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RESEARCH ARTICLE

Canonical signaling by TGF family members in mesenchymal stromal cells is dispensable for hematopoietic niche maintenance under basal and stress conditions

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Abstract

Mesenchymal stromal cells are an important component of the bone marrow hematopoietic niche. Prior studies showed that signaling from members of the transforming growth factor (TGF) superfamily in mesenchymal stromal cells is required for normal niche development. Here, we assessed the impact of TGF family signaling on niche maintenance and stress responses by deleting Smad4 in mesenchymal stromal cells at birth, thereby abrogating canonical TGF signaling. No alteration in the number or spatial organization of CXCL12-abundant reticular (CAR) cells, osteoblasts, or adipocytes was observed in Osx-Cre, Smad4fl/fl mice, and expression of key niche factors was normal. Basal hematopoiesis and stress erythropoiesis responses to acute hemolytic anemia were normal. TGF-β potently inhibits stromal CXCL12 expression in vitro; however, G-CSF induced decreases in bone marrow CXCL12 expression and subsequent hematopoietic stem/progenitor cell mobilization were normal in Osx-Cre, Tgfbr2fl/fl mice, in which all TGF-β signaling in mesenchymal stromal is lost. Finally, although a prior study showed that TGF-β enhances recovery from myeloablative therapy, hematopoietic recovery following single or multiple doses of 5-fluorouracil were normal in Osx-Cre, Tgfbr2fl/fl mice. Collectively, these data suggest that TGF family member signaling in mesenchymal stromal cells is dispensable for hematopoietic niche maintenance under basal and stress conditions.

Introduction

The bone marrow contains a complex dynamic population of stromal and hematopoietic cells that together generate a unique microenvironment, or niche, to support hematopoiesis. Mesenchymal stromal cells (MSCs) are an important component of the bone marrow hematopoietic niche and include CXCL12-abundant reticular (CAR) cells, adipocytes, osteolineage cells, arteriolar pericytes, and mesenchymal stem cells, all of which have been implicated in
hematopoietic stem/progenitor cell (HSPC) maintenance.\[1–7\] The signals that regulate MSCs and their impact on hematopoiesis are not well characterized.

There is evidence that TGF-\(\beta\) signaling regulates MSCs in the bone marrow. TGF-\(\beta\) has complex stage-specific effects on bone marrow MSCs. It stimulates osteoprogenitor proliferation and induces mesenchymal stem cell migration, while inhibiting terminal osteoblast differentiation.\[8\] In vitro modeling of the interaction between TGF-\(\beta\) and bone marrow MSCs reveal its potential to negatively regulate adipocyte and osteoblast differentiation while promoting osteoblast progenitor proliferation.\[9–11\] In addition, genetic abrogation of TGF-\(\beta\) signaling in mesenchymal progenitor cells during development results in impaired osteoblast differentiation and a marked expansion of CAR cells and bone marrow adiposity.\[12,13\] These stromal alterations are associated with a shift in hematopoiesis from lymphopoiesis to myelopoiesis.\[12\] In contrast, abrogation of TGF-\(\beta\) signaling in mesenchymal progenitor cells at birth (using a doxycycline-inducible Osx-Cre transgene) resulted in no discernable alterations in the niche or basal hematopoiesis. Thus, TGF-\(\beta\) signaling in mesenchymal cells during development is required for the establishment of a normal hematopoietic niche but is dispensable for niche maintenance in adults under steady-state conditions.

