 cGy in vitro and then plated them in noncontact culture with FL− endothelial cells,
we found significant increases in the recovery of total cells, colony-forming cells (CFCs) and colony-forming units, spleen, day 12 (CFU-S12) at day +7 compared to when we plated them in noncontact FL/+ endothelial cell cultures (Fig. 1a). These results suggest that bone marrow endothelial cells from Tie2Cre; Bak1−/−; Bax1flox/flox mice produce soluble factors that promote hematopoietic stem and progenitor cell regeneration after radiation injury. In a complementary experiment, the addition of bone marrow serum from irradiated Tie2Cre; Bak1−/−; Bax1flox/flox mice to cultures of irradiated bone marrow KSL cells promoted the recovery of total cells and CFCs in 7-d culture, whereas bone marrow serum from Tie2Cre; Bak1−/−; Bax1flox/flox mice had no beneficial effect (Supplementary Fig. 1a).

To identify paracrine factors in the bone marrow of Tie2Cre; Bak1−/−; Bax1flox/flox mice that might contribute to the radioprotection of hematopoietic stem and progenitor cells in vivo, we assessed cytokine levels using a cytokine array on the bone marrow serum from Tie2Cre; Bak1−/−; Bax1flox/flox; Tie2Cre; Bak1−/−; Bax1flox/flox wild-type C57BL/6 mice before and after 750-cGy TBI. Within the list of cytokines that we described to be significantly enriched in bone marrow serum from Tie2Cre; Bak1−/−; Bax1flox/flox compared to Tie2Cre; Bak1−/−; Bax1flox/flox mice14, EGF was approximately 18-fold increased in concentration in the bone marrow of irradiated Tie2Cre; Bak1−/−; Bax1flox/flox mice compared to irradiated Tie2Cre; Bak1−/−; Bax1flox/flox mice (Fig. 1b). EGF was also expressed at threefold higher levels in Bak1−/−; Bax1flox/flox compared to Bak1−/−; Bax1flox/flox+ endothelial cells (Fig. 1b). Furthermore, we did not detect EGF by ELISA of supernatants of bone marrow KSL cells from Tie2Cre; Bak1−/−; Bax1flox/flox mice (data not shown), suggesting that EGF is not produced in an autocrine manner by bone marrow stem and progenitor cells. Notably, Tie2Cre; Bak1−/−; Bax1flox/flox mice had an increased density of mouse endothelial cell antigen (MECA)± vessels in the bone marrow compared to Tie2Cre; Bak1−/−; Bax1flox/flox+ or C57BL/6 mice (Supplementary Fig. 1b). Therefore, the increased concentrations of EGF in the bone marrow serum of Tie2Cre; Bak1−/−; Bax1flox/flox− mice compared to the controls may be due, in part, to the increased density of EGF-secreting bone marrow endothelial cells in Tie2Cre; Bak1−/−; Bax1flox/flox− mice.

We next performed fluorescence-activated cell sorting (FACS) analysis to determine whether EGFR is expressed by wild-type bone marrow HSCs and progenitor cells. Less than 2% of bone marrow Lin+ and Lin− cells expressed EGFR, but 7.9% of bone marrow KSL cells and 9.2% of bone marrow signaling lymphopoeitc activation molecule family (SLAM)+ KSL cells, which are enriched for long-term HSCs, expressed EGFR (Fig. 1c). EGFR surface expression increased fourfold and sixfold in bone marrow KSL and SLAM+ KSL cells, respectively, at 4 h after 300-cGy irradiation (Fig. 1c, d), indicating that EGFR expression is induced in HSCs after irradiation. After EGFR treatment, EGFR phosphorylation levels were increased in bone marrow KSL cells and bone marrow SLAM+ KSL cells in vitro and in bone marrow KSL cells in vivo (Fig. 1e), demonstrating that EGFR is functional in bone marrow hematopoietic stem and progenitor cells.

We next performed gain-of-function studies to determine whether treatment of irradiated bone marrow KSL cells with EGF could support bone marrow stem and progenitor cell regeneration in vitro. When we added 20 nM EGF to irradiated (300 cGy) bone marrow KSL cells cultured with cytokines (thrombopoietin, stem cell factor, Flt-3 ligand (TFS)), we found significant increases in the recovery of CFCs and CFU-S12 compared to cultures with cytokines alone (Fig. 1f). The addition of EGF to noncontact FL/+ endothelial cell cultures of irradiated bone marrow KSL cells also increased CFC and CFU-S12 recovery compared to culture with FL/+ endothelial cells alone, whereas the addition of EGF-specific antibody to FL/+ endothelial cell cultures caused a substantial decrease in the recovery of CFC and CFU-S12 compared to FL/+ endothelial cell cultures alone (Supplementary Fig. 2). These data suggest that FL/+ endothelial cell–mediated regeneration of irradiated bone marrow progenitor cells is dependent on EGF. Using competitive repopulation assays, we also found that mice transplanted with the product of of irradiated, EGF-treated bone marrow CD34+ KSL cells showed threefold and fivefold higher donor hematopoietic cell engraftment at 8 and 12 weeks after transplant, respectively, compared to mice transplanted with the progeny of cytokine cultures alone (Fig. 1g).

Treatment of nonirradiated bone marrow KSL cells with EGF in vitro also caused a significant expansion of bone marrow KSL cells and CFU-S12 recovered compared to the progeny of cytokine cultures alone (Fig. 1h). Mice competitively transplanted with the progeny of bone marrow CD34+ KSL cells cultured with cytokines plus EGF showed more than tenfold increased donor hematopoietic cell repopulation at 12 weeks after transplant compared to mice transplanted with the progeny of bone marrow CD34+ KSL cells cultured with cytokines alone (Fig. 1h). These results suggest that EGF also promotes the maintenance of nonirradiated HSCs in culture.

EGF treatment promotes HSC regeneration in vivo

To determine whether EGF treatment can promote HSC regeneration in vivo, we measured hematopoietic reconstitution in C57BL/6 mice after 700-cGy TBI and subsequent intraperitoneal treatment with EGF or saline (control) beginning at 2 h after irradiation and then daily for 7 d (Fig. 2a). At day 7 after TBI, the EGF-treated mice had increased bone marrow cellularity compared to controls (Fig. 2b), as well as significantly increased numbers of bone marrow cells, bone marrow KSL cells, CFCs and CFU-S12 (Fig. 2b–d).

