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Activation of Wnt Signaling in Hematopoietic Regeneration

KENDRA L. CONGDON, CARLIJN VOERMANS, EMILY C. FERGUSON, LEAH N. DIMASCIO, MWEIA UQOEZWA, CHEN ZHAO, TANNISHTHA REYA

Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina, USA

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ABSTRACT

Hematopoietic stem cells (HSCs) respond to injury by rapidly proliferating and regenerating the hematopoietic system. Little is known about the intracellular programs that are activated within HSCs during this regenerative process and how this response may be influenced by alterations in signals from the injured microenvironment. Here we have examined the regenerating microenvironment and find that following injury it has an enhanced ability to support HSCs. During this regenerative phase, both hematopoietic and stromal cell elements within the bone marrow microenvironment show increased expression of Wnt10b, which can function to enhance growth of hematopoietic precursors. In addition, regenerating HSCs show increased activation of Wnt signaling, suggesting that microenvironmental changes in Wnt expression after injury may be integrated with the responses of the hematopoietic progenitors. Cumulatively, our data reveal that growth signals in the hematopoietic system are re-activated during injury, and provide novel insight into the influence of the microenvironment during regeneration. STEM CELLS *2008;26:1202–1210*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Along with maintaining a steady state of hematopoietic cell production for the lifetime of an organism, hematopoietic stem cells (HSCs) also have the ability to proliferate rapidly and repair loss of the hematopoietic compartment in response to injury. Loss of cells due to events such as chemotherapy, infection, and radiation exposure ablates rapidly cycling cells, which includes much of the developing hematopoietic system. In response to such injury, HSCs initiate a program of rapid proliferation in order to regenerate the lost hematopoietic compartment. Presently, neither the changes in the extracellular microenvironment nor the intracellular signals activated by HSCs during the proliferative phase of regeneration are known. Furthermore, it is also unknown whether the signals that modulate regeneration after injury are the same as or distinct from those implicated in the homeostatic maintenance and growth of HSCs and progenitors.

Any injury to the hematopoietic system results in a significant loss of cells and consequent changes in the microenvironmental milieu of HSCs. It is likely that HSCs sense these changes and in turn activate intracellular signals to initiate the proliferation process that ultimately leads to successful regeneration of the hematopoietic compartment. Studies examining the bone marrow environment following injury have revealed changes in mRNA levels of multiple growth factors, including stem cell factor, stromal cell derived factor 1, and transforming growth factor β1 [1]. In addition, recent work has shown that the bone marrow microenvironment becomes increasingly hypoxic after treatment with granulocyte colony stimulating factor (G-CSF) or injury induced by a chemotherapeutic agent [2]. Although these point to a changing microenvironment that could potentially influence HSC regeneration, the significance of these changes for HSCs remains unclear. Comparison of gene expression profiles of human and mouse progenitor and HSC populations before and during regeneration have begun to identify the intrinsic changes in regenerating HSCs [3, 4]. However, the critical issue of whether the extrinsic microenvironmental changes that occur initiate the intrinsic genetic changes in HSCs and how these signals may be integrated remains unresolved.

To gain an understanding of these issues in regeneration, we have focused on how the Wnt signaling pathway may influence regeneration. Wnt proteins are a large family of secreted signaling molecules that are expressed in diverse tissues and have been shown to influence multiple processes in vertebrate and invertebrate development (reviewed in [5]). In addition, a role for Wnt signaling during hematopoiesis is beginning to emerge, as modulation of Wnt signaling can affect HSC function. Activation of the Wnt pathway can increase proliferation of HSC-enriched cells [6] and the ability of HSCs to reconstitute the hematopoietic system of lethally irradiated mice after bone marrow transplantation, whereas overexpression of Axin, a Wnt signaling inhibitor, has the opposite effect [7]. In addition, ectopic activation of the Wnt pathway in committed hematopoietic progenitors can generate cells with stem cell-like properties [8]. A supportive effect for Wnt signaling in human hematopoietic stem cells has also been demonstrated [9], as delivery of Wnt5a in vivo leads to increased reconstitution efficiency of human progenitors/stem cells [10]. Interestingly, constitutive activation of Wnt signaling in a transgenic mouse model causes a block in differentiation; as a consequence, there is an expansion of the phenotypic HSC pool but an inability of these cells to transplant effectively [11, 12]. In an inducible model of β-catenin deletion, HSCs demonstrate normal functional capacity [13]. In contrast, in a model with a genetic deletion of β-catenin using Vav-Cre, HSC function is compromised as hematopoietic reconstitution after transplant is decreased [14]. These data may

Correspondence: Tannishtha Reya, Ph.D., C333 LSRC Building, Research Drive, Durham, North Carolina 27710, USA. Telephone: 919-613-8756; Fax: 919-668-3556; e-mail: t.reya@duke.edu Received September 12, 2007; accepted for publication February 12, 2008; first published online in STEM CELLS *EXPRESS* February 28, 2008; available online without subscription through the open access option.
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indicate that the potency and mode of activation/inhibition of Wnt signaling may determine its influence on HSCs.

Although the effect of Wnt signaling in HSC homeostasis has been investigated using a number of different approaches, as described above, whether the pathway is reactivated after injury in the hematopoietic system is unknown. To address this issue, we modeled hematopoietic injury using the chemotherapeutic drug cyclophosphamide (Cy) in conjunction with the cytokine G-CSF (G). Although treatment with either Cy or G-CSF can induce the HSC regenerative response, treatment with both yields the greatest number of HSCs as a response to the loss of the hematopoietic compartment [15], and it was thus chosen to observe maximal regeneration. Because of the significant HSC expansion and subsequent mobilization that occurs, this approach is commonly used clinically to harvest stem cells for transplants in clinical therapy [16–20], as well as to study migration [21–24]; however, less is known about how changes in the microenvironment may be coordinated with the intrinsic changes in HSCs.