There also is evidence that other members of the TGF family of cytokines may contribute to the development, maintenance, and/or function of MSCs in the bone marrow. The TGF superfamily consists of approximately 45 ligands divided into four subgroups: TGF-\(\beta\)s, decapentaplegic-Vg-related (DVR), activins/inhibins, and other distant TGF members.\[14,15\] The DVR subgroup consists of bone morphogenetic proteins (BMPs) and growth differentiation factors (GDFs) which play a critical role in skeletal patterning and soft and hard tissue development.\[8,16–20\] Deletion of \(Bmpr1a\) (Alk3) in hematopoietic and stromal cells using \(Mx1-Cre\), which targets osteoblast lineage cells, is associated with an increase in N-cadherin positive osteoblasts and a modest increase in HSCs, suggesting that BMP signaling in stromal cells may negatively regulate the stem cell niche.\[21\] Multiple groups have also shown that inhibition of activin signaling by treating with an activin receptor 2 alpha (ACVR2a) ligand trap stimulates erythropoiesis in vivo.\[22–25\] Indeed, clinical trials have demonstrated improvements in anemia in patients with myelodysplastic syndrome treated with sotatercept, a ACVR2a antagonist.\[23,26\] Although ACVR2a signaling in erythroid progenitors contributes to this effect,\[22,24\] two groups showed that inhibition of ACVR2a signaling in bone marrow stromal cells also stimulates erythropoiesis.\[23,27\]

Myelosuppressive therapy induces marked alterations in the bone marrow microenvironment that includes an expansion of osteolineage cells and adipocytes, which have been linked to hematopoietic recovery.\[28,29\] Relevant to this study, myelosuppressive chemotherapy activates TGF-\(\beta\) in the bone marrow, and inhibition of TGF-\(\beta\) signaling enhances recovery from chemotherapy.\[30\] Whether TGF-\(\beta\) or other TGF family member signaling in mesenchymal cells contributes to stromal and hematopoietic responses to myeloablative therapy or other stressors is an open and clinically relevant question. Of note, recent single cell RNA sequencing of bone marrow stromal cells shows that \(Tgfb1, Tgfb2, Tgfb3,\) and \(Smad4\) are expressed in osteolineage cells and perivascular stromal cells.\[31\]

TGF family ligands bind to their cognate type I and type II serine/threonine kinase receptors to phosphorylate and active pathway-restricted SMADs (R-SMADs), which in turn complex with SMAD4 to active target genes. Thus, SMAD4 is required for all canonical TGF family signaling. Here, we show that deletion of \(Smad4\) in bone marrow MSCs at birth results in no discernible alteration in the bone marrow hematopoietic niche. Indeed, basal and stress hematopoiesis are normal. These data suggest that canonical TGF-\(\beta\) family signaling is not required for hematopoietic niche maintenance or niche response to certain hematopoietic stressors.
Results

Post-natal loss of Smad4 in MSCs does not alter the bone marrow stromal microenvironment

To investigate the role of canonical TGF family member signaling in bone marrow MSCs to hematopoietic niche maintenance, we deleted Smad4 in mesenchymal cells using a doxycycline-repressible Sp7 (osterix)-Cre transgene (Osx-Cre). Prior studies have shown that Osx-Cre targets the majority of MSCs in the bone marrow, including osteoblasts, adipocytes, pericytes, and CXCL12-abundant reticular (CAR) cells, but not endothelial cells or hematopoietic cells. [5,32,33] We previously reported that constitutive activation of Osx-Cre, by maintaining Osx-Cre, Smad4<sup>fl/fl</sup> mice off of doxycycline throughout embryonic development, results in a loss of osteoblasts, a marked increase in adiposity, and severe runting.[12] Thus, in this study, we activated the Osx-Cre transgene post-natally by removing doxycycline 1–2 days after birth. To confirm targeting, we performed lineage mapping studies on 6-to-8-week-old Osx-Cre, ROSA26<sup>Ai9/+</sup> (Ai9), Smad4<sup>fl/fl</sup> mice, in which tdTomato expression is induced in cells that have undergone Cre-mediated recombination. As expected, in control Osx-Cre, Ai9 mice tdTomato<sup>+</sup> perivascular CAR cells and osteocalcin<sup>+</sup> endosteal osteoblasts were observed; a similar pattern was observed in Osx-Cre, Ai9, Smad4<sup>fl/fl</sup> mice (Fig 1A and S1A Fig). To assess Smad4