To determine whether treatment with EGF can rescue the functional bone marrow HSC pool in irradiated mice, we competitively transplanted lethally irradiated (CD45.1+) recipient mice with bone marrow cells from nonirradiated donor mice or from donor mice irradiated with 700 cGy and treated with either EGF or saline (CD45.2+). At 12 weeks after transplant, mice transplanted with 5 × 106 bone marrow cells from nonirradiated donors showed high donor-cell engraftment in the bone marrow (mean, 74.9%; Fig. 2e). Conversely, mice transplanted with the same dose of bone marrow cells from irradiated and saline-treated donors demonstrated much lower donor-cell engraftment (mean, 0.4%). Notably, mice transplanted with an identical dose of bone marrow cells from irradiated and EGF-treated mice showed significantly increased donor-cell engraftment (mean, 8.5%) compared to mice transplanted with bone marrow from irradiated and saline-treated donors (Fig. 2e). This difference in total engraftment was a function primarily of increased donor myeloid cell recovery in the EGF treatment group, suggesting that EGF treatment may have specifically augmented the recovery of short-term HSCs with myeloid reconstituting potential (Fig. 2e). We also found a close correspondence between total donor-cell engraftment and donor-cell chimerism within bone marrow KSL cells in each group of mice (Fig. 2e,f). Donor-cell engraftment within bone marrow KSL cells in recipients of bone marrow cells from irradiated and EGF-treated mice was sixfold higher than that in recipients of bone marrow cells from irradiated and saline-treated mice but was approximately 11% of the engraftment observed in mice transplanted with nonirradiated bone marrow cells. Therefore, although EGF treatment significantly increased the recovery of bone marrow HSCs after TBI, it did not fully restore HSC function compared to nonirradiated donor mice.
We also performed competitive secondary transplants in which we transplanted bone marrow cells from primary recipient mice into lethally irradiated syngeneic mice (Fig. 2a). At 12 weeks after transplant, secondary recipient mice receiving cells from the nonirradiated donor group showed high donor-cell engraftment (mean, 53.4%; Fig. 2g). Conversely, mice receiving cells from the irradiated and saline-treated donor group had very low donor-cell engraftment (mean, 0.5%). Secondary recipients receiving cells from the irradiated

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**Figure 1** TIE2-expressing bone marrow endothelial cells produce EGF, and EGF mediates HSC regeneration after irradiation. (a) Mean numbers (± s.e.m.) of total cells (left), CFCs (middle) and CFU-S12 (right) at day 7 from noncontact cultures of 300 cGy–irradiated bone marrow KSL cells with FL+/– bone marrow endothelial cells compared to culture with FL+/– bone marrow endothelial cells or cytokines alone (TSF). Input, population of KSL cells at day 0. *P = 0.003, **P = 0.04 compared to TSF and FL+, respectively, for total cells (n = 3–7 experiments per condition); *P < 0.0001, ^P < 0.0001 compared to TSF and FL+, respectively, for CFCs (n = 3 experiments per condition, t test); *P = 0.04, ^P = 0.02 compared to TSF and FL+, respectively, for CFU-S12 (n = 3–5 experiments per condition). (b) Left, concentrations of EGF in the bone marrow serum of Tie2Cre; Bak1−/−; Bak1lox/lox (red line), Tie2Cre; Bak1−/−; Bak1lox/lox (blue line) and C57BL/6 mice (black line) before irradiation (Nonirradiated) and at 6 h and 7 d after 750-cGy irradiation. *P = 0.02, **P = 0.04, ***P = 0.04 compared to Tie2Cre; Bak1−/−; Bak1lox/lox mice determined by one-tailed t test. n = 3 mice per condition; data are shown as the means ± s.e.m. Right, EGF expression in FL−, FL+ and C57BL/6 (BL6) endothelial cells by quantitative RT-PCR. *P = 0.002, ^P = 0.003 compared to BL6 and FL+/– endothelial cells, respectively, determined by two-tailed t test. Data are shown as the means ± s.e.m. n = 3 experiments per group. (c) Left, representative EGFR surface expression on bone marrow Lin+, Lin− and KSL cells from C57BL/6 mice. Right, representative EGFR surface expression on nonirradiated bone marrow SLAM+ KSL cells and at 4 h after 700-cGy irradiation. The numbers shown indicate the percentage of EGFR surface expression on the indicated cell population. (d) Left, EGFR surface expression in each bone marrow subset. *P = 0.009, ^P < 0.001 for bone marrow KSL cells compared to bone marrow Lin+ and Lin− cells, respectively. *P = 0.02, **P = 0.004 for bone marrow SLAM+ KSL cells compared to Lin+ and Lin− cells, respectively (n = 3–9 experiments per group). Data are shown as the means ± s.e.m. Right, EGFR expression in nonirradiated bone marrow SLAM+ KSL (red line), KSL (blue line), Lin− (black line) and Lin+ cells (green line, along x axis) and at 4 h after 300-cGy and 700-cGy TBI. *P = 0.002 for the difference in SLAM+ KSL cells between the 300 cGy–irradiated and nonirradiated groups, ^P = 0.001 for the difference between the 700 cGy–irradiated and nonirradiated groups (n = 3–7 experiments per group). Data are shown as the means ± s.e.m. (e) Representative FACS analyses of phosphorylation of EGFR at Tyr1173 (pEGFR) in bone marrow KSL (left) and bone marrow SLAM+ KSL (middle) cells in serum-free culture (blue curve) or in the same conditions plus EGF (red curve). Right, pEGFR in bone marrow KSL cells in mice treated with EGF (red curve) or saline (blue). Isotype-stained cells are shown in black. Percentages of EGFR phosphorylation are shown for each group at right. *P = 0.008 for EGFR compared to control cultures of KSL cells, *P < 0.001 for EGF compared to control cultures of bone marrow SLAM+ KSL cells (n = 5 experiments per group). Data are shown as the means ± s.e.m. *P = 0.03 for EGF compared to saline treatment of KSL cells in vivo (n = 4 experiments per group). (f) CFC and CFU-S12 content of cultures of irradiated bone marrow KSL cells with TSF or TSF plus EGF. *P = 0.0002, ^P = 0.0003 for CFCs (n = 8 experiments per group) and CFU-S12 (n = 9–12 experiments per group), respectively, compared to TSF alone. Data are shown as the means ± s.e.m. (g) Peripheral blood donor CD45.1+ cell, myeloid (Mac-1/Gr-1), B cell (B220) and T cell (Thy1.2) engagement at 8 weeks after transplant in CD45.2+ mice after transplantation, TSF-cultured CD34+ KSL cells or irradiated, TSF- and EGF-cultured CD34+ KSL cells (n = 7–9 mice per group). *P = 0.002 for CD45.1+ cell engagement, ^P = 0.002 for myeloid engagement. Right, total peripheral blood CD45.1+ cell engagement over time (red line, TSF plus EGF culture, black line, TSF alone). *P = 0.002. Data are shown as the means ± s.e.m. (h) Numbers of KSL cells and CFU-S12 from cultures of irradiated bone marrow KSL cells with TSF or TSF plus EGF. *P = 0.03, ^P = 0.004. Data are shown as the means ± s.e.m. n = 4–6 experiments per group. Right, percentage donor CD45.1+ cell engagement at 12 weeks in mice transplanted with nonirradiated and TSF-cultured CD34+ KSL cells or nonirradiated and TSF- and EGF-cultured CD34+ KSL cells. *P = 0.04 determined by two-tailed t test. Data are shown as the means ± s.e.m. n = 4–5 mice per group. A Mann-Whitney test was used for all statistical analyses unless otherwise noted.
and EGF-treated donor group showed significantly higher total and multilineage donor engraftment (mean, 24%; Fig. 2g) compared to mice receiving cells from the irradiated and saline-treated donor group. Secondary recipient mice receiving cells from the irradiated and EGF-treated donor group showed a corresponding increase in donor-cell chimerism within bone marrow KSL cells compared to mice receiving cells from the irradiated and saline-treated donor group (Fig. 2h). These results suggest that EGF treatment promotes the recovery of long-term HSCs in mice after TBI.