Using this model, we demonstrate that following injury the soluble fraction of the bone marrow microenvironment develops an enhanced ability to support HSCs. Examination of the injured bone marrow microenvironment revealed that both hematopoietic and stromal cells specifically upregulate their expression of Wnt10b. Furthermore, regenerating HSCs show increased activation of the Wnt signaling pathway. Since we find that Wnt10b functions as an HSC growth factor, we propose that elevated levels of Wnt10b after Cy/G-mediated injury may serve to extrinsically activate the Wnt pathway in regenerating HSCs. These findings are the first to demonstrate that the canonical Wnt cascade is activated by regenerating HSCs and that this activation coincides with an increase in the microenvironmental availability of a specific Wnt ligand, thus providing novel insight into both the microenvironmental changes that occur after injury and how these changes are integrated with the regeneration of hematopoietic cells. Importantly, the finding that renewal signals are reactivated during tissue injury lends support to the proposal that recurrent activation of renewal signals during tissue injury may form the basis of oncogenic transformation [25].

MATERIALS AND METHODS

Mice

C57BL/6J, C57BL/Ka CD45.2 Thy1.1 and β-actin green fluorescent protein (GFP) mice were used at 1–6 months of age. Mice were bred and maintained on acidified water in the animal care facility at Duke University Medical Center. Cyclophosphamide was injected intraperitoneally on day 1 at 200 mg/kg in phosphate-buffered saline (PBS). G-CSF (Filgrastim, Amgen Inc., Thousand Oaks, CA, http://www.neupogen.com) was injected subcutaneously on day 2 and/or day 3 at 250 μg/kg per day in 0.1% bovine serum albumin in PBS. All animal experiments were performed according to protocols approved by the Duke University Institutional Animal Care and Use Committee.

Cell Isolation and Fluorescence-Activated Cell Sorting Analysis

HSCs were sorted and analyzed from mouse bone marrow on the basis of surface marker expression of c-Kit and Sca-1, low expression of Thy1.1, and low to negative expression of lineage markers (Lin) using an antibody cocktail as previously described [26, 27]. All antibodies were purchased from BD Pharmingen (San Diego, http://www.bdbiosciences.com/index_us.shtml) or eBioscience Inc. (San Diego, http://www.ebioscience.com). Analysis and cell sorting were carried out on a FACSVantage (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com) at the Duke Cancer Center fluorescence-activated cell sorting (FACS) facility. KFLS cells (A2F10 anti-Flk-2) were isolated from GFP transgenic and C57BL/6J mice as an alternate method for isolating long-term HSCs, as these animals do not possess Thy1.1.

Viral Production and Infection

Wnt10b was cloned into the murine stem cell virus-internal ribosomal entry site-green fluorescent protein (MSCV-IRES-GFP) retroviral expression vector. MSCV-IRES-GFP alone was used as control vector. Virus was produced and HSCs were infected as previously described [27]. Infected cells were then sorted on the basis of their GFP expression for in vitro and in vivo assays. All cytokines were purchased from R&D Systems Inc. (Minneapolis, http://www.rndsystems.com).

Real-Time Polymerase Chain Reaction Analysis

Hematopoietic cell fractions were isolated from wholebone marrow (WBM) by FACS. RNA was isolated using RNAqueous-Micro (Ambion, Austin, TX, http://www.ambion.com) and converted to cDNA using Superscript II or III (Invitrogen, Carlsbad, CA, http://www.invitrogen.com). Absolute cDNA concentrations were measured with a fluorometer (Turner Designs, Sunnyvale, CA, http://www.turnerdesigns.com) using RiboGreen reagent (Molecular Probes, Eugene, OR, http://probes.invitrogen.com). Quantitative real-time polymerase chain reaction (PCR) was performed using an iCycler (Bio-Rad, Hercules, CA, http://www.bio-rad.com), with iQ SYBR Green Supermix (Bio-Rad) and gene-specific primers.

To calculate the fold increase in gene expression, equivalent amounts of cDNA (5 ng/μl) were used to assess gene expression. These data were then normalized to expression of the housekeeping gene β2-microglobulin from the same sample. These values were then used to determine the relative difference between control and regenerating cell fractions.

In Vitro Proliferation and Colony Forming Assays

Freshly purified ckit$^+$, Thy1.1^{lo}, Lin$^{neg/lo}$, Sca1$^+$ (KTLS) or ckit$^+$, Flk-2^{neg}, Lin$^{neg/lo}$, Sca1$^+$ (KFLS) cells were infected for 48–72 hours with Wnt10b-IRES-GFP or control GFP retrovirus. Either lineage-negative ckit$^+$ Sca1$^+$ GFP$^+$ or lineage-negative GFP$^+$ cells were sorted by FACS at 5 or 15 cells per well, respectively, into Terasaki plates containing serum-free medium (XVivo15; BioWhittaker, Walkersville, MD, http://www.cambrex.com) supplemented with 50 μM βM 2-mercaptoethanol, 5% fetal calf serum, and SCF (50 ng/ml). After 5 days in culture, cells were visually counted and stained with ckit$^+$, Lin$^{neg/lo}$, Sca1$^+$ KLS antibody cocktail.

Bone marrow serum was isolated by flushing four long bones into 350 μl of XVivo15 with 50 μM βM 2-mercaptoethanol and penicillin/streptomycin. Sera were spun for 10 minutes at 4°C at 13,200 rpm. The soluble fraction was aspirated off, and 65 μl was cultured with 10 μl of freshly purified KLS or KFLS cells (4,000–12,000 cells) in a 96-well U-bottomed plate in 75 μl of sera at 37°C for 48 hours. Percentage viability via propidium iodide (PI) exclusion, as well as the absolute number of live cells at the end of assay, was determined by the number of GFP$^+$ propidium iodide-negative cells in the appropriate forward scatter/side scatter gate, as determined by FACS analysis.

To assess the colony-forming ability of KLS cells after bone marrow serum incubation, 20,000–30,000 KLS cells were incubated in 75 μl of control or injury-derived bone marrow serum for approximately 48 hours. Cells were sorted at a density of one cell per well into 96-well plates and cultured and scored as described previously [27].

In Vivo Analysis of HSC Function

CD45.2 KLS cells (20,000–30,000 cells) were incubated in 75 μl of control or injury-derived bone marrow serum for approximately 48 hours. PI-negative cells were sorted by FACS after incubation, and 1,200 cells were injected retro-orbitally per CD45.1 recipient along with 300,000 Sca1-depleted CD45.1 unirradiated WBM cells. Recipient mice were lethally irradiated with 10 Gy using a 200 kV x-ray machine 4–24 hours prior to transplantation, obtained by two doses of 5 Gy each, and they were maintained on antibiotic water (sulfamethoxazole and trimethoprim) following irradiation. Donor and host cells were distinguished by expression of CD45.1 (A20; eBioscience) and CD45.2 (104; eBioscience).

