
The authors note that both Fig. 1M and its legend appeared incorrectly. The corrected figure and its corresponding legend appear below.

Fig. 1. H-Cx43 deficiency does not impair serial competitive repopulation but impairs the hematopoietic recovery after 5-FU administration. (A) Representative example of a longitudinal section of β-galactosidase staining of a femur (original magnification, 40x) from Vav1-Cre; Rosa-loxP-Stop-loxP-LacZ.
The authors note that the x/y/z coordinates listed for brain regions in Table 1 appeared incorrectly. The corrected table appears below. This error does not affect the conclusions of the article.

<table>
<thead>
<tr>
<th>Region (BA)</th>
<th>L/R/B</th>
<th>Volume (mm$^3$)</th>
<th>x</th>
<th>y</th>
<th>z</th>
<th>Baseline, phobogenic vs. neutral</th>
<th>Baseline vs. posttherapy</th>
<th>Posttherapy vs. follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>t</td>
<td>P</td>
<td>t</td>
</tr>
<tr>
<td>Baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>t</td>
<td>P</td>
<td>t</td>
</tr>
<tr>
<td>Baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>t</td>
<td>P</td>
<td>t</td>
</tr>
</tbody>
</table>

For each activity cluster, listed are Brodmann areas (BA), hemisphere [left (L), right (R), or bilateral (B)], the volume (mm$^3$), and stereotactic coordinates for the centrally activated voxel (x, y, z mm). Statistics are reported for all P values ≤ 0.10, and otherwise listed as nonsignificant (NS).

**PSYCHOLOGICAL AND COGNITIVE SCIENCES**

**Table 1. Summary of fMRI activity clusters**

<table>
<thead>
<tr>
<th>Region (BA)</th>
<th>L/R/B</th>
<th>Volume (mm$^3$)</th>
<th>x</th>
<th>y</th>
<th>z</th>
<th>Baseline, phobogenic vs. neutral</th>
<th>Baseline vs. posttherapy</th>
<th>Posttherapy vs. follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>t</td>
<td>P</td>
<td>t</td>
</tr>
<tr>
<td>Baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>t</td>
<td>P</td>
<td>t</td>
</tr>
<tr>
<td>Baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>t</td>
<td>P</td>
<td>t</td>
</tr>
</tbody>
</table>

Fig 1. regions shown in blue

Posterior cingulate (BA 31) B 8,343 4 −40 49 1.77 0.10 −2.82 0.02 2.03 0.07
Anterior cingulate/ vmPFC (BA 24, 32) B 7,955 −3 20 27 3.19 0.01 −3.11 0.01 NS
Anterior insula (BA 13) L 2,187 −53 8 −1 2.09 0.06 −2.70 0.02 NS
Anterior insula (BA 13) L 1,782 37 6 6 3.91 0.00 −2.46 0.03 NS
Posterior insula (BA 13, 19) R 8,478 47 −45 13 3.33 0.04 −2.81 0.02 NS
Middle temporal gyrus (BA 39) L 1,134 −41 −51 7 3.77 0.00 −2.71 0.02 NS
Medial frontal gyrus (BA 6) R 1,809 6 −15 67 2.68 0.02 −2.48 0.03 NS
Amygdala (anatomically defined) R 891 n/a n/a n/a 3.65 0.00 −4.59 0.00 NS

Fig 1. regions shown in red
dIPFC (BA 6, 8) R 918 36 15 61 −2.38 0.04 8.27 0.00 −2.63 0.02
Superior parietal lobule (BA 7) R 1,458 25 −72 57 NS 3.12 0.01 NS

Fig 2. regions shown in gray

Fusiform/lingual gyrus (BA 18, 19) L 8,046 −34 −81 −9 4.91 0.00 NS −5.10 0.00
Fusiform/lingual gyrus (BA 18, 19) R 11,367 36 −78 −11 5.14 0.00 NS −5.36 0.00
Connexin-43 prevents hematopoietic stem cell senescence through transfer of reactive oxygen species to bone marrow stromal cells

Eri Taniguchi Ishikawa, Daniel Gonzalez-Nieto, Gabriel Ghiaur, Susan K. Dunr, Ashley M. Ficker, Bhuvana Murali, Malav Madhu, David E. Gutstein, Glenn I. Fishman, Luis C. Barrio, and Jose A. Cancelas