**EGFR inhibition impairs HSC regeneration in vivo**

To determine whether EGFR inhibition can suppress HSC regeneration in vivo, we irradiated mice with 700 cGy and then treated them with erlotinib, an EGFR antagonist, or water (control) by oral gavage from day 0 to day 14 (Fig. 3a). At day 7, both the erlotinib-treated and control mice showed depletion of bone marrow stem and progenitor cells (data not shown). At day 14, irradiated control mice showed recovery of bone marrow cellularity, CFCs and CFU-S12, whereas erlotinib-treated mice showed depletion of bone marrow CFCs and CFU-S12 (Fig. 3b,c). The erlotinib-treated mice also had a deficit in short-term and longer-term HSCs after TBI as compared to irradiated control mice as measured by competitive repopulation assay (Fig. 3d). These results suggest that EGFR inhibition impairs hematopoietic stem and progenitor cell regeneration after TBI.

To determine whether EGFR signaling is involved in mediating the radioprotection we observed in Tie2Cre; Bak1<sup>−/−</sup>; Bax<sup>flox/−</sup> mice,

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**Figure 2** Systemic administration of EGF promotes HSC regeneration in vivo.

(a) Schematic diagram of intraperitoneal treatment of mice after TBI with either EGF or normal saline (NS) for 7 d and subsequent analysis of bone marrow (BM) progenitor cell content and competitive HSC repopulation assays. (b) Representative H&E-stained femurs from EGF- and saline-treated mice. Scale bar, 250 μm. Right, bone marrow cell counts. *P = 0.003. Data are shown as the means ± s.e.m. n = 6 experiments per group. (c) Representative FACS analysis of bone marrow c-Kit<sup>+</sup>Sca-1<sup>+</sup> cells within the Lin<sup>−</sup> gate (KSL) from nonirradiated (Nonirrad) mice and at day 7 from irradiated mice treated with either saline or EGF. The numbers shown indicate the percentage of c-Kit<sup>+</sup>Sca-1<sup>+</sup> cells within the Lin<sup>−</sup> population. (d) Bone marrow KSL cells, CFCs and CFU-S12 at day 7 in irradiated mice treated with either saline or EGF. *P = 0.008 for KSL cells (means ± s.e.m., n = 6 experiments per group); *P < 0.0001, *P = 0.03 for CFCs and CFU-S12 (means ± s.e.m., n = 3–5 experiments per group). (e) Percentage total donor CD45.2<sup>+</sup> cell, myeloid (Mac-1/Gr-1<sup>+</sup>), B cell (B220) and T cell (Thy1.2) engraftment in the bone marrow of CD45.1<sup>+</sup> mice at 12 weeks after transplantation of 5 x 10<sup>5</sup> bone marrow cells from nonirradiated donor (NI), irradiated and saline-treated or irradiated and EGF-treated donor mice. *P = 0.0006 for EGF compared to saline; *P = 0.0001 for EGF compared to NI (CD45.2<sup>+</sup> engraftment); *P = 0.002 for EGF compared to saline (myeloid engraftment) (n = 7–10 mice per group). Horizontal lines represent mean percentage of donor cell engraftment. Percentages of donor CD45.2<sup>+</sup> cells within the bone marrow KSL population are also shown. *P = 0.001, *P = 0.0001 for EGF compared to the saline and NI groups, respectively (n = 7–10 mice per group, means ± s.e.m.). (f) Representative FACS plots of total donor CD45.2<sup>+</sup> cells and CD45.2<sup>+</sup> cell chimerism within bone marrow KSL cells at 12 weeks in mice transplanted with bone marrow from NI mice or irradiated mice treated with either saline or EGF. The numbers shown indicate the percentage of each annotated cell population within the total bone marrow cell population. (g) Percentage donor CD45.2<sup>+</sup> cell, myeloid, B cell and T cell engraftment at 12 weeks in the bone marrow of secondary transplant recipient mice (CD45.1<sup>+</sup>) receiving cells from mice in the NI donor group, the irradiated and saline-treated donor group and the irradiated and EGF-treated donor group. *P = 0.03, *P = 0.01, *P = 0.001 for differences in total CD45.2<sup>+</sup> cell engraftment, myeloid cell, B cell and T cell engraftment, respectively, between the EGF- and saline-treated donor groups (n = 5–8 mice per group, means ± s.e.m.). The mean percentages of donor CD45.2<sup>+</sup> cells within the bone marrow KSL population are also shown. *P = 0.009 for the EGF compared to saline-treated donor group (n = 5–8 mice per group, means ± s.e.m.). (h) Representative FACS plots of CD45.2<sup>+</sup> cell chimerism within bone marrow KSL cells in secondary transplanted mice receiving cells from mice in the NI, saline- and EGF-treated donor groups at 12 weeks after transplantation. The numbers in the bottom row indicate the percentage of donor CD45.2<sup>+</sup> cells within the bone marrow c-Kit<sup>+</sup>Sca-1<sup>+</sup>Lin<sup>−</sup> cell population. A Mann-Whitney test was used for all statistical analyses.
we administered erlotinib or water (control) to Tie2Cre; Bak1+/−; Baxlox/fl mice beginning 3 d before 300-cGy TBI and evaluated bone marrow HSC and progenitor cell content at 2 h after TBI (Fig. 3f). These decreases corresponded with decreased EGFR phosphorylation in bone marrow KSL cells in the erlotinib-treated Tie2Cre; Bak1+/−; Baxlox/fl mice (Fig. 3g). Irradiated Tie2Cre; Bak1+/−; Baxlox/fl mice showed relative protection of bone marrow HSCs with multilineage repopulating capacity after TBI (Fig. 3h). Conversely, erlotinib-treated irradiated Tie2Cre; Bak1+/−; Baxlox/fl mice had a marked deficit in bone marrow HSCs capable of multilineage reconstitution in vivo (Fig. 3h). These results suggest that EGFR signaling is important for the radioprotection of bone marrow stem and progenitor cells in Tie2Cre; Bak1+/−; Baxlox/fl mice.