Fig 1. Mesenchymal stromal cell number and organization are normal in Osx-Cre Smad4<sup>fl/fl</sup> mice. (A) Representative photomicrographs of femur sections from Osx-Cre Ai9 or Osx-Cre, Ai9, Smad4<sup>fl/fl</sup> mice showing DAPI stained nuclei (blue), osteocalcin (OCN) expressing mature osteoblasts (green), and tdTomato<sup>+</sup> mesenchymal stromal cells (red). (B) Relative Smad4 mRNA expression of sorted tdTomato<sup>+</sup> CD45<sup>−</sup> Ter119<sup>−</sup> stromal cells normalized to β-actin mRNA. (C) Representative flow plots showing the gating strategy used to identify CD31<sup>−</sup> Sca1<sup>−</sup> sinusoidal endothelial cells (SE) and CD31<sup>−</sup> Sca1<sup>−</sup> arteriolar endothelial cells (AE, left panel); data are gated on CD45<sup>−</sup> Ter119<sup>−</sup> cells. The right panel shows PDGFRβ<sup>−</sup> Sca1<sup>−</sup> mesenchymal stromal (PBS<sup>−</sup>) cells and PDGFRβ<sup>+</sup> Sca1<sup>−</sup> (PBS<sup>+</sup>) mesenchymal stem cells. (D) Number of SE, AE, PBS<sup>−</sup>, and PBS<sup>+</sup> cells per femur is shown for Osx-Cre (control) or Osx-Cre Smad4<sup>fl/fl</sup> mice. (E) Relative mRNA expression in whole bone marrow compared to β-actin mRNA of the indicated gene. Data represent the mean ± SEM. ***P < 0.0001 by unpaired student t-test.

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deletion, we sorted tdTomato⁺ stromal cells from the bone marrow of Osx-Cre, Ai9, Smad4⁺/⁻ mice. Expression of full length Smad4 mRNA was essentially absent, confirming efficient deletion of Smad4 in bone marrow MSCs (Fig 1B).

Immunostaining of bone sections from Osx-Cre, Ai9, Smad4⁺/⁻ mice suggested that the number and organization of bone marrow MSCs was comparable to control mice (Fig 1A, and S1 Fig). To further characterize the hematopoietic niche, we quantified the number of PDGFRβ⁺ Sca1⁺ CD31⁻ lineage cells (mesenchymal stem/progenitor cells) and PDGFRβ⁺ Sca1⁺ CD31⁻ lineage cells (a mixture of CAR cells and osteoblasts) by flow cytometry (Fig 1C). We also quantified CD31⁺ Sca1⁺ lineage venous sinusoidal cells and CD31⁺ Sca1⁺ lineage arteriolar endothelial cells. In each case, the number of stromal cells was similar to control mice (Fig 1D). We previously reported that loss of TGF-β signaling in MSCs during development results in a marked increase in bone marrow adiposity. However, no increase in bone marrow adipocytes was observed in Osx-Cre, Smad4⁺/⁻ mice (S1B Fig). Finally, total bone marrow expression of key niche factors, including Spp1, Scf, and Cxcl12, was comparable in Osx-Cre, Smad4⁺/⁻ and control mice (Fig 1E). Together, these data suggest SMAD4 signaling in bone marrow MSCs is not required for their maintenance under steady-state conditions.

Post-natal loss of Smad4 in MSCs does not alter basal hematopoiesis

We next examined basal hematopoiesis in Osx-Cre, Smad4⁺/⁻ mice. Peripheral blood counts and the level of circulating T cells, B cells, and neutrophils were normal (Fig 2A and 2B). Likewise, the number of myeloid, B-cell, and T-cell lineage cells in the bone marrow was comparable to control mice (Fig 2C and 2D). The number of phenotypic hematopoietic stem cells (HSCs), common myeloid progenitors (CMPs), granulocyte-macrophage progenitors (GMPs), megakaryocyte-erythocyte progenitors (MEPs), and LSK cells in the bone marrow of Osx-Cre, Smad4⁺/⁻ also was similar to control mice (Fig 2E–2G). Finally, no perturbation in

![Fig 2. Basal hematopoiesis is normal in Osx-Cre Smad4⁺/⁻ mice.](https://doi.org/10.1371/journal.pone.0233751.g002)
the number of mature hematopoietic cells or HSPCs in the spleen of Osx-Cre, Smad4<sup>fl/fl</sup> mice was observed (Fig 2H–2L). Collectively, these data show that SMAD4 signaling in bone marrow mesenchymal cells is not required to maintain basal hematopoiesis.