Hematopoietic stem cell (HSC) aging has become a concern in chemotherapy of older patients. Humoral and paracrine signals from the bone marrow (BM) hematopoietic microenvironment (HM) control HSC activity during regenerative hematopoiesis. Connexin-43 (Cx43), a connexin constituent of gap junctions (GJs) is expressed in HSCs, down-regulated during differentiation, and postulated to be a self-renewal gene. Our studies, however, reveal that hematopoietic-specific Cx43 deficiency does not result in significant long-term competitive repopulation deficiency. Instead, hematopoietic Cx43 (H-Cx43)-deficient HSCs and progenitors (HSC/P) cells display decreased survival and fail to enter the cell cycle to proliferate. Cell cycle quiescence is associated with down-regulation of cyclin D1, up-regulation of the cyclin-dependent kinase inhibitors, p21wp1 and p16INK4a, and Forkhead transcriptional factor 1 (Foxo1), and activation of p38 mitogen-activated protein kinase (MAPK), indicating that H-Cx43-deficient HSCs are prone to senescence. The mechanism of increased senescence in H-Cx43-deficient HSC/P cells depends on their inability to transfer reactive oxygen species (ROS) to the HM, leading to accumulation of ROS within HSCs. In vivo antioxidant administration prevents the defective hematopoietic regeneration, as well as exogenous expression of Cx43 in HSC/P cells. Furthermore, ROS transfer from HSC/P cells to BM stromal cells is also rescued by reexpression of Cx43 in HSC/P. Finally, the deficiency of Cx43 in the BM phenocopies the hematopoietic defect in vivo. These results indicate that Cx43 exerts a protective role and regulates the HSC/P ROS content through ROS transfer to the HM, resulting in HSC protection during stress hematopoietic regeneration.

Results
To clarify whether Cx43 plays a crucial role in HSCs and to elucidate the mechanism of impaired hematopoietic recovery after in vivo 5-FU challenge, we have generated a mouse model with constitutive deficiency of Cx43 in hematopoiesis [Vav1-Cre/Cx43flox/flox; hematopoietic specific (H)-Cx43-deficient]. As reported by others (12-15), we confirmed that Vav1-Cre expression is extremely efficient, inducing recombinant of either a reporter gene (Fig. 1A) or the floxed Gja1 (Cx43flox/flox) gene (Fig. 1B and C). The mRNA expression of Cx43 in HSCs from Vav1-Cre;Cx43flox/flox mice was practically abolished in BM HSCs (defined as lineage-/c-kit+Sca-1+/CD34-) and multipotential progenitors (MPPs) (defined as LSK/CD34+/c-kit+Sca-1+; Fig. 1D). Furthermore, we analyzed the protein expression of Cx43 in wild-type (WT) and Vav1-Cre;Cx43flox/flox HSCs. Confocal microscopy detected Cx43 protein expression in the membrane of isolated HSCs from WT but not from Vav1-Cre;Cx43flox/flox mice (Fig. 1F). Expression of Cx45 (Gja1), another connexin family protein involved in hematopoiesis, was detected in both WT and Vav1-Cre;Cx43flox/flox HSCs (Fig. S1) and was found not to be significantly up-regulated in H-Cx43-deficient HSCs (Table S1). Cx37 (Gja8) and Cx50 (Gja4) showed minimal mRNA expression up-regulation trends (Table S1). Thus, the deficiency of Cx43 in HSCs was not associated with significant compensatory up-regulation of other connexins.