Immunofluorescence Staining of Cytospins

Freshly purified KLS cells were sorted by FACS, and cytospins were fixed in 4% paraformaldehyde for 20 minutes. Slides were then washed in PBS + 0.1% Tween, blocked with 20% normal goat serum, and stained with mouse anti-active β-catenin (Upstate, Charlottesville, VA, http://www.upstate.com) or mouse IgG isotype control (BD Pharmingen). Slides were washed in PBS-Tween (T) and stained with donkey anti-mouse IgG-Tetramethyl rhodamine isothiocyanate (Jackson Immunoresearch Laboratories, West Grove, PA, http://www.jacksonimmuno.com) and 4,6-diamidino-2-phenylindole (DAPI) (Molecular Probes). Confocal images were taken with a Zeiss 410 Axiovert microscope (Carl Zeiss, Jena, Germany, http://www.zeiss.com).

Immunofluorescence Staining of Bone Sections

Fresh bone specimens from control and injured mice were decalcified, infiltrated with sucrose, and embedded in Tissue-Tek OCT Compound (Sakura Finetek, Torrance, CA, http://www.sakura.com). Frozen sections were fixed in acetone, washed in PBS-T, and blocked with 20% normal donkey serum (Jackson Immunoresearch Laboratories). Primary antibodies were as follows: Wnt3a-specific antisera from immunized rabbits [28] (generous gift of Roel Nusse), anti-Wnt5a (Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com), anti-Wnt10b (Santa Cruz), and/or biotin-conjugated mouse panendothelial cell antigen (MECA)-32 (BD Pharmingen) or the appropriate isotype controls; normal rabbit serum (Jackson Immunoresearch Laboratories), normal goat IgG (Santa Cruz), or biotin-conjugated rat IgG2a,k (BD Pharmingen). Secondary antibodies were as follows: donkey anti-rabbit Cy3 (Jackson Immunoresearch Laboratories), donkey anti-goat Alexa 594 (Molecular Probes), and/or streptavidin-Alexa 488 (Molecular Probes). The nuclear dye DAPI (Molecular Probes) was included in all stains. Wnt10b levels within $MECA^+$ cells were quantified with Metamorph software. A Wnt10b-positive endothelial cell was one in which staining was above background, as determined by isotype controls.

Statistical Analysis

Two-tailed Student's *t* test was used to determine statistical significance. *p* values less than .05 were considered significant.

RESULTS

Enhanced HSC Support by the Microenvironment During Regeneration

In mice, as in humans, Cy/G-CSF treatment promotes the expansion of HSCs in the marrow followed by their release into the peripheral blood, thus providing an in vivo model of hematopoietic regeneration in response to injury [29]. To determine whether the microenvironmental changes that occur following injury may contribute functionally to the regeneration response, we examined the soluble fraction of bone marrow from untreated or Cy/G-treated mice 48 hours (on day 3) after cyclophosphamide administration. Whole bone marrow was flushed into a small volume (350 μl per four long bones) of serum-free medium, and the cells were removed by centrifugation. We postulated that this supernatant (referred to here as bone marrow serum) should contain soluble factors secreted by the bone marrow and should recapitulate the soluble milieu that hematopoietic cells may see in vivo. The experiments described below were carried out either with a population enriched for HSCs (KLS cells: $ckit^+$, $Lin^{neg/lo}$, $Sca1^+$) when higher cell numbers were needed or with populations more stringently purified for HSCs (KTLS [30] or KFLS [31], referred to as HSCs) when cell number was not as limiting. Cells were cultured with bone marrow sera from injured or uninjured mice without the addition of any exogenous serum or growth factors to stringently recapitulate the bone marrow microenvironment. As shown in Figure 1A, analysis of PI exclusion revealed a significant decrease in relative death of HSCs after exposure to injury-derived serum (average control PI^{pos}, 23.3%; average injury-derived PI^{pos}, 11.7%; $n = 5$; $p = .01$). In addition, after 2 days of bone marrow serum culture, we found significantly higher numbers of HSCs in cultures exposed to bone marrow serum derived from injured mice compared with serum from uninjured mice (Fig. 1B). To confirm that the presence of G-CSF in the Cy/G serum was not directly responsible for the differential support observed, cultures were performed with bone marrow serum derived from mice injected with Cy alone. KLS cells in the Cy-only bone marrow serum demonstrated an increase in number equivalent to that of Cy/G bone marrow serum cultures (data not shown). The fact that increased cell support was not dependent on cell-cell contact suggested that the injured bone marrow milieu contains soluble factors that are qualitatively or quantitatively different from those in uninjured bone marrow. Examination of the $Lin^{neg/lo}$ $Sca1^+$ phenotype after control or injury-derived bone marrow sera culture revealed equivalent frequencies of progenitor cells, suggesting that the enhanced support observed affected viability but not differentiation (data not shown). Our data indicate that in vitro, microenvironmentally derived soluble factors may partially contribute to the regenerative process by enhancing the survival of stem and progenitor cells.

We next determined whether the increase in cell numbers reflected an increase in the support of functional HSCs or an increase in numbers of differentiated cells. Thus, we isolated KLS cells, incubated them in sera from injured and uninjured mice, and tested the frequency of differentiated versus undifferentiated cells using both in vitro colony forming and in vivo transplantation assays. As shown in Figure 1C, cells cultured in serum from control mice gave rise to a significant predominance of committed single-lineage colonies in methylcellulose cultures. In contrast, KLS cells cultured in serum from injured mice gave rise to equivalent numbers of multilineage and single-lineage colonies, indicating that KLS from injury-derived serum retain a similar or improved stem cell functionality on a single-cell basis in comparison with control cultures. To test this in vivo, we isolated KLS cells, incubated them in sera from injured and uninjured mice, and transplanted equal numbers of the cultured cells into a total of 13 lethally irradiated hosts over two independent experiments. After 14 weeks, recipient WBM was analyzed for donor chimerism to determine the HSC contribution after bone marrow serum exposure. Of the total WBM KLS population, the donor chimerism was equivalent from stem cells cultured with injury-derived or uninjured sera (Fig. 1D). Although peripheral blood donor chimerism from injury-derived cultures displays some reduction compared with control chimerism, this difference was not significant (supplemental online Fig. 1a). The total donor chimerism within the WBM compartment was once again equivalent, as was seen within the KLS population (supplemental online Fig. 1b). Analysis of donor PB chimerism demonstrated normal lineage contribution from both sets of donor stem cells, indicating the full developmental capacity of transplanted stem cells (supplemental online Fig. 1c). Cumulatively, these data indicate that KLS cells cultured in control or injury-derived sera possess a similar heterogeneity of stem cell multipotency after culture. However, although KLS cells exposed to control serum may possess a frequency of HSCs similar to that of KLS cells exposed to injury-derived serum, there is an increased absolute number of HSCs after exposure to serum from Cy/G-treated mice. Thus, when the level of reconstitution of donor KLS cells (Fig. 1D) was multiplied by the average increase in cell number observed with injury-derived versus control sera (Fig. 1B), a two-fold increase in functional chimerism from KLS cells exposed to injury-derived serum was observed (Fig. 1E). These data indicate