**EGFR deficiency inhibits hematopoietic recovery after TBI**

Erlotinib has been shown to inhibit kinases other than EGFR, including JAK2 and Src kinases. To determine whether the effects of erlotinib on HSCs were specific to EGFR or were through off-target effects, we generated VavCre; Egfrlox/lox (Egfrlox/lox) and VavCre; Egfr+/+/ (Egfr+/+) mice and verified the lack of EGFR mRNA expression in bone marrow lineage-negative (Lin−) cells (Fig. 4a). We cultured bone marrow Lin− cells from Egfrlox/lox or Egfr+/+ mice in cytokine medium for 72 h and found decreased total cell growth and CFC production in the EGFR-deficient cells (Fig. 4b). Treatment of Egfrlox/lox bone marrow Lin− cells with erlotinib caused no significant effect on total cell expansion or CFC production compared to Egfrlox/lox bone marrow Lin− cells cultured with cytokines alone (Fig. 4c). Conversely, erlotinib treatment of Egfr+/+ bone marrow Lin− cells decreased total cell expansion and CFC production compared to Egfr+/+ bone marrow Lin− cells cultured with cytokines alone or compared to Egfrlox/lox bone marrow Lin− cells cultured with erlotinib. These data suggest that erlotinib acts specifically on EGFR in bone marrow progenitor cells.

We also compared the in vivo recovery of bone marrow hematopoietic stem and progenitor cells in Egfr+/+ and VavCre; Egfrlox/lox (Egfrlox/lox) mice after 500-cGy TBI. At baseline, Egfrlox/lox mice showed decreased EGFR expression in bone marrow Lin− cells relative to Egfr+/+ mice (Fig. 4d) and had no differences in complete blood counts or bone marrow CFCs compared to Egfr+/+ mice (Fig. 4e,f). However, at day 7 after TBI, Egfrlox/lox mice had fivefold decreased bone marrow CFC content and 30-fold decreased numbers...
of bone marrow SLAM+ KSL cells compared to Egfr+/+ mice (Fig. 4g). These data suggest that EGFR may be necessary for normal bone marrow stem and progenitor cell regeneration after TBI.

**EGF inhibits HSC apoptosis by repression of PUMA.**

We performed annexin and 7-aminoactinomycin D (7-AAD) staining to assess the effects of EGF on HSC survival after irradiation. At 72 h after 300-cGy irradiation, EGF-treated cultures contained twofold decreased numbers of annexin-positive KSL cells compared to cytokine cultures (Fig. 5d). C57BL/6 mice irradiated with 700 cGy and then treated with EGF for 7 d also contained fourfold decreased numbers of annexin-positive bone marrow hematopoietic cells compared to saline-treated controls (Fig. 5d). EGF treatment also decreased the numbers of annexin-positive cells in cultures of irradiated bone marrow KSL cells from Tie2Cre; Bak1−/−; Baxlox/lox−/− mice (Supplementary Fig. 3b). This result suggests that EGF may mediate HSC survival after irradiation through additional mechanisms, such as DNA repair, that are not directly related to inhibition of apoptosis.23

PUMA is an essential mediator of radiation-induced hematopoietic toxicity.24,25 Because EGF promoted HSC survival after irradiation, we examined whether EGF causes such effects through inhibition of PUMA. PUMA mRNA expression increased significantly in bone marrow HSCs from Tprp53+/+ mice after 300-cGy irradiation, whereas PUMA mRNA expression did not change in irradiated HSCs from Tprp53−/− mice, indicating that PUMA induction in HSCs is p53 dependent (Fig. 5e). EGF treatment repressed radiation-induced PUMA expression in bone marrow KSL cells from Tprp53+/+ mice but had no effect on those from Tprp53−/− mice (Fig. 5e). PUMA protein expression in bone marrow KSL cells correspondingly increased in response to irradiation in a p53-dependent manner, and EGF treatment significantly decreased PUMA protein expression in these cells after irradiation (Fig. 5e).

Bone marrow KSL cells from PUMA-deficient mice had a lower percentage of apoptotic cells and increased CFC content at 72 h after 300-cGy irradiation compared to PUMA-expressing bone marrow KSL cells (Fig. 5f–h). EGF treatment increased HSC survival and CFC regeneration in PUMA-expressing KSL cell cultures after irradiation but had no effect on HSC survival or CFC production in PUMA-deficient KSL cell cultures after irradiation (Fig. 5f–h). These results suggest that EGF-mediated inhibition of radiation-induced HSC apoptosis is dependent on inhibition of PUMA.