To examine whether the loss of Cx43 expression in HSCs impairs the BM HSC content and function, we first analyzed the peripheral blood (PB) counts (Fig. S2A) and content of...
phenotypically identifiable BM HSC/P populations. We found negative control of BM from non-Cre transgenic littermates is presented (Lower). (B and C) Genomic recombination of Vav1-Cre;Cx43floxedLSK CD34+ BM cells (n = pool of 3 mice per group). (B) Cx43 floxed vs. WT allele PCR. Cx43 floxed band is practically abrogated in sorted LSK CD34+. Controls of genomic DNA from source animals are also presented for reference. (C) Cx43 floxed-out PCR. (D and E) mRNA expression of Cx43 in HSC/P cells isolated from control and H-Cx43-deficient mice (n = pool of 3 mice per group). (D) Semiquantitative RT-PCR for Cx43 expression in LSK CD34+ BM cells. (E) Quantitative RT-PCR (Q-RT-PCR) for Cx43 expression in BM MPP. (F) Immunofluorescence pictures showing Cx43 (green) along with DAPI (blue) in WT or H-Cx43-deficient HSCs. Cx43 is detected around the cell membrane with asymmetric distribution in most of WT HSCs (n = 20 HSCs from each individual mouse, n = 3 mice per group). (Scale bar, 5 μm.) (G–I) Serial competitive repopulation assays. Lethally irradiated primary recipient CD45.1+ mice were transplanted with a mixture of Vav1-Cre; H-Cx43-deficient BM cells (CD45.2+) and WT BM (CD45.1+) cells (solid circle). Control group was transplanted with a mixture of Vav1-Cre; WT BM cells (CD45.2+) and WT BM (CD45.1+) cells (open circle). (G) PB chimera analysis was performed at 1, 2, 3, and 4 mo after transplant in primary recipients. (H) PB chimera of secondary recipients transplanted with 10 x 10⁶ BM cells obtained from primary mice. (I) PB chimera of tertiary recipients transplanted with 10 x 10⁶ BM cells obtained from secondary mice. For competitive repopulation assay, 8–16 mice per group were transplanted and analyzed from 2 independent experiments. (I and K) PB counts of WT (open circle) or H-Cx43-deficient mice (solid circles) after 5-FU administration. Counts were performed at the indicated days after 5-FU administration. (J) Absolute neutrophil counts (ANC). (K) Platelet counts. *P < 0.05 (n = 3 mice per group in each of two independent experiments). (L) HSC and MPP content of BM on day 21 after 5-FU treatment. (M) Transplant of HSC/P cells transduced with a Cx43-expressing lentiviral vector rescues PB recovery after 5-FU administration in H-Cx43-deficient mice. Recipient mice were transplanted with LSK BM cells transduced with an empty vector or a Cx43-expressing lentiviral vector. After 4 wk posttransplantation, recipient mice were administered S-FU and PB cell counts and flow cytometric analysis of myeloid (CD11b) recovery were performed at the indicated days after 5-FU administrations. ○, WT with empty vector transduction (WT + Mock); ●, WT with Cx43 transduction (WT + Cx43); □, H-Cx43-deficient with empty vector transduction (H-Cx43-deficient + Mock); ■, H-Cx43-deficient with Cx43 transduction (H-Cx43-deficient + Cx43). *P < 0.05 (n = 4 mice/group in each of two independent experiments). Values represent means ± SD. (N and O) Lethally irradiated primary recipient CD45.1+ mice were transplanted with H-Cx43-deficient BM cells (CD45.2+) or Vav1-Cre; WT BM cells (CD45.2+). PB count of WT (open circle) or H-Cx43-deficient mice (solid circle) after 5-FU administration were analyzed after 4 wk post transplant. (K) Absolute neutrophil count (ANC). (O) Platelet count. *P < 0.05 (n = 5 mice per group). Values represent means ± SD.
first analyzed the frequency of HSCs in DNA synthesis phase in vivo at 0, 24, 48, and 96 h after 5-FU administration. Whereas WT BM HSCs showed a ~fourfold increase in the frequency of HSCs in S phase between days 2 and 4 after 5-FU administration (Fig. 2A), H-Cx43-deficient HSCs did not significantly cycle, as assessed by lack of increase in bromodeoxyuridine (BrdU) uptake (Fig. 2A) or expression of Ki67 (Fig. 2B and Fig. S4A), by 96 h after 5-FU administration compared with WT HSCs, which confirmed an impaired cell cycle entry in response to chemotherapeutic stress. Pyrionin/7-aminoactinomycin D (7-AAD) staining showed accumulation of H-Cx43-deficient HSCs in the G0 phase of the cell cycle (Fig. 2C and Fig. S4B), indicating that 5-FU treated H-Cx43-deficient HSCs also failed to transition through the G0/G1 checkpoint. Pathway analysis of the differential transcriptional expression of 5-FU (96 h)-treated H-Cx43-deficient HSCs suggested significant impairment of the transition through the cell cycle checkpoints (Table S2), and Q-RT-PCR confirmed the up-regulation of the cyclin dependent kinase p21 (p21) and down-regulation of cyclin D1 mRNA levels in 5-FU (96 h)-treated H-Cx43-deficient HSCs (Fig. 2D). In addition, HSCs (and MPPs) from H-Cx43-deficient mice showed increased apoptosis in vivo (Fig. 2E) and activation of cell death genes (Table S2).