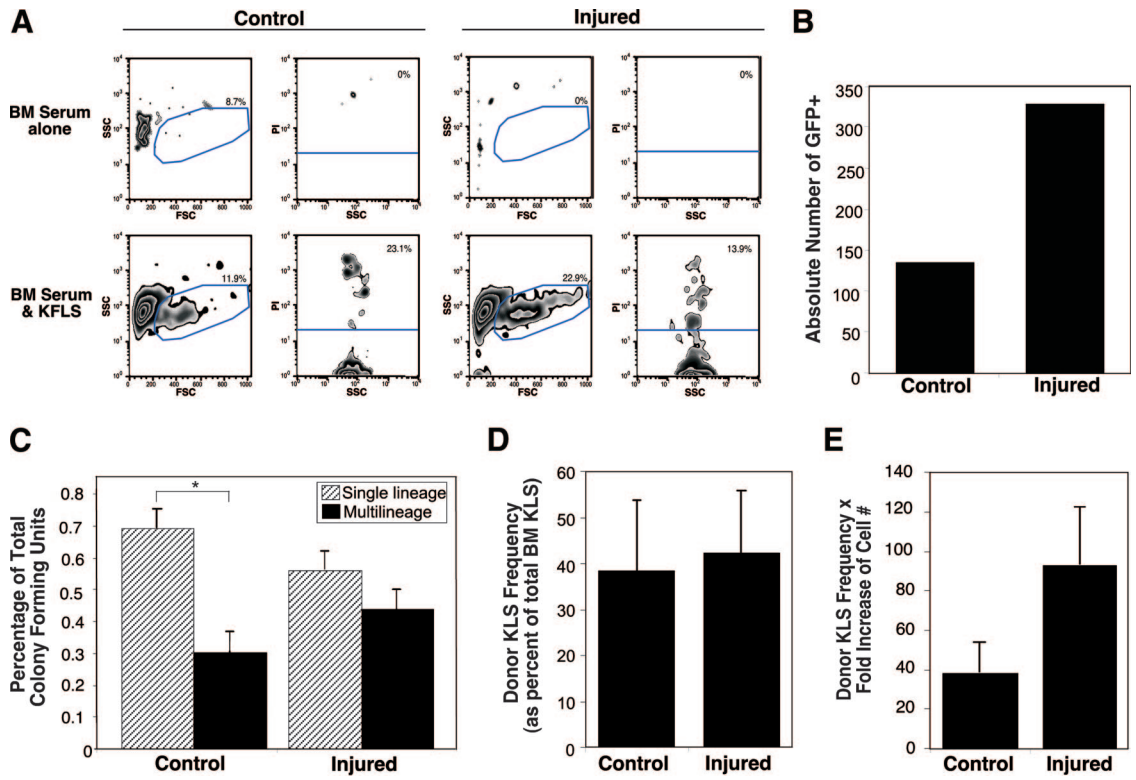

Figure 1. BM serum from injured mice has an enhanced capacity to support hematopoietic stem cells. **(A):** β-Actin GFP$^+$ KFLS cells were incubated in injury or control-derived BM sera for 48 hours. Fluorescence-activated cell sorting plots of BM serum assay show gating strategy to determine live cell numbers. GFP$^+$ cells were selected prior to forward scatter/side scatter and propidium iodide exclusion to remove noncellular debris introduced from BM serum. **(B):** Representative graph shows absolute cell numbers from injury-derived BM serum compared with control BM serum ($n = 5$; $p = .005$). **(C):** KLS cells were incubated in control or injury-derived BM sera, and single cells cultured in methylcellulose to assess colony-forming ability ($n = 6$; $p = .02$). **(D):** KLS cells were incubated in control or injury-derived BM sera. Following incubation, 1,200 cells were injected into lethally irradiated recipients. Two separate transplants were performed using a total of 13 recipient mice ($n = 6$ for control serum and $n = 7$ for injury-derived serum). Graph shows average frequency of donor KLS cells detected in BM after 14 weeks (control engraftment range, 2.48%–85.6%; injury engraftment range, 8.58%–86.9%). **(E):** As equal numbers of KLS from control or injury-derived sera were transplanted, the chimerism observed was multiplied by the fold increase of cell number that occurs with exposure to injury-derived serum. This indicates the total increase of functional KLS cells that occurs following incubation with injury-derived serum. Abbreviations: BM, bone marrow; GFP, green fluorescent protein.

that culture with serum from Cy/G-injured mice leads to an increase in the absolute number of functional HSCs in comparison with culture with control serum.

Increased Expression of Wnt10b in the Microenvironment During Regeneration

In light of our previous finding that Wnt signaling can promote HSC growth [7], we tested whether the injured microenvironment upregulates Wnt proteins, which could mediate the increased support observed. To visualize Wnt ligands in the bone marrow microenvironment, we analyzed bone sections by immunofluorescence analysis. We have previously shown that Wnt10b, Wnt3a, and Wnt5a are expressed in the mouse bone marrow [32]. Therefore, we tested whether any of these candidate Wnts were upregulated in the bone marrow on day 3 after Cy/G treatment. Although we could detect low levels of Wnt3a and Wnt5a in bone sections from both control and injured mice, there was no detectable difference between the two conditions (Fig. 2A). In contrast, we found a dramatic increase in Wnt10b expression in the injured microenvironment compared with control (Fig. 2A; supplemental online Fig. 2).