**Post-natal loss of Smad4 in MSCs does not alter basal or stress erythropoiesis**

There is evidence that activin signaling in bone marrow stromal cells may contribute to the regulation of erythropoiesis.[23] Flow cytometry was used to identify and quantify phenotypic erythroid progenitors in bone marrow and spleen (Fig 3A). The number of stage I proerythroblasts (CD44<sup>high</sup>, Ter119<sup>int</sup> cells), stage II basophilic erythroblasts (CD44<sup>high</sup>, Ter119<sup>high</sup> forward scatter<sup>high</sup> cells), stage III (polychromatic erythroblasts, CD44<sup>high</sup>, Ter119<sup>high</sup> forward scatter<sup>int</sup> cells), and stage IV orthochromatic erythroblasts (CD44<sup>high</sup>, Ter119<sup>high</sup> forward scatter<sup>high</sup> cells).

![Flow cytometry plots showing the gating strategy to identify different stages of erythroid development.](https://doi.org/10.1371/journal.pone.0233751.g003)
scatter\textsuperscript{high} cells) in the bone marrow or spleen was similar in control and Osx-Cre, Smad4\textsuperscript{fl/fl} mice (Fig 3B–3D).

To assess stress erythropoiesis, we characterized the response of mice to the induction of acute hemolytic anemia after phenylhydrazine treatment. As expected, in control mice, treatment with phenylhydrazine induced an acute fall in the hematocrit followed by recovery over a 10-day period (Fig 3E). The magnitude of anemia and kinetics of recovery were similar in Osx-Cre, Smad4\textsuperscript{fl/fl} mice. These data show that SMAD4-dependent signaling in mesenchymal stromal cells is not required for the suppressive effect of activins on erythropoiesis.

Post-natal loss of Smad4 in mesenchymal stromal cells does not alter hematopoietic recovery following myeloablative chemotherapy

A prior study suggested that TGF-β contributes to hematopoietic recovery following myeloablative therapy. However, the contribution of TGF-β signaling in MSCs to hematopoietic recovery is unknown. To address this question, we characterized the hematopoietic response to 5-fluorouracil (5-FU) in Osx-Cre, Tgfbr2\textsuperscript{0/0} mice, in which both canonical and non-canonical TGF-β signaling is abrogated in MSCs. Of note, we previously reported that basal hematopoiesis was normal in these mice when Osx-Cre was activated on day 1–2 after birth.[12] In control mice, treatment with a single dose of 5-FU induced neutropenia with a nadir on day 7 and complete recovery by day 9 (Fig 4A). Both the magnitude of neutropenia induced by 5-FU and kinetics of neutrophil recovery were similar in Osx-Cre, Tgfbr2\textsuperscript{0/0} mice. Repeated doses of 5-FU induce HSC exhaustion and death in mice due to hematopoietic failure. Similar median survival after weekly 5-FU was observed in Osx-Cre, Tgfbr2\textsuperscript{0/0} and control mice (Fig 4B).

These data show that TGF-β signaling in bone marrow MSCs does not contribute to hematopoietic recovery following myeloablative therapy. Of note, survival following repeated treatment with 5-FU in Osx-Cre, Smad4\textsuperscript{0/0} mice was comparable to control mice, suggesting that
canonical signaling by other TGF-β family members in MSCs also does not contribute to hematopoietic stress responses to myeloablative therapy (Fig 4C).