We next determined the molecular mechanisms associated with HSC impaired cell cycle entry in the live gated cell fraction of 5-FU-treated H-Cx43-deficient HSCs. We analyzed the expression and activation through Ser-10 phosphorylation of p53 in H-Cx43-deficient HSCs after 5-FU administration, which is associated with HSC quiescence. We found that H-Cx43-deficient HSCs from unchallenged mice expressed a ~2.5-fold higher level of activated p53. In 5-FU-treated H-Cx43-deficient HSCs, the activation of p53 (Fig. 3A and Fig. S5) or its downstream targets Gadd45a, Pimpl, and Bmi1 (Fig. 3B) was similar to WT HSCs. In contrast to p53-dependent HSC quiescence, HSC senescence depends on up-regulated expression of the cyclin-dependent kinase inhibitor p16 (p16), a hallmark of stem cell aging (22). There was a ~twofold increase in the expression of nuclear p16 (p16), which is up-regulated during cell senescence (22), in both unchallenged and in vivo 5-FU-challenged H-Cx43-deficient HSCs (Fig. 3C). In addition, there was a ~twofold up-regulation of the expression of Rb1, a central regulator of the G1 phase of the cell cycle and a regulator of interactions between HSCs and the HM (Table S2) (23). These results indicate that the H-Cx43-deficient HSCs are prone to senescence under stress.

A major pathway of p16 up-regulation in HSC senescence is ROS-dependent activation of p38 (24). Pathway analysis of the top signaling pathways differentially expressed by 5-FU-treated H-Cx43-deficient HSCs showed a statistically significant activation of oxidative damage in 5-FU-treated HSCs from H-Cx43-deficient mice (Table S2). Analysis of the intracellular levels of ROS (H2O2 and O2-) in WT and H-Cx43-deficient HSCs after 5-FU administration, showed that H-Cx43-deficient HSCs after 5-FU administration had an ~1.8- to 2.1-fold increase in intracellular ROS content compared with WT HSCs (Fig. 3D and E). The production of ROS is one of the byproducts of mitochondrial respiration, and mitochondriads have frequently been considered as the main source of cellular-
two independent experiments). Q-RT-PCR for Foxol expression in BM-Lin~CD41~CD48~CD150+ were measured from a minimum of 2 mice per group). In Intracellular H2O2 level measured with DCF-DA (D), intracellular O2~ reporter that binds to DNA irreversibly upon oxidation becoming unavailable to be transferred from WT HSC/P cells to BM stromal cells. Before adhesion to HSCs (29). To address whether the contact of HSCs with BM stromal cells was causal in the control of HSC ROS levels, we cocultured HSC/P cells derived from WT or H-Cx43-deficient mice with preplated FBMD-1 stromal cells, a well-recognized model of heterocellular hematopoiesis-supporting stroma (30) composed of ROS+ and ROS− cell populations (Fig. S6). We then analyzed whether ROS could be efficiently transferred from WT HSC/P cells to BM stromal cells. Before culture, primary sorted HSC/P cells were treated with LY83583 (6-anilino-5,8-quinolinequinone), a generator of superoxide anions (31), to model increased ROS production as seen in vivo after 5-FU administration; and BM stromal cells were treated with NAC, to diminish basal ROS levels. The intracellular concentration of ROS in HSC/P cells was measured in sorted HSC/P cells by flow cytometric analysis of fluorescence intensity of dihydroethidium (DHE), an O2~ reporter that binds to DNA irreversibly upon oxidation becoming unavailable to be transferred through GJs. When HSC/P cells were plated without FBMD-1 stroma as a control, LY83583-treated WT or H-Cx43-deficient HSC/P cells showed high intracellular ROS levels (Fig. 3C). However, the mitochondrial function of Cx43-dependent channels (28). We hypothesized that Cx43 deficiency would lead to accumulated levels of intracellular ROS in HSCs, resulting in cell cycle arrest, apoptosis, and senescence. To test this hypothesis, we performed a set of mechanistic experiments to address the role of Cx43 in the control of HSC ROS content. First, we tested whether antioxidant therapy with N-acetyl-L-cysteine (NAC), a reducing agent that diminishes the endogenous level of intracellular ROS, could reverse the impaired hematopoietic regeneration of H-Cx43-deficient mice after 5-FU administration. WT or H-Cx43-deficient animals were treated daily with NAC or control vehicle starting 1 d before 5-FU administration. There was a complete restoration of the neutrophil and platelet count recovery in H-Cx43-deficient mice after in vivo treatment with NAC to the levels seen in WT mice treated with PBS or NAC (ANOVA; P < 0.05 for both neutrophil and platelet counts) (Fig. 4 A and B). These data prove that oxidative stress is causal in the hematopoietic recovery delay of H-Cx43-deficient HSCs after 5-FU administration.