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**Figure 4** Deficiency of EGFR inhibits hematopoietic progenitor cell regeneration. (a) EGFR mRNA expression in bone marrow Lin− cells from VegfCre; Egfrlox/lox (Egfrlox/lox) and VegfCre; Egfr+/+ mice. *P = 0.008 (means ± s.e.m., n = 5 experiments per group). (b) Numbers of total cells and CFCs at 72 h of cytokine culture of bone marrow Lin− cells from VegfCre; Egfrlox/lox and VegfCre; Egfr+/+ mice. *P = 0.03, *P = 0.002 for total cells and CFCs, respectively (means ± s.e.m., n = 5–6 experiments per group). (c) Numbers of total cells and CFCs at 72 h of culture of bone marrow KSL cells from VegfCre; Egfrlox/lox and VegfCre; Egfr+/+ mice with erlotinib. *P = 0.004, *P = 0.04 for total cells and CFCs, respectively (means ± s.e.m.; n = 8 experiments per group). (d) EGFR expression in bone marrow Lin− cells from VegfCre; Egfrlox/lox (Egfrlox/lox) and VegfCre; Egfr+/+ mice. *P = 0.008 (means ± s.e.m., n = 5 experiments per group). (e,f) Complete blood counts (n = 10–15 mice per group) and bone marrow CFCs (f) in VegfCre; Egfrlox/lox and VegfCre; Egfrlox/lox mice (n = 6 experiments per group); means ± s.e.m. (g) Numbers of bone marrow CFCs and bone marrow SLAM+ KSL cells in VegfCre; Egfrlox/lox and VegfCre; Egfr+/+ mice at day 7 after 500-cGy TBI. *P = 0.002 (n = 6 experiments per group), *P = 0.004 (n = 6–11 experiments per group) for CFCs and SLAM+ KSL cells, respectively; means ± s.e.m. A Mann-Whitney test was used for all statistical analyses.
EGF treatment improves the survival of lethally irradiated mice

To test whether EGFR signaling affects the survival of mice after TBI, we treated C57BL/6 mice with 10 µg per g body weight erlotinib or water (control) from day –3 to day +14 after 700-cGy TBI. Fifty-three percent (8 of 14) of control irradiated mice remained alive through day +30. In contrast, none (0 of 15) of the erlotinib-treated mice survived through day +30. Conversely, 93% of EGF-treated mice (14 of 15) remained alive through day +30. These results suggest that pharmacologic modulation of EGFR signaling alters survival after TBI.

Figure 5 EGF promotes HSC cycling and survival after irradiation. (a) Representative FACS analysis of cell-cycle status of bone marrow KSL cells at day 0 and 72 h after irradiation in cells cultured with either TSF or TSF plus EGF, G0 white bar; G1, light gray bar; G2/S/M, gray bar. *P = 0.002, ^P = 0.002 compared to TSF for G0 and G2/S/M, respectively (means ± s.e.m., n = 3–5 experiments per group). The numbers shown indicate the percentage of each annotated population within bone marrow KSL cells. (b) Representative BrdU incorporation in bone marrow KSL cells in vivo at day 7 after 700-cGy TBI and treatment with EGF or saline (left). Right, mean BrdU incorporation. *P = 0.02 (means ± s.e.m., n = 3 experiments per group, two-tailed t test). The numbers shown indicate the percentage of BrdU+ cells within the total bone marrow KSL population. (c) Left, percentage phosphorylated AKT (pAKT) in bone marrow KSL cells after 300-cGy irradiation and the culture conditions shown. *P = 0.002 for TSF compared to EGF, ^P = 0.0006 for EGF compared to EGF plus Ly294002 (LY29) (means ± s.e.m., n = 7–8 experiments per group). Middle, FACS analysis of cell-cycle status of KSL cells from PUMA-expressing (+PUMA) and PUMA-deficient (PUMA-def) mice at day +30. In contrast, none (0 of 15) of the erlotinib-treated mice survived through day +30. Fifty-three percent (8 of 15) of control irradiated mice remained alive through day +30 after 700-cGy TBI. Fifty-seven percent of saline-treated mice (8 of 14) survived through day +30 (Fig. 6). Conversely, 93% of EGF-treated mice (14 of 15) remained alive through day +30. These results suggest that pharmacologic modulation of EGFR signaling alters survival after TBI.

A Mann-Whitney test was used for all statistical analyses.
DISCUSSION

Recent studies suggest that hematopoietic regeneration in vivo is regulated by bone marrow endothelial cells\(^6\). However, the mechanisms through which these cells regulate hematopoietic regeneration remain largely unknown. Identification of the mechanisms that govern hematopoietic regeneration could have broad implications for the treatment of patients receiving myelosuppressive chemotherapy or radiotherapy or those undergoing stem cell transplantation\(^6\). Here we demonstrate that EGF, which we identified using a screen of bone marrow serum from radioprotected mice bearing deletion of \(Bax\) and \(Bak\) in Tie2-expressing endothelial cells, mitigates radiation injury to HSCs, and systemic administration of EGF can improve the survival of irradiated mice. Of note, EGF is a mitogen for nonhematopoietic cells that are affected by radiation injury (for example, intestinal epithelium), and such effects may have contributed to the EGF-mediated improvement in mouse survival. Nonetheless, our results suggest that systemic administration of EGF could have therapeutic potential to accelerate hematopoietic recovery in patients who have received TBI, as well as in victims of acute radiation sickness\(^27,28\). In that regard, it is worth noting that EGF treatment caused a significant enhancement in myeloid reconstitution in primary transplanted mice, suggesting a specific effect on short-term HSCs with myeloid repopulating potential. Furthermore, whereas EGF treatment was associated with improved multilineage hematopoietic reconstitution in secondary transplanted mice, stronger myeloid recovery was observed, and T cell reconstitution was relatively low. This skewing toward myeloid recovery at the expense of T lymphoid recovery in recipients of bone marrow cells from irradiated donors mimics some aspects of the HSC aging phenotype and may reflect TBI-mediated DNA damage to the HSC pool\(^29,30\).