Loss of Smad4 in mesenchymal stromal cells does not affect HSPC mobilization by granulocyte-colony stimulating factor

There is strong evidence that G-CSF induced HSPC mobilization is mediated, at least in part, by the downregulation of CXCL12 expression in bone marrow MSCs.[34–39] Treatment of bone marrow stromal cell lines with TGF downregulates CXCL12 expression in a SMAD4-dependent fashion.[40,41] There also is evidence that G-CSF treatment is associated with increased circulating TGF-β1 levels.[42] Together, these observations suggest the hypothesis that canonical TGF-β family signaling in bone marrow MSCs, by downregulating CXCL12 expression, may contribute to G-CSF induced HSC mobilization. To test this hypothesis, we first asked whether TGF-β1 regulates CXCL12 in primary murine bone marrow MSCs. Indeed, treatment of cultures of primary bone marrow MSC with TGF-β1 for 3 days resulted in a significant decrease in CXCL12 mRNA expression (Fig 5A). We next characterized G-CSF induced HSPC mobilization in Osx-Cre, Smad4<sup>fl/fl</sup> mice. Treatment of control mice with G-CSF for 5 days induced robust mobilization of colony-forming cells (CFU-C) and Lineage<sup>-</sup> Sca<sup>+</sup> Kit<sup>+</sup> (LSK) cells into the blood and spleen (Fig 5B–5F). A similar level of HSPC mobilization was observed in Osx-Cre, Smad4<sup>fl/fl</sup> mice. Consistent with prior studies, G-CSF induced a marked decrease in total bone marrow CXCL12 mRNA expression, with a similar decrease observed in Osx-Cre, Smad4<sup>fl/fl</sup> mice (Fig 5G). We also analyzed G-CSF induced HSPC mobilization in Osx-Cre, Tgfbr2<sup>fl/fl</sup> mice, where all TGF-β1 signaling in MSCs is abrogated. Again, HPSC mobilization by G-CSF, as measured by CFU-C levels in blood and spleen, was comparable to control mice (S2 Fig). Collectively, these data show that neither TGF-β1 signaling nor canonical TGF family signaling in bone marrow MSCs is required for efficient HSPC mobilization by G-CSF.

Discussion

Recent single cell RNA sequencing studies of murine bone marrow stromal cells show that most of the receptors for TGF family members are expressed at low levels on mesenchymal...
stromal cells, including osteolineage cells, isolated from adult mice under steady state conditions. Prior studies have established that TGF family members play an important role during development in the generation of the hematopoietic niche in the bone marrow. In particular, we recently showed that TGF-β signaling plays a key role in the specification of fetal mesenchymal progenitor cells to a non-adipocyte fate. There also is evidence, mainly from cell culture systems, that TGF family members can regulate the function of mesenchymal stromal cells. In contrast, our data suggest that canonical SMAD4-dependent TGF family member signaling in mesenchymal stromal cells is dispensable for their maintenance in the bone marrow hematopoietic niche, once the niche is established. Of note, since Osx-Cre only targets mesenchymal stromal cells in the bone marrow, the contribution of TGF family member signaling in other stromal cells, such as endothelial cells, to basal and stress hematopoiesis remains uncertain.

There is evidence that alterations in mesenchymal stromal cells contribute to stress hematopoiesis responses. G-CSF induced HSPC mobilization is associated with a loss of active osteoblasts and decreased CXCL12 expression in bone marrow MSCs. Myeloablation due to chemotherapy or radiation therapy is associated with an expansion of bone marrow adipocytes, which support hematopoietic recovery through secretion of stem cell factor.[29] Myeloablative radiation also induces a loss of osteoblasts, which is followed by an expansion of osteolineage cells.[28] Our data suggest that SMAD-dependent signaling by TGF family members in bone marrow MSCs is not a major contributor to hematopoiesis responses to certain stressors, including myeloablation therapy with 5-FU, acute anemia, and G-CSF treatment. Whether TGF family member signaling contributes to hematopoiesis responses after other stressors, such as irradiation needs further study.

TGF family members activate multiple intracellular signaling pathways besides SMADs, including Erk[43–45], Rho-like GTPases[46–51], JNK/p38[52,53], and PI3K/Akt[54–56]. Our prior and current data suggest that both SMAD-dependent and -independent TGF-β signaling in bone marrow MSCs is dispensable for hematopoietic niche maintenance and response to certain stressors. However, for the other TGF family members, there is a possibility that non-SMAD dependent signaling may regulate bone marrow MSCs. Indeed, a prior study showed that loss of BMP receptor type 1A (Bmpr1a) in bone marrow MSCs results in an expansion of N-cadherin+ osteoblastic cells and an increase in HSCs.[21] Our data suggests that non-SMAD4 dependent BMP signaling likely mediates this response. Further studies are needed to test this hypothesis and characterize the contribution of non-canonical signaling by TGF family members in bone marrow MSCs to the regulation of basal or stress hematopoiesis.