Second, it has been shown that Cx43 mediates BM stromal cell adhesion to HSCs (29). To address whether the contact of HSCs with BM stromal cells was causal in the control of HSC ROS levels, we cocultured HSC/P cells derived from WT or H-Cx43-deficient mice with preplated FBMD-1 stromal cells, a well-recognized model of heterocellular hematopoiesis-supporting stroma (30) composed of ROS+ and ROS− cell populations (Fig. S6). We then analyzed whether ROS could be efficiently transferred from WT HSC/P cells to BM stromal cells. Before culture, primary sorted HSC/P cells were treated with LY83583 (6-anilino-5,8-quinolinequinone), a generator of superoxide anions (31), to model increased ROS production as seen in vivo after 5-FU administration; and BM stromal cells were treated with NAC, to diminish basal ROS levels. The intracellular concentration of ROS in HSC/P cells was measured in sorted HSC/P cells by flow cytometric analysis of fluorescence intensity of dihydroethidium (DHE), an O2~ reporter that binds to DNA irreversibly upon oxidation becoming unavailable to be transferred through GJs. When HSC/P cells were plated without FBMD-1 stroma as a control, LY83583-treated WT or H-Cx43-deficient HSC/P cells showed high intracellular ROS levels (Fig. 3C). However, the mitochondrial function of Cx43-dependent channels (28). We hypothesized that Cx43 deficiency would lead to accumulated levels of intracellular ROS in HSCs, resulting in cell cycle arrest, apoptosis, and senescence. To test this hypothesis, we performed a set of mechanistic experiments to address the role of Cx43 in the control of HSC ROS content. First, we tested whether antioxidant therapy with N-acetyl-L-cysteine (NAC), a reducing agent that diminishes the endogenous level of intracellular ROS, could reverse the impaired hematopoietic regeneration of H-Cx43-deficient mice after 5-FU administration. WT or H-Cx43-deficient animals were treated daily with NAC or control vehicle starting 1 d before 5-FU administration. There was a complete restoration of the neutrophil and platelet count recovery in H-Cx43-deficient mice after in vivo treatment with NAC to the levels seen in WT mice treated with PBS or NAC (ANOVA; P < 0.05 for both neutrophil and platelet counts) (Fig. 4 A and B). These data prove that oxidative stress is causal in the hematopoietic recovery delay of H-Cx43-deficient HSCs after 5-FU administration.