As erlotinib has recently been shown to mediate cellular effects through inhibition of targets other than EGFR\(^17,18\), we used \(Vav^{Cre}\); \(Egf\text{-}\text{flkx/flox}\) mice to determine the specific role of EGFR in regulating the hematopoietic response to radiation. In vitro studies demonstrated that erlotinib treatment had no effect on EGFR-deficient hematopoietic cells in culture, whereas erlotinib treatment of \(Egf^{+/+}\) hematopoietic cells significantly inhibited both cell expansion and CFC production in culture. Therefore, erlotinib acts specifically through EGFR inhibition to diminish hematopoietic progenitor cell recovery in our model. Notably, \(Vav^{Cre}\); \(Egf^{flkx/+}\) mice, which are heterozygous for EGFR expression, had significantly decreased bone marrow HSC and progenitor cell recovery early after TBI compared to \(Vav^{Cre}\); \(Egf^{+/+}\) mice, which retain both EGFR alleles. These results suggest that EGFR signaling has an important role in regulating hematopoietic regeneration after radiation injury.

Gamma radiation causes direct and indirect DNA damage that can result in cell-cycle arrest or apoptosis of hematopoietic progenitor cells\(^31\). In the presence of radiation-induced DNA damage, cell-cycle arrest can occur through p53-dependent or independent mechanisms\(^32,33\). Cell-cycle arrest of hematopoietic cells can be overridden by treatment with cytokines\(^34\), and cytokine-mediated induction of hematopoietic progenitor cell proliferation early after radiation exposure may have beneficial effects toward promoting short-term hematopoietic recovery and improved near-term survival\(^10,15,36\). Although the mechanism behind these effects is not clear, cytokine treatment may induce synchronous entry of hematopoietic stem and progenitor cells into late S phase, a more radioresistant phase of the cell cycle\(^36,37\). Cytokine-driven differentiation of HSCs into myeloid progenitor cells could also provide short-term benefit, as systemic infusion of myeloid progenitor cells alone can completely radioprotec mice after lethal-dose TBI\(^38\). In nonhematopoietic tissues, EGFR signaling can regulate cell proliferation in a context-dependent manner, producing both cell-cycle arrest and augmented proliferation\(^22,39\).

Here we show that EGF treatment induces early HSC cycling after radiation exposure and that this effect is mediated through activation of the PI3K-AKT pathway. Although EGF can modulate radiation responses through additional mechanisms such as induction of DNA repair mechanisms\(^23\), our data suggest that EGF-mediated induction of HSC proliferation contributes to the early recovery of the hematopoietic progenitor pool after irradiation.

Deletion of PUMA has been shown to protect bone marrow hematopoietic stem and progenitor cells from radiation-induced death and confer a survival advantage in mice after TBI\(^24,25\). We have shown that EGF treatment represses radiation-induced expression of PUMA in HSCs. Furthermore the effects of EGF are dependent largely on repression of PUMA. This result is consistent with previous studies that have shown that cytokines such as interleukin-3 (IL-3) can inhibit PUMA expression in hematopoietic cells and that cytokine withdrawal mediates hematopoietic cell death in a PUMA-dependent manner\(^40,41\). Of note, AKT, which is induced by EGF treatment of HSCs, was reported to suppress p53-dependent induction of hematopoietic progenitor cell death in a PUMA-dependent manner\(^40,41\). Our data suggest that EGF-mediated induction of HSC apoptosis is dependent on repression of PUMA, it remains possible that changes in the expression of other cell-cycle regulators (for example, induction of p21) in PUMA-deficient mice\(^43\) may contribute to the effects of PUMA deficiency on the regenerative effects of EGF.

EGF has been implicated in regulating cell survival and stem cell fate outside the hematopoietic system. EGF is an established mitogen and prosurvival factor for epithelial cells and endothelial cells, and mutations in EGFR can be tumorigenic\(^44-48\). EGF also regulates stem cell functions in nonhematopoietic tissues such as the brain and liver\(^49-51\). Previous studies have suggested that EGFR is not expressed in hematopoietic stem cells\(^52-54\), although these studies did not use multiparametric flow cytometry to isolate bone marrow HSCs as we performed here. Although EGFR was recently shown to be expressed in bone marrow c-Kit\(^+\) Lin\(^-\) progenitor cells\(^55,56\), EGFR was not previously known to regulate HSC self renewal or regeneration. In a previous study, the addition of EGF to stromal cell cocultures was shown to...
inhibit hematopoietic progenitor cell growth in vitro, but these effects could be attributed to indirect effects on stromal cells57. It was also recently reported that EGFR inhibition facilitates granulocyte colony-stimulating factor (GCSF)-mediated mobilization of hematopoietic progenitor cells in mice55. In this study, no effects were demonstrated in the absence of G-CSF and no effects on HSC content, proliferation or function were described55.

We have elucidated a previously unknown function of EGF in promoting HSC regeneration after radiation-induced myelosuppression. We show that bone marrow HSCs express functional EGFR and that EGF acts directly on HSCs to increase HSC cycling and survival after irradiation. These observed effects of EGF on HSC growth are comparable to those described for fibroblast growth factor (FGF) stimulation. We show that bone marrow HSCs express functional EGFR, which also activates PI3K-AKT signaling, suggesting a possible convergence of action of EGF and FGF1 on crucial signaling pathways in HSCs58,59. Transcriptionally, these results suggest that EGF may have therapeutic potential in patients undergoing hematopoietic cell transplant who receive TBI-based conditioning before transplant. In light of a recent report of a deleterious bystander effect of TBI-induced reactive oxygen species on transplanted donor HSCs, it will be interesting to determine whether EGF administration can ameliorate such effects to augment hematopoietic reconstitution60. EGF also has potential utility for the treatment of acute radiation sickness, which can cause life threatening bone marrow failure and for which few treatments exist.