We then examined the kinetics of Wnt10b upregulation to determine whether it correlated with the timing of HSC regeneration. We isolated bones from control and injured mice 3, 5, and 7 days after damage and stained for the presence of Wnt10b (Fig. 2B). Interestingly, we found that the highest upregulation of Wnt10b occurred at day 3 after injury and that this increase was reduced by day 5 and even further reduced, down to control levels, by day 7. This temporal analysis suggested that Wnt10b upregulation is controlled in a dynamic manner and that its peak coincides with the peak of HSC proliferation, which also occurs at day 3 after Cy/G-induced injury.

To determine which cells were responsible for the upregulated production of Wnt10b, we analyzed both hematopoietic and stromal cell populations. Recently, a large fraction of HSCs have been shown to be associated with endothelial cells in vivo [33, 34]. Using confocal microscopy to determine colocalization, we stained bone sections with the endothelial marker MECA, as well as Wnt10b, and found that Wnt10b expression was associated with endothelial cells in vivo (Fig. 3A, arrows). Quantification of Wnt10b revealed a significant increase in the frequency of Wnt10b-positive endothelial cells after Cy/G-mediated injury versus control cells (Fig. 3B). In addition, there was a small but significant increase in the intensity of Wnt10b within endothelial cells after injury (data not shown). In addition, the Wnt10b increase was also associated with small cells that had a hematopoietic morphology (Fig. 3A, arrowhead). To identify the hematopoietic cells, we fractionated bone marrow cells on the basis of lineage-specific cell-surface markers and analyzed the populations for Wnt10b expression using quantitative real-time PCR. We found that although multiple hematopoietic cell fractions expressed Wnt10b following injury, the highest upregulation of Wnt10b after

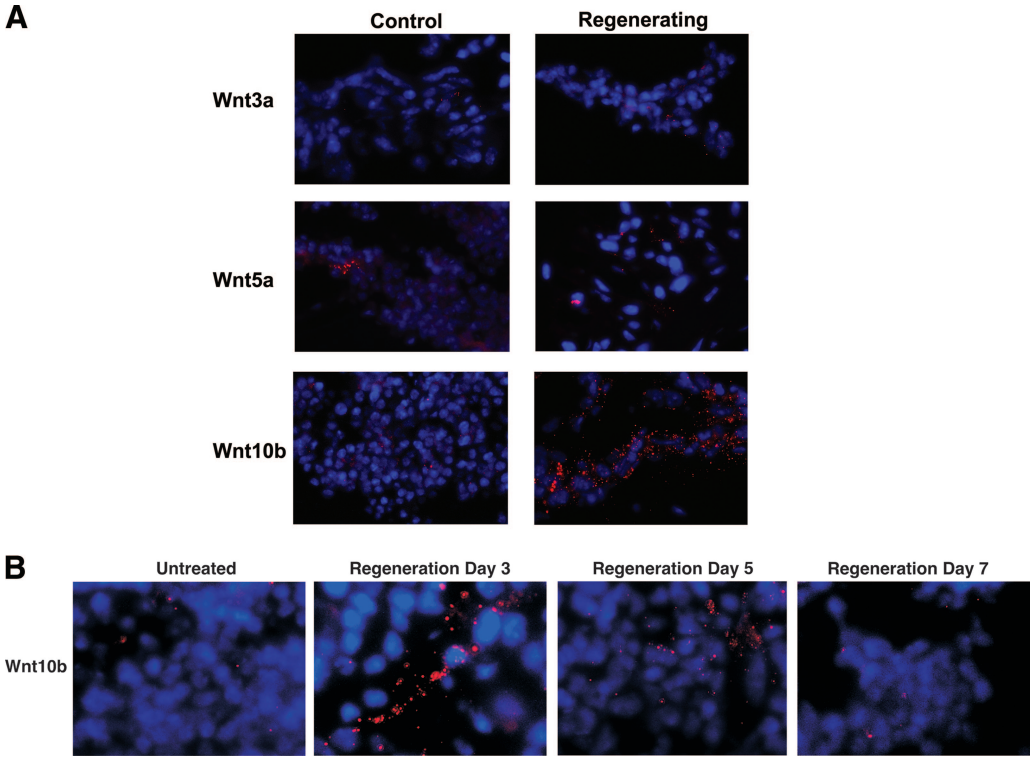

Figure 2. Wnt10b is temporally regulated in the bone marrow microenvironment after injury. **(A):** Bone sections of control or Cy/G-injured mice were stained for the expression of Wnt3a, Wnt5a, or Wnt10b (red) and 4,6-diamidino-2-phenylindole (blue) ($n = 2$ for Wnt3a and Wnt5a and $n = 5$ for Wnt10b). **(B):** Kinetics of Wnt10b expression levels were monitored for 1 week following injury by analyzing bone sections at days 3 ($n = 3$), 5 ($n = 2$), and 7 ($n = 3$) after Cy/G delivery. All images were acquired with a ×40 objective.

injury was derived from $B220^{lo}$ developing B cells [35] (3.93-fold) and $Ter119^{+}$ erythroid cells (3.31-fold) (Fig. 3C–3E). Overall, these data indicate that there is a significant upregulation of Wnt10b following injury in the bone marrow and that this upregulation is derived from a combination of both stromal cell elements and specific hematopoietic cells.

The elevation of Wnt10b after injury suggested that it might contribute to the growth of HSCs during regeneration. Although we have previously shown that Wnt3a can induce growth of HSCs [36], it was unclear whether this ability is shared with other Wnt family members. Wnt10b is not available in its purified form; however, soluble Wnt10b has been studied in vitro via conditioned media generated from cells expressing Wnt10b [6]. Therefore, to study the effects of Wnt10b in vitro, we generated retroviruses engineered to express Wnt10b and infected HSCs to test their ability to induce HSC expansion in an autocrine/paracrine manner. Wnt10b stimulation resulted in a statistically significant growth of HSCs in comparison with vector-infected control cells (Fig. 4A). However, it is possible that the effect of Wnt10b is underestimated because of the need to infect these cells retrovirally in the presence of other growth factors and the background proliferation that occurs as a consequence. Additionally, Wnt10b-induced expansion was not due to elevated differentiation since control and Wnt10b-transduced cells possessed equivalent frequencies of $Lin^{neg/lo}$ $Sca1^{+}$ cells after culture. Thus, the absolute number of KLS cells was increased in response to Wnt10b (Fig. 4B). These data show that Wnt10b, which is elevated in the bone marrow following injury, can act to enhance the growth of HSCs.