It has been shown that Cx43 mediates BM stromal cell adhesion to HSCs (29). To address whether the contact of HSCs with BM stromal cells was causal in the control of HSC ROS levels, we cocultured HSC/P cells derived from WT or H-Cx43-deficient mice with preplated FBMD-1 stromal cells, a well-recognized model of heterocellular hematopoiesis-supporting stroma (30) composed of ROS+ and ROS− cell populations (Fig. S6). We then analyzed whether ROS could be efficiently transferred from WT HSC/P cells to BM stromal cells. Before culture, primary sorted HSC/P cells were treated with LY83583 (6-anilino-5,8-quinolinequinone), a generator of superoxide anions (31), to model increased ROS production as seen in vivo after 5-FU administration; and BM stromal cells were treated with NAC, to diminish basal ROS levels. The intracellular concentration of ROS in HSC/P cells was measured in sorted HSC/P cells by flow cytometric analysis of fluorescence intensity of dihydroethidium (DHE), an O2~ reporter that binds to DNA irreversibly upon oxidation becoming unavailable to be transferred through GJs. When HSC/P cells were plated without FBMD-1 stroma as a control, LY83583-treated WT or H-Cx43-deficient HSC/P cells showed high intracellular ROS levels (Fig. 3C). However, the mitochondrial function of Cx43-dependent channels (28). We hypothesized that Cx43 deficiency would lead to accumulated levels of intracellular ROS in HSCs, resulting in cell cycle arrest, apoptosis, and senescence. To test this hypothesis, we performed a set of mechanistic experiments to address the role of Cx43 in the control of HSC ROS content. First, we tested whether antioxidant therapy with N-acetyl-L-cysteine (NAC), a reducing agent that diminishes the endogenous level of intracellular ROS, could reverse the impaired hematopoietic regeneration of H-Cx43-deficient mice after 5-FU administration. WT or H-Cx43-deficient animals were treated daily with NAC or control vehicle starting 1 d before 5-FU administration. There was a complete restoration of the neutrophil and platelet count recovery in H-Cx43-deficient mice after in vivo treatment with NAC to the levels seen in WT mice treated with PBS or NAC (ANOVA; P < 0.05 for both neutrophil and platelet counts) (Fig. 4 A and B). These data prove that oxidative stress is causal in the hematopoietic recovery delay of H-Cx43-deficient HSCs after 5-FU administration.

Third, if ROS transfer is the mechanism of ROS scavenging, then culture of high ROS-containing HSC/P cells onto FBMD-1 cells should increase the intracellular levels of ROS in the

derived ROS (25). Mitochondrial Cx43 has been shown to play a role in mediating the cardioprotective effect of ischemic preconditioning through modification of the mitochondrial content and membrane potential (26). Analysis of O2− generated by mitochondrial activity showed that, similarly to overall intracellular ROS levels, mitochondrial-derived superoxide levels were increased in 5-FU-treated H-Cx43-deficient HSCs compared with WT HSCs (Fig. 3F). However, the mitochondrial mass was not affected and the mitochondrial membrane potential only marginally decreased in unchallenged H-Cx43-deficient HSCs but not after 5-FU administration (Fig. 5 G and H), indicating that the deficiency of Cx43 does not correlate with significant modifications in mitochondrial mass or membrane potential. Moreover, increased ROS levels correlated with increased p38 activation (Fig. 3I) but not extracellular signal-regulated kinase (Erk) activation (Fig. 3J). Activation of p38 correlated with Foxo1 expression (Fig. 3K), which, in addition to p16INK4a up-regulation, have been shown to be hallmarks of ROS-dependent HSC repopulation loss-of-function (24) and HSC resistance to physiologic oxidative stress (27), respectively.