METHODS
Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
P.L.D. performed experiments, analyzed data and wrote the paper. H.A.H., K.H. and J.L.R. performed experiments and analyzed data. E.F., M.Q., I.R.H. and D.D. performed experiments. J.M.S. provided reagents. N.J.C. analyzed data and wrote the paper. D.G.K. designed experiments, analyzed data and wrote the paper. J.P.C. conceived of the study, designed experiments, analyzed the data and wrote the paper.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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online methods

animals. ten- to 12-week-old female C57BL/6 (CD45.2) mice and B6.SJl
(CD45.1) mice were obtained from Jackson Laboratory (Bar Harbor, ME).
Tie2Cre; Bakt1/2-; BaxBos-1 and Tie2Cre; Bakt1/2-; BaxBos-1 mice were generated as previously described3. Egrflox/crox mice4 (Mutant Mouse Regional Resource
Centers, Chapel Hill, NC) were bred with VavCre mice (Jackson Laboratory) to
generate VavCre; Egrflox mice. In VavCre mice, floxed alleles are excised by Cre
in Vav-expressing cells and their progeny62. To generate VavCre; Egrflox mice,
we mated VavCre; Egrflox mice with Egrflox mice. Mice were geno-
typed for the cre allele by Transnetyx, Inc. (Cordova, TN) and for the Egrflox allele as previously described4. Egrf deletion in bone marrow cells was quanti-
ified using RT-PCR (Applied Biosystems, Carlsbad, CA). Egrflox littermate
mice were used as controls in some experiments. Tbp35+ and Tbp35– mice64
were purchased from Jackson Laboratories and genotyped through Transnetyx
Inc. Bhc3+/+ (PUMA-expressing) and Bhc3–/– (PUMA-deficient) mice were pur-
chased from Jackson Laboratory65. Tbp53 and Bhc3 deletions were quantified
with RT-PCR (Applied Biosystems, Carlsbad, CA). All animal studies described
here were approved by the Duke University Animal Care and Use Committee.
The background strain of the Tbp53, Bhc3 and Egrf floxed mice was C57BL/6J.

Hematopoietic progenitor cell assays. Bone marrow cells were collected into
PBS (Cellgro, Manassas, VA) with 10% FBS (Hyclone, Logan, UT) and 1% penicil-
in and streptomycin (GIBCO, Grand Island, NY). Viable bone marrow cells were
quantified using Trypan blue stain (Lonza, Basel, Switzerland) to exclude apo-
totic and dead cells. Cells were then incubated with antibodies to c-Kit, Sca-1,
a Lin-specific antibody cocktail, CD41 (1% dilution, clone eBioscience, San Jose, CA), CD48 (1% dilution, clone HM48-1, eBioscience) and
2% FBS in 1× PBS and then labeled with antibodies to Ki67–phycoerythrin
(BD). Cell proliferation was measured in C57BL/6 mice exposed to 700-cGy TBI
and administered BrdU-FITC (1% dilution, catalog number 559619, BD) and
BrdU–FITC (1% dilution, catalog number 559619, BD). Incorporation of BrdU was analyzed by flow cytometry according to the
manufacturer’s staining protocol (BD).

Because 300-cGy irradiation in vitro induces apoptosis in bone marrow
hematopoietic progenitor cells, we irradiated 3 × 105 C57BL/6KSL cells with 300
cGy and then placed them in culture with TSF alone, TSF with 20 ng/ml EGF or
TSF, EGF and 1 μM Ly294002 (Cell Signaling Technology, Danvers, MA) for
72 h. Cell apoptosis and necrosis were analyzed by flow cytometry according to
manufacturer’s protocols with annexin V–FITC and 7-AAD staining (BD,
San Jose, CA). For analysis of phosphorylation AKT at Ser473, bone marrow KSL
were collected for 15 min with TSF or TSF plus 20 ng/ml EGF or with 20 μM
Ly294002. Cells were fixed and permeabilized with Fix Buffer I and Perm Buffer
III (BD) and then stained with mouse antibody to pAKT (Ser473)-PE (BD)
(1% dilution, catalog number 560378, BD) or isotype control antibody.

Generation and culture of primary bone marrow endothelial cells from
Tie2Cre; Bakt1/2-; BaxBos-1 mice. For isolation and generation of primary bone
marrow endothelial cells from FL– and FL+/+ mice, whole bone marrow was
collected from bilateral femurs and passed through a 70-
µm filter. Bone marrow
vessel fragments were then plated, rinsed with 10% FBS, washed in PBS, and
treated with 0.25% trypsin–EDTA. Bone marrow vessel explants were cultured on
10% gelatin-coated wells (Sigma-Aldrich) with EGM-2 (endothelial cell
collected from bilateral femurs and passed through a 70-
marrow endothelial cells from FL– and FL/+ mice, whole bone marrow was
collected from donor mice and
alone or in noncontact culture with FL– or FL/+ endothelial cells. In some
experiments, cells were cultured daily for 7–10 d and primary cells were passaged
when confluent.

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vessel fragments were then plated, rinsed with 10% FBS, washed in PBS, and
treated with 0.25% trypsin–EDTA. Bone marrow vessel explants were cultured on
10% gelatin-coated wells (Sigma-Aldrich) with EGM-2 (endothelial cell
growth medium-2) (Lonza) as previously described2,3,9,10,15. Wells were washed
daily for 7–10 d and primary cells were passaged when confluent.

CD34+ cells were isolated by negative selection on a HemaVet 950 (Drew Scientific, Dallas, TX).

Bone marrow KSL cells from adult C57BL/6 mice were exposed to 300 cGy
in vitro and then cultured with TSF (20 ng/ml thrombopoietin, 125 ng/ml stem
cell factor and 50 ng/ml Flt-3 ligand; TSF; R&D Systems, Minneapolis, MN)
alone or in noncontact culture with FL– or FL+/+ endothelial cells. In some
experiments, cultures were supplemented with 20 ng/ml EGF or 1 μg/ml of a
blocking antibody to EGF (1% dilution, catalog number AF2028, R&D Systems,
Minneapolis, MN). After 7 d in culture, cell progeny were collected and CFC
and CFU–S12 assays were performed as previously described2.