Activation of Wnt Signaling During Regeneration

The elevated levels of Wnt10b in the bone marrow and the ability of Wnt10b to induce HSC proliferation suggested that Wnt signaling may be used by HSCs during regeneration. To test whether Wnt signaling is used by HSC-enriched fractions during regeneration, we first examined the stabilization and activation of β-catenin, a key mediator in the Wnt pathway. When Wnt signaling is inactive, β-catenin levels are kept low by glycogen synthase kinase 3β (GSK3-β)-mediated phosphorylation and subsequent degradation. On the other hand, when Wnt signaling is activated, β-catenin is no longer phosphorylated, and its levels are stabilized. Using an antibody demonstrated to be specific for the nonphosphorylated form of β-catenin [37], we found that a significantly higher number of KLS cells from treated mice contain activated β-catenin in comparison with cells from control mice (Fig. 5A, 5B). To test whether the elevated levels of β-catenin reflected increased transcriptional activation of the Wnt pathway in HSCs, we analyzed expression of c-Myc and Axin-2, two known direct targets of Wnt signaling [38, 39]. Both c-Myc and Axin-2 were upregulated 2.8-fold and 3.6-fold, respectively, in regenerating HSCs in comparison with quiescent HSCs (Fig. 5C). In addition, induction of regeneration by 5-fluorouracil administration also generated a significant increase in activated β-catenin levels in KLS cells (data not shown), indicating that reactivation of Wnt signaling by the stem and progenitor compartment may potentially be a conserved phenomenon during regeneration.

Collectively, our data demonstrate that after injury, the microenvironment changes to be more supportive of HSCs. We demonstrate that the soluble fraction of the regenerating bone marrow microenvironment possesses an enhanced ability to support HSCs. In addition, Wnt ligand presentation in the form of Wnt10b, an HSC growth factor, is increased by both stromal and hematopoietic cell subsets after Cy/G treatment. Concomitant with the increased expression of Wnt ligand, regenerating stem and progenitor cells demonstrate increased activation of the Wnt signaling pathway. We propose that the microenvironmental upregulation of Wnt10b after injury may serve to extrinsically activate the Wnt signaling pathway in regenerating HSCs.