Fig. 3. Cell cycle arrest in H-Cx43-deficient after 5-FU treatment is associated with increased levels of intracellular ROS and activation of quiescence markers p16 and p38. (A) Immunofluorescence intensity of anti-phopho-p38 immunostaining of BM-Lin~CD41~CD48~CD150+ cells from WT (open bars) or H-Cx43-deficient mice (solid bars) after 5-FU administration was measured by using computer-imaging software (Axiovision; Zeiss). *P < 0.05 (n = 20–35 cells per group; were measured from a minimum of two mice per group). (B) Q-RT-PCR of p38 downstream targets in BM-Lin~CD41~CD48~CD150+ cells, isolated from WT (open bars) and H-Cx43-deficient mice (solid bars) after 5-FU administration. (C) Immunofluorescence intensity of anti-p16 Immunostaining of BM-Lin~CD41~CD48~CD150+ cells from WT (open bars) or H-Cx43-deficient mice (solid bars) after 5-FU administration. *P < 0.05 (n = 20–35 cells per group; were measured from a minimum of two mice per group). (D–F) Intracellular H2O2 level measured with DCF-DA (D), Intracellular O2~ level measured with DHE (E) and mitochondrial-derived superoxide levels (F) in BM-Lin~CD41~CD48~CD150+ cells from WT (open bars) or H-Cx43-deficient mice (solid bars) after 5-FU administration. *P < 0.05 (n = 3 mice per group in each of two independent experiments). (G) Mitochondrial size (MitoTracker Green FM) (G) and mitochondrial potential (MitoTracker Red FM) (H) of BM-Lin~CD41~CD48~CD150+ cells after 5-FU administration in WT (open bar) or H-Cx43-deficient mice (solid bars) were measured in vivo. *P < 0.05 (n = 3 mice per group). (J and K) Mean fluorescent intensity of phospho-p38 (J) and phospho-MAPK (J) of BM-Lin~CD41~CD48~CD150+ cells from WT (open bars) or H-Cx43-deficient mice (solid bars) after 5-FU administration. *P < 0.05 (n = 3 mice per group in each of two independent experiments). (K) Q-RT-PCR for Foxo1 expression in BM-Lin~CD41~CD48~CD150+ cells, isolated from WT (open bars) and H-Cx43-deficient mice (solid bars) after 5-FU administration. *P < 0.05 (pool of three mice per group). Values represent means ± SD.
sternal cells. As a control, we checked that overnight NAC treatment of FBMD-1 cells significantly reduces the intracellular ROS levels (Fig. 4D). A 3-h coculture of WT HSC/P cells onto FBMD-1 reversed the effect of NAC, returning intracellular ROS levels similar to those in untreated FBMD-1 cells (Fig. 4D). However, H-Cx43-deficient HSC/P cells were unable to increase the transfer of ROS to FBMD-1 cells beyond the basal levels of NAC-treated FBMD-1 cells (Fig. 4D). Furthermore, exogenous expression of WT Cx43 in lentivirus-transduced Cx43-deficient HSCs rescued the decreased transfer of ROS into the stromal cells in H-Cx43-deficient HSC/P cells to the same level of WT-Mock HSC/P cells (Fig. 4E and Fig. S6). Together, these data indicate that Cx43 mediates the transfer of ROS from HSC/P cells to hematopoiesis-supporting BM stromal cells.

Finally, to address whether Cx43 homotypic interactions between HSCs and BM stroma were at play, we analyzed whether Cx43 deficiency in the HM phenocopies the deficiency of Cx43 in the HSC compartment with respect to its inability to regenerate stress hematopoiesis. For this purpose, we induced Cx43 deficiency in the HM (10) using Mx1-Cre transgenic mice as shown previously (23, 32). Mx1-Cre;WT and Mx1-Cre;Cx43lox/lox mice were treated with polyinositide:polycytidine (polyI:C). One week after the last injection of polyI:C, the mice were submitted to lethal irradiation, followed by transplantation of WT CD45.1+ BM. Chimeric mice (>90% CD45.1+ hematopoietic chimeras) were challenged with 5-FU in the same way as in primary Vav1-Cre;Cx43lox/lox mice. The myeloid regeneration of H1 Cx43-deficient mice phenocopied the defective regeneration observed in H-Cx43-deficient mice, as assessed by neutrophil counts in the PB (Fig. 4F) and reduced BM cellularity and progenitor content (Fig. 4G and H). This suggests that Cx43 expression in the HM is similarly required for hematopoietic regeneration and Cx43-Cx43 heterologous interactions between HSC/P cells and the cellular HM are required for an adequate regenerative response after chemotherapy.

Discussion

HSCs are responsible for sustaining blood formation and regeneration after injury for the entire lifespan of an organism through self-renewal, survival, proliferation, and differentiation. HSC aging has become a concern in chemotherapy of older patients. HSC function declines with age, and prolonged myelo-suppression in response to cytotoxic chemotherapy drugs suggests a reduced narrow regenerative capacity in older individuals (33–36). However, the number of HSCs does not necessarily decline, but it can also increase (37, 38). There is evidence to indicate a distinct role for intrinsic and extrinsic factors in HSC aging to explain this apparent discrepancy (39). However, the molecular mechanisms that regulate the HM control on HSC function during aging are poorly understood.