Materials and methods

Contact for reagents and resource sharing

Further information and requests for resources and reagents should be directed to Daniel C. Link (danielclink@wustl.edu).

Mice and animal housing

Osx1-GFP::Cre[57], A19[58], Smad4fl/fl[59], and Tgfbr2fl/fl[60] mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were crossed to generate Osx-Cre Tgfbr2fl/fl, Osx-Cre A19 Tgfbr2fl/fl, Osx-Cre Smad4fl/fl, and Osx-Cre A19 Smad4fl/fl mice on a C57Bl/6 background. To suppress the Osx-Cre transgene throughout embryonic development, mice were maintained on doxycycline chow (200 mg/serving) until post-natal day 0. All experiments were done using 6-8-week-old mice. An equal number of male and female mice were used. Mice were maintained under standard pathogen-free conditions, and all of the procedures
performed in this study were approved by the Washington University Animal Studies Committee (approval number 20180018). Mice were anesthetized using isoflurane for all procedures, and they were euthanized using CO₂ asphyxiation followed by cervical dislocation.

**Flow cytometry**

Peripheral blood, bone marrow, and spleen mononuclear cell (MNC) preparations were suspended in Tris-buffered ammonium chloride (pH 7.2) buffer for 15 minutes at room temperature (RT) to lyse red blood cells. MNCs were then incubated with target antibodies at 4°C for 30 minutes in phosphate buffered saline (PBS) containing 1mM ethylenediaminetetraacetic acid (EDTA) and 0.2% (weight/volume) bovine serum albumin (BSA). The HSPC panel included Pe-Cy7-conjugated CD117 (2B8); BV711-conjugated Ly-6A/E; BV605-conjugated CD150 (TC15-12F12.2) BV421-conjugated CD48 (HM48-1); APC-conjugated CD16/32 (2.4G2); FITC-conjugated CD34 (RAM34); and PE-conjugated CD135 (A2F10.1) and the following APC-Cy7-conjugated antibodies recognizing lineage markers: CD3e (145-2C11), B220 (RA3-6B2), Gr1 (RB6-8C5), Ter119 (TER-119), and CD11b (M1/70). The mesenchymal cell panel included PerCP-Cy5.5-conjugated Ly-6A/E (D7), BV421-conjugated CD31 (390), biotin-conjugated PDGFRβ (APB5), PE-conjugated streptavidin, APC-Cy7-conjugated CD45 (30-F11) and Ter119 (TER-119) antibodies. The lineage cell panel included PE-conjugated CD115 (CSF-1R), FITC-conjugated Ly-6G/Ly-6C (RB6-8C5), BV421-conjugated CD45R/B220 (RA3-6B2), and APC-Cy7-conjugated CD3e (145-2C11). The erythroid panel included FITC-conjugated Ter119 (TER-119), PE-conjugated CD44 (IM7) and the following APC-Cy7-conjugated antibodies: Ly-6G/Ly-6C (RB6-8C5), CD45R/B220 (RA3-6B2), and CD3e (145-2C11). All antibodies were obtained from BioLegend (San Diego, CA), unless otherwise indicated. Data were acquired using a FACS Aria III flow cytometer (BD biosciences, San Jose, CA) and analyzed using FlowJo™ v10.6.1 software (BD biosciences).