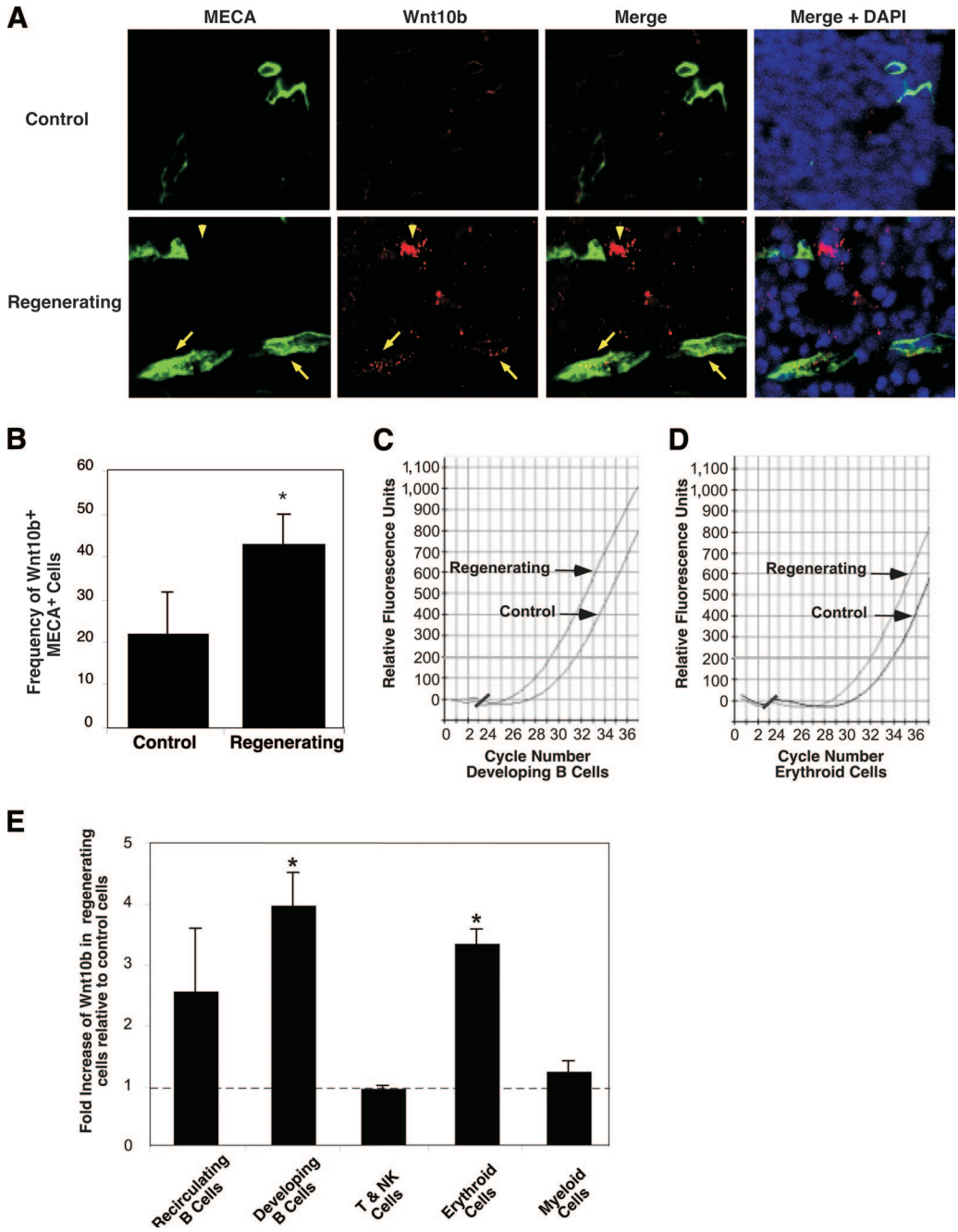

Figure 3. Wnt10b is upregulated by stromal and hematopoietic cells after injury. **(A):** Bone sections from control and injured mice were stained for Wnt10b (red) and MECA (green) and visualized by confocal microscopy with a ×40 objective. Yellow arrows indicate colocalization of endothelial cells ($MECA^+$) and Wnt10b. Yellow arrowheads indicate Wnt10b staining associated with nonendothelial cells. **(B):** The frequency of Wnt10b-positive endothelial cells was determined by quantification with Metamorph software ($n = 2$; $p = .04$). **(C):** Hematopoietic lineages from control or injured mice were sorted by fluorescence-activated cell sorting from bone marrow on the basis of cell surface markers, and polymerase chain reaction (PCR) was carried out for Wnt10b. Shown are recirculating B cells ($B220^{Hi}$), developing B cells ($B220^{Lo}$), T and NK cells ($CD3e^+$,$CD4^+$, $CD8^+$, and $NK1.1^+$), erythroid cells ($Ter119^+$), and myeloid cells ($Mac1^+$) and a representative plot of Wnt10b real-time PCR in control and regenerating $B220^{lo}$ cells. **(D):** Representative plot of Wnt10b real-time PCR in control and regenerating $Ter119^+$ cells. **(E):** Data represent fold increase ± SEM of Wnt10b expression after Cy/G-induced injury in comparison with control samples ($B220^{Lo}$, $p = .03$; erythroid, $p = .01$) after normalization to β2-microglobulin ($n = 3$). Abbreviations: DAPI, 4,6-diamidino-2-phenylindole; NK, natural killer.

DISCUSSION

A powerful feature of the hematopoietic system is its ability to repair itself after injury. However, little is known about how the microenvironment changes to influence hematopoietic repair. Our data suggest that injury to the hematopoietic system causes elevated levels of soluble mediators that enhance HSC growth, as determined by in vitro and in vivo assays, and that one of these mediators is Wnt10b. Furthermore, we found increased levels of Wnt10b in the bone marrow and a concomitant rise in

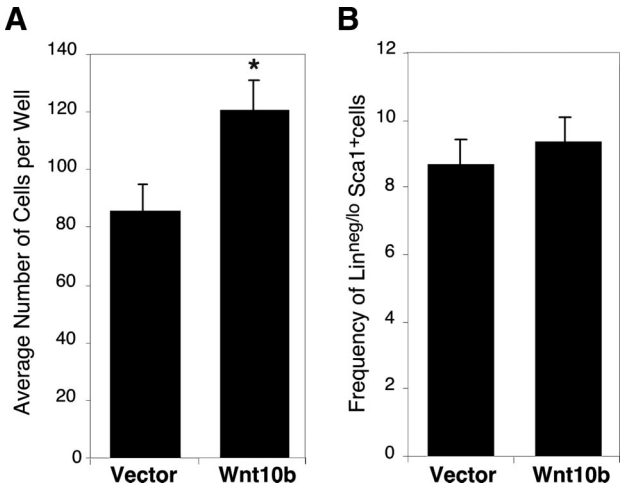

Figure 4. Wnt10b induces proliferation of hematopoietic stem cells (HSCs). **(A):** HSCs infected with Wnt10b demonstrated increased proliferation at day 5 (range, 1.0–2.3-fold increase) in comparison with vector-infected control cells ($n = 13$; $p = .00005$). **(B):** On day 5 of culture, both Wnt10b- and vector-transduced cells possessed equivalent progenitor cell frequencies ($n = 4$).

the activation of Wnt signaling within stem and progenitor cells. These data suggest that after injury, the microenvironment and hematopoietic cells act in concert, possibly to drive efficient repair. However, we found that although inhibition of Wnt signaling via Axin overexpression inhibits HSC reconstitution [36], it did not inhibit the regenerative capacity of HSCs in response to Cy/G (data not shown). Considering Axin overexpression within HSCs is dependent upon retroviral delivery and transplantation prior to Cy/G induced damage, the complexity of this model may not accurately reflect physiological stem cell requirements. However, injury and regeneration during Wnt inhibition can also be examined within our mouse model of β-catenin null hematopoiesis. Since β-catenin deficient KLS cells have a reduced capacity to regenerate the hematopoietic compartments of lethally irradiated recipients [14], it will be important to test whether they also possess defects in their regenerative response after exposure to injury. It is possible that the requirement for Wnt signaling may depend on the type and severity of hematopoietic damage. This would parallel work by Ito et al. wherein Wnt driven de novo hair follicle regeneration from wounded epidermis only occurs if the size of the wound is large enough [40]. It is also worth noting that exogenous activation of Wnt signaling may boost the regenerative response. Administration of a GSK-3 inhibitor after HSC transplantation increases the repopulating ability of transplanted HSCs; this may be due in part to activation of the Wnt signaling pathway [41]. In addition, recent work demonstrates that mice deficient in Hmgb3 have increased levels of Wnt reporter expression within HSCs [42]. After injury with 5-fluorouracil, these Hmgb3-deficient mice recover the phenotypically normal HSC population faster than their wild-type counterparts. The authors attribute this enhanced regenerative capacity to the increased levels of Wnt signaling within the stem cell compartment,