HSC functions can be affected by the intracellular level of ROS that are produced endogenously through cellular metabolism or directly after exposure to exogenous stress, and ROS levels have long been associated with aging (40). At physiological levels, low and moderate levels of ROS appear to be required for HSC activity (41–43), including early hematopoietic reconstitution after transplantation (44). However, a sustained, abnormal increase in ROS production occurs under aging (24) and genotoxic stress (45), including 5-FU chemotherapy (46), which can inhibit HSC self-renewal and induce HSC senescence and hematopoietic dysfunction (24). Mimicking the situation in aged individuals, the HSC BM content of H-Cx43-deficient old mice is increased over aged-matched controls (47), whereas their ability to regenerate after 5-FU administration is diminished (Ref. 10 and Fig. 1J and K). Followed by 5-FU administration, HSCs from H-Cx43-deficient mice showed decreased ability to enter the cell cycle and survive, as well as an increased intracellular ROS content.
In this report, we demonstrate a function of the HM as a scavenger of ROS from stressed HSC cells through Cx43. Our data provide evidence that Cx43 deficiency cannot be significantly compensated by other connexins, at either expression or functional levels, and Cx43 is a major mediator of ROS scavenging through transfer from HSCs to stromal cells.

It has been shown that ROS can regulate HSC function in a concentration-dependent manner. High levels of ROS can induce HSC senescence and apoptosis secondary to DNA damage (24). Whereas Cx43 deficiency induces increased apoptosis, surviving HSCs from H-Cx43-deficient mice display the hallmark features of senescence, including hyporegenerative capacity and cell cycle arrest after chemotherapy, and up-regulation of p16INK4a. Hyporegenerative/senescent HSCs are induced by high levels of ROS/p38MAPK/Foxo1 signal activation, and HSC loss-of-function can be reversed by NAC administration in vivo or by the reintroduction of Cx43, confirming the expected role of HSC Cx43 in ROS scavenging by the HM. Finally, the deficiency of Cx43 in the HM in chimeric mice generated by transplanting WT hematopoiesis (>90%) into an inducible murine model of Cx43 deficiency (10) significantly phenocopies the deficiency of Cx43 in HSCs.

Altogether, our data provide insights into the homeostatic regulation of ROS content in BM HSCs and present a mechanistic model of BM microenvironment control on HSC activity through indispensable expression of Cx43 in both HSCs and the cellular HM.

Materials and Methods

Information on generation of H-/-/CM-Cx43-deficient and chimeric mice, repopulation experiments, drug administration and cell sorting, may be found in SI Materials and Methods. For HSCC assays, including proliferation, cell cycle, survival and lentiviral transduction, see SI Materials and Methods. ROS transfer, genomic PCR, RT-PCR analysis, and statistical analysis are included in SI Materials and Methods.

Acknowledgments. We thank Dr. Hartmut Geiger (University of Ulm) for helpful comments and Ms. Margaret O'Leary for editing the manuscript. We also thank Jorden Arnett, Jeff Bailey, and Victoria Summey for technical assistance and the Mouse and Research Flow Cytometry Core Facilities, both supported by National Institutes of Health Centers of Excellence for Molecular Hematology Grant 1R01DK60972-01. This project was funded by the Heimlich Institute of Cincinnati (J.A.C.), US Department of Defense Grant 10580355 (to J.A.C.), National Institutes of Health Grants R01-HL087159 and HL087159X1, the National Blood Foundation (D.G.-N.), Spanish Technology Science Council CSD2008-00005 (to J.C.B.), Community of Madrid Grant S2010/BMD-2460 (to D.G.-N.), and funds from the Hoxworth Blood Bank and Cincinnati Children's Hospital Medical Center (to J.A.C.).

4. Chen J, et al. (2008) Enrichment of hematopoietic stem cells with SLAM and LSK scavenging by the HM. Finally, the deficiency of Cx43 in the HM in chimeric mice generated by transplanting WT hematopoiesis (>90%) into an inducible murine model of Cx43 deficiency (10) significantly phenocopies the deficiency of Cx43 in HSCs.