**Cell sorting**

Mouse femurs, tibias, and pelvises were homogenized in PBS using a mortar and pestle. The cell suspension was then incubated in PBS containing 1.7 mg/ml collagenase type 1 (#17100017, ThermoFisher), 1.7 mg/ml collagenase type 2 (#LS004174, Worthington, Lakewood, NJ), and 1.7 mg/ml collagenase type 4 (#LS004188, Worthington) at 37°C while shaking for 15 minutes. The resulting cell suspension was filtered through CellTrics 50μm filters (Sysmex, Goerlitz, Germany) to remove debris, creating a single-cell suspension. The samples were then incubated at 4°C for 30 minutes in PBS containing 1mM EDTA and 0.2% BSA with the following panel of BV421-conjugated lineage antibodies: CD45, Gr1, CD11b, and B220. A19⁺ lineage⁻ cells were sorted using a Sony iCyt Synergy SY3200 (Synergy) cell sorter (Sony, San Jose, CA).

**Immunostaining of bone sections.** Mouse femurs were fixed in PBS containing 4% paraformaldehyde, pH 7.4, for 24 hours at 4°C. Bones were then decalcified in PBS containing 14% EDTA, pH 7.4, for 14 days at 4°C, changing buffer every 24 hours. Following incubation in PBS containing 30% sucrose for 1 hour at room temperature, bones were embedded in optimal cutting temperature compound (OCT) (Sakura Finetek, Torrance, CA). The tissue blocks were cut into 12μm sections using a Leica Cryo-Jane system (Leica Biosystems, Wetzlar, Germany). For immunostaining, the slides were first incubated in 0.1M Tris-Cl pH 7.5, 150mM NaCl, and 0.1% Tween 20 (TNT) buffer containing 10% donkey serum for 1 hour at room temperature. Sections were then incubated for 15 minutes at room temperature using the Avi-din/Biotin Blocking Kit (SP-2001; Vector Laboratories, Burlingame, CA). Sections were incubated with the primary antibody overnight at 4°C and, where applicable, then incubated with the secondary antibody at a 1 to 100 dilution for 1 hour at room temperature. The following
antibodies were used: rabbit anti-osteocalcin (FL-95) at a 1:50 dilution (Santa Cruz Biotechnology Dallas, TX) and goat anti-VE-cadherin (AF1002) at a 1:25 dilution (R&D Systems Minneapolis, MN). Finally, slides were mounted with ProLong Gold antifade reagent with DAPI (Life Technologies, Inc., Grand Island, NY). Images were acquired using an LSM 700 confocal microscope (Carl Zeiss Microscopy, Peabody, MA) and processed using Velocity™ v6.5.1 software (PerkinElmer, Waltham, MA). Oil red staining was performed using the Sigma Oil Red O kit per manufacturer’s recommendations (Millipore Sigma Darmstadt, Germany). Sections were mounted with Organo/Limonene Mount™ (Millipore Sigma), and images were acquired using a Hamamatsu Nanozoomer (Hamamatsu Photonics, Hamamatsu City, Japan).

Quantitative reverse-transcription PCR
Total bone marrow RNA was obtained by flushing femurs with 1 ml of Trizol (Invitrogen). RNA was prepared according to the manufacturer’s specification. One-step quantitative reverse-transcription PCR was performed using 50 ng of total RNA and the iTaq™ Universal Probes One-Step Kit (Bio Rad, Hercules, CA) with no template and no reverse transcriptase controls. mRNA expression is normalized to β-actin mRNA expression. Data was collected on a StepOnePlus™ Real-Time PCR System (Thermo Fisher). The following quantitative PCR primer/probe sets were used:
  TaqMan® Assays and Arrays:
  Actb, Mus_musculus VIC–spanning exons 1–2 Mm04394036_g1
  Kit ligand, Mus_musculus FAM–spanning exons 2–3 Mm00442972_m1
  Cxcl12, Mus_musculus FAM–spanning exons 2–3 Mm00445553_m1
  Spp1, Mus_musculus FAM–spanning exons 3–4 Mm00436767_m1
  IDT PrimeTime Std® qPCR Assay:
  Smad4, Mus_musculus FAM–spanning exons 8–9 Mm.PT.58.31543505

Mesenchymal stromal cell culture
C57BL/6 mice were sacrificed at 3-to-4-weeks-old, and hindlimb bones were harvested and mechanically disrupted in complete Dulbecco’s modified eagle medium (DMEM) containing 20% fetal bovine serum (FBS) and penicillin-streptomycin using a mortar and pestle. The resulting cell suspension was cultured overnight at 37°C with 5% CO₂, and the following day non-adherent cells removed by gentle aspiration. Adherent cells were cultured until reaching approximately 40% confluence (generally, 5–6 days). 100,000–200,000 cells were plated in 6 well plates 24 hours prior to treatment. TGFβ1 (14-8342-80, Life Technologies) was added every 24 hours for 72 hours at a dose of 10ng/mL.