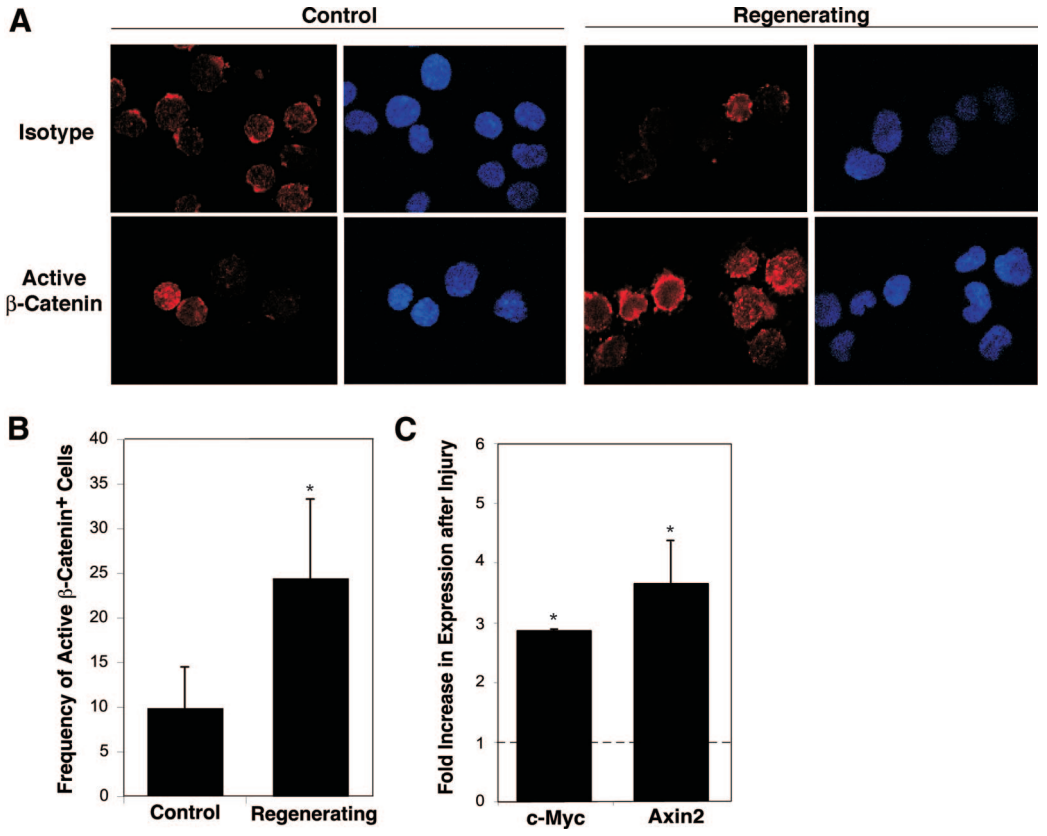

Figure 5. Wnt signaling is activated in hematopoietic stem and progenitor cells after injury. **(A):** KLS-sorted cells from untreated or Cy/G/G-treated mice were spun onto slides and stained for activated β-catenin (red) and 4,6-diamidino-2-phenylindole (blue) ($n = 7$). All images were acquired by confocal microscopy with a ×40 objective. **(B):** Frequency of active β-catenin-positive cells ($p = .03$). **(C):** KTLS cells from Cy/G/G or control mice were isolated and analyzed for c-Myc, Axin-2, and β2-microglobulin expression by real-time polymerase chain reaction. Average fold increase of c-Myc and Axin-2 expression over β2-microglobulin in response to Cy/G/G treatment was 2.8-fold and 3.6-fold, respectively ($p = .02$ and .03, respectively; $n = 6$).

suggesting that activation of the canonical Wnt pathway may be sufficient to augment regeneration [42].

Our identification of specific molecular changes in the microenvironment could provide insight into the behavior of stem and progenitor cells during injury in a variety of systems. Stem and progenitor cells possess a well-documented ability to respond to microenvironmental changes induced by stresses. For example, hematopoietic cells possess an enhanced ability to migrate and engraft into nonhematopoietic tissues, such as the liver, skeletal muscle, and brain, following injury [43–46]. Although it remains controversial whether hematopoietic cells in these altered environments integrate and change fate, undergo fusion, or simply integrate and remain hematopoietic [45–49], it is clear that the injured microenvironment possesses some ability to support and attract immature hematopoietic cells, and in many cases this can lead to improved function after damage [50–53]. It thus becomes important to define precisely the microenvironmental changes after stress or injury that can modulate stem and progenitor cell activity. Our work identifying Wnts as a specific cue that is upregulated early after injury in the bone marrow suggests that Wnts and other, similar developmental pathways should be investigated as possible players in repair after injury in other organs and tissues as well.

Our findings implicating Wnt signaling in regeneration after Cy/G treatment offer a parallel to regeneration in other systems [40, 54–62]. In the zebrafish, canonical Wnt signaling is required for tail fin regeneration, and this regeneration is accompanied by upregulation of the ligand Wnt10a [57]. In murine muscle, several Wnt transcripts are upregulated after injury induction, and in vivo inhibition of Wnt signaling via secreted frizzled related proteins impairs muscle regeneration by decreasing the proliferation of resident adult stem cells [56]. Currently, little is known about the mechanisms by which Wnt expression may be enhanced to promote regeneration. During *Drosophila* wing development, high levels of apoptosis are accompanied by the induction of wingless (Wg) and the compensatory proliferation of remaining wing disc cells [63, 64]. In the *Drosophila* wing, Wg is produced in apoptotic cells in response to c-Jun N-terminal kinase signaling, so that the dying cell itself provides the signal for initiating compensatory proliferation. In contrast, our data in the hematopoietic system show that after injury Wnt10b is specifically upregulated by distinct stromal and hematopoietic cell populations. In fact, examination of apoptotic (Annexin V^+) hematopoietic cells revealed no differential increase in Wnt10b expression (K.L.C. and T.R., unpublished observations). These data suggest that divergent mechanisms may be in place in different organisms to elevate Wnt levels after injury or loss to influence the repair process. It is also worth noting that Wnt ligands appear to be differentially regulated by injury, as the levels of Wnt3a and Wnt5a remained unchanged during hematopoietic regeneration. It is possible that some Wnts, such as 3a and 5a, may be more important under homeostatic conditions, whereas Wnt10b becomes more relevant under conditions of stress and repair. However, as Wnt signaling is upregulated in diverse systems and organisms besides the hematopoietic system, the data suggest that that although each system may use distinct strategies to upregulate Wnt signaling, activation of the pathway itself may be a conserved element of injury repair. In the future, it will be important to determine whether similar activation of Wnt signaling occurs after injury in specific organs and tissues in humans. If so, this would raise the possibility that modulating Wnt signaling may be effective as a therapy to accelerate recovery after injury.

Finally, it has recently been proposed that cancers may arise as a consequence of tissue injury if homeostatic stem cell renewal signals are continually reactivated during regeneration [25]. Our data support this idea by demonstrating the recurrent activation of Wnt signaling at high levels after injury. Thus, our findings may provide insight into how classic developmental signals such as Wnt can fuel cancer progression.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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