Cytokine array and EGF and EGRF expression analyses. Whole bone
marrow was collected from adult, nonirradiated Tie2Cre; Bakt1/2-; BaxBos-1 and Tie2Cre; Bakt1/2-; BaxBos-1 mice and C57BL/6 mice and at 6 h and 7 d after
750-cGy TBI. After centrifugation, bone marrow supernatants were collected into
Iscove’s modified Dulbecco’s medium and analyzed for cytokine concentrations
using the Quantikine mouse cytokine array 1000 according to manufacturer’s
guidelines (RayBiotech, Inc., Norcross, GA). For analyses of protein expres-
sion of EGRF by C57BL/6 bone marrow Lin–, Lin–, KSL and SLAM+ KSL cells,
bone marrow Lin+ cells were isolated using MACS microbeads and LS columns
according to the manufacturer’s specifications (Miltenyi Biotec, Auburn, CA). Bone
marrow Lin– cells were stained with fluorescein isothiocyanate (FITC)–labeled
EGFR antibodies (1% dilution, catalog number ab11400, Abcam, Cambridge,
MA) and antibodies to SLAM and KSL markers, as noted above. For analysis of
EGFR expression after irradiation, C57BL/6 mice were exposed to 300–cGy or
700–cGy TBI and then euthanized at 4 h. Bone marrow Lin– cells were isolated
and stained with 7-AAD and antibodies to SLAM and KSL markers, as noted
above. For analysis of phosphorylation of the Tyr1173 residue of EGRF, bone
marrow Lin+ and Lin– cells were isolated using MACS Lin depletion columns
as described above. Bone marrow KSL and SLAM+ KSL cells were isolated using
FACS. Cells were cultured for 45 min in X-Vivo medium (Lanza) alone or in
medium supplemented with 20 ng/ml EGF and then fixed in 4% paraformal-
dehyde and permeabilized in 0.25% saponin. Cells were stained with rabbit
polyclonal antibody to mouse pTyr1173 (1% dilution, catalog number ab5652,
Abcam) or isotype control antibody and then incubated with a secondary goat
antibody to rabbit Alexa Fluor 488 (Life Technologies). Functional studies were also performed using bone
marrow Lin+ and Lin– cells with TSF, TSF plus 20 ng/ml EGF or
TSF, 20 ng/ml EGF and then stained with mouse antibody to pAKT (Ser473)-PE (BD)
(1% dilution, catalog number 560378, BD) or isotype control antibody.

VavCre; Egrflox mice were cultured
with TSF, TSF plus 10
µg per g body weight EGF (R&D Systems,

Competitive repopulation assays and survival studies. Competitive repopulation
assays were performed using bone marrow cells harvested at day +7 from donor
C57BL/6 mice (CD45.2) that had been irradiated with 700-cGy TBI and given
daily intraperitoneal injections of 0.5 µg per g body weight EGF (R&D Systems,

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Minneapolis, MN) or 200 µl PBS starting at 2 h after TBI on day 0 through day +7. Competitive repopulation assays were also performed using bone marrow cells from nonirradiated C57BL/6 mice as positive controls to compare with irradiated and saline-treated and irradiated and EGFR-treated donors. Competitive repopulation assays were also performed with bone marrow harvested at day +14 from donor C57BL/6 mice (CD45.2^+) that had been irradiated with 700-cGy TBI and gavaged daily with 10 µg per g body weight erlotinib (Genentech, San Francisco, CA) or 150 µl water beginning on day 0 and continued through day +14. Donor bone marrow cells were injected by tail vein into recipient B6.SJL mice (CD45.1^+) at a dose of 5 × 10^5 cells with a competing dose of host 1 × 10^5 bone marrow mono- nuclear cells (MNCs). Primary total CD45.2^+ donor cell engraftment, multilineage engraftment and donor chimerism within bone marrow KSL cells were measured in recipient mice at 12 weeks after transplant. Secondary competitive repopulation assays into lethally irradiated B6.SJL mice were performed using 75% of the total whole bone marrow cells from a group of primary recipient mice, transplanted as described above, and a competing dose of host 1 × 10^5 bone marrow MNCs. Measurement of donor cell chimerism within the bone marrow KSL population was performed at 12 weeks after transplantation in primary and secondary transplanted mice, as previously described. Tie2Cre; Bak1^−/−; Bax^flox/− mice were gavaged daily with 10 µg per g body weight erlotinib or water starting day −3 and given 300-cGy TBI on day 0. Erlotinib administration continued until the time point of donor bone marrow cell collection and analysis. Tie2Cre; Bak1^−/−; Bax^flox/− bone marrow cells were injected by tail vein into recipient CD45.1^+ mice at a cell dose of 3 × 10^5 cells with a competing dose of host 1 × 10^5 CD45.1^+ cells. Multilineage hematopoietic reconstitution was measured in the peripheral blood of recipient mice by flow cytometry at weeks 4, 8 and 12 after transplant. For survival studies with erlotinib administration, C57BL/6 mice were exposed to 700-cGy irradiation and then given 10 µg per g body weight erlotinib or saline starting on day −3 and continuing daily through day +14. Age-matched adult C57BL/6 mice were also exposed to 700-cGy TBI and then given tail vein injections with 0.5 µg per g body weight EGF or saline beginning at +2 h after TBI and then daily through day +7. We chose 700 cGy for lethality studies because this radiation dose causes 50% lethality by day +30 in adult C57BL/6 mice at Duke University Medical Center (Cs137 irradiator).

**Immunohistochemical analyses.** Femurs were decalcified and embedded in optimal cutting temperature (OCT) medium (Sakura Finetek, Torrance, CA), as previously described, on day 7 or 14 after 700-cGy TBI with daily administration of EGF or erlotinib. Ten-micrometer sections were cut using the CryoJane tape system (Instrumedics Inc, Hackensack, NJ, USA). Femurs were stained with H&E or antibody to MECA-32 (1% dilution, catalog number 550563, BD) as previously described to assess bone marrow cellularity and the bone marrow vasculature after irradiation. Images were obtained using an Axiosvert 200 microscope (Carl Zeiss Microscopy, Thornwood, NY) or a Leica SP5 confocal microscope (Leica Microsystems Inc, Buffalo Grove, IL). Adobe Photoshop software (version 9.0.2, Adobe Systems, San Jose, CA) was used to quantify a positive signal as a measure of the total number of pixels.

**Statistical analyses.** Data are shown as means ± s.e.m. We used the Mann-Whitney test (two-tailed nonparametric analysis) for the majority of comparisons, along with the Student’s t test (two-tailed or one-tailed distribution with unequal variance). Comparisons of overall survival were performed using a log-rank test.

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