Colony-forming unit assay
25μL of peripheral blood, or 50,000 bone marrow MNCs, or 50,000 spleen MNCs were suspended in DMEM containing 2% FBS and 1% penicillin-streptomycin. The cell suspensions were mixed with 2 mL of methocult (M3434, Stemcell Technologies, Vancouver, Canada) prior to plating 1.1 mL of the methocult-cell mix in one 35 mm dish, repeating with the remaining mix for two technical replicates. The dishes were placed in a 100 mm petri dish with a third 35 mm dish containing 3 mL of sterile water. The samples were incubated at 37°C, 5% CO₂ in air and ≥ 95% humidity for 7–10 days prior to colony counting.
Phenylhydrazine, 5-FU, and G-CSF treatments
Phenylhydrazine (30 mg/kg, Sigma) was given daily for 2 days by intraperitoneal injection. A single injection of 5-FU (150 mg/kg, F6627-5G, Sigma) was given by intraperitoneal injection. For the HSC exhaustion experiments, mice were treated with weekly 150mg/kg doses of 5-FU. Human G-CSF (125 mg/kg, Amgen, Thousand Oaks, CA) was given twice daily for 7 days via intraperitoneal injection. Complete blood counts were obtained using the HV950 hemavet (Drew Scientific, Miami Lakes, FL).

Quantification and statistical analysis
Significance was determined using Prism v8.1.2 (GraphPad, San Diego, CA, USA). For single parameter analysis, unpaired t-test were used to assess statistical significance. For multiple parameter data, statistical significance was calculated using one-way or two-way analysis of variance (ANOVA). P values less than 0.05 were considered significant. Expression data are log transformed prior to statistical analysis.

Supporting information
S1 Fig. Characterization of mesenchymal stromal cells in Osx-Cre, Ai9, Smad4<sup>fl/fl</sup> mice. (A) Representative photomicrographs of femur sections from Osx-Cre Ai9 (control) or Osx-Cre, Ai9, Smad4<sup>fl/fl</sup> (Smad4<sup>fl/fl</sup>) mice showing DAPI stained nuclei and tdTomato (Ai9) mesenchymal stromal cells with morphologic similarities to CAR cells. (B) Representative images of femurs stained with oil red o (purple/red staining) to identify adipocytes (black arrowheads). Femur sections from Osx-Cre Tgfbr2<sup>fl/fl</sup> mice are included as a positive control, since we previously showed that constitutive deletion of Tgfbr2 in mesenchymal stromal cells is associated with a marked increase in bone marrow adiposity (Abou-Ezzi, Stem Cell Reports, 2019).
(TIF)

S2 Fig. G-CSF induced HSPC mobilization is normal in Osx-Cre Tgfbr2<sup>fl/fl</sup> mice. (A-C) Osx-Cre Tgfbr2<sup>fl/fl</sup> or Osx-Cre (control mice) were treated with 125 mg/kg of granulocyte-colony stimulating factor (G-CSF) twice daily for 7 days (). Shown are the number of colony forming cells (CFU-C) in bone marrow (A), blood (B) or spleen (C). D-E) Shown are the number of LSK cells in bone marrow (D) and spleen (E) after 7 days of G-CSF. Data represent the mean ± SEM. ***P < 0.001 and ****P < 0.0001 by two-way ANOVA with an alpha of 0.05 and Sidak’s multiple comparisons test. The saline treated cohort is the same as in Fig 5.
(TIF)

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Conceptualization: Joseph Ryan Krambs, Grazia Abou Ezzi, Daniel C. Link.
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