Corrections

MEDICAL SCIENCES


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

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**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, 40×) from Vav1-Cre; Rosa-loxP-Stop-loxP-LacZ BM transduced with an empty vector at day 0.

Legend continued on following page.
PSYCHOLOGICAL AND COGNITIVE SCIENCES


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 1. Summary of fMRI activity clusters

<table>
<thead>
<tr>
<th>Region (BA)</th>
<th>L/R/B</th>
<th>Volume (mm³)</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>
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<tbody>
<tr>
<td></td>
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<td>t</td>
<td>P</td>
<td>t</td>
<td>P</td>
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<td><strong>Fig. 1. regions shown in blue</strong></td>
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<tr>
<td>Posterior cingulate (BA 31)</td>
<td>B</td>
<td>8,343</td>
<td>4</td>
<td>−40</td>
<td>49</td>
<td>1.77</td>
<td>0.10</td>
<td>−2.82</td>
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<tr>
<td>Anterior cingulate/vmPFC (BA 24, 32)</td>
<td>B</td>
<td>7,965</td>
<td>−3</td>
<td>20</td>
<td>27</td>
<td>3.19</td>
<td>0.01</td>
<td>−3.11</td>
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<tr>
<td>Anterior insula (BA 13)</td>
<td>L</td>
<td>2,187</td>
<td>−53</td>
<td>8</td>
<td>−1</td>
<td>2.09</td>
<td>0.06</td>
<td>−2.70</td>
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<tr>
<td>Anterior insula (BA 13)</td>
<td>R</td>
<td>1,782</td>
<td>37</td>
<td>6</td>
<td>6</td>
<td>3.91</td>
<td>0.00</td>
<td>−2.46</td>
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<tr>
<td>Posterior insula (BA 13, 19)</td>
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<td>8,478</td>
<td>47</td>
<td>−45</td>
<td>13</td>
<td>2.33</td>
<td>0.04</td>
<td>−2.81</td>
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<tr>
<td>Middle temporal gyrus (BA 39)</td>
<td>L</td>
<td>1,134</td>
<td>−41</td>
<td>−51</td>
<td>7</td>
<td>3.77</td>
<td>0.00</td>
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<tr>
<td>Medial frontal gyrus (BA 6)</td>
<td>R</td>
<td>1,809</td>
<td>6</td>
<td>−15</td>
<td>67</td>
<td>2.68</td>
<td>0.02</td>
<td>−2.48</td>
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<tr>
<td>Amygdala (anatomically defined)</td>
<td>R</td>
<td>891</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>3.85</td>
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<td><strong>Fig. 1. region shown in red</strong></td>
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<tr>
<td>dIPFC (BA 6, 8)</td>
<td>R</td>
<td>918</td>
<td>36</td>
<td>15</td>
<td>61</td>
<td>−2.38</td>
<td>0.04</td>
<td>8.27</td>
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<tr>
<td>Superior parietal lobule (BA 7)</td>
<td>R</td>
<td>1,458</td>
<td>25</td>
<td>−72</td>
<td>57</td>
<td>NS</td>
<td>3.12</td>
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<tr>
<td><strong>Fig. 2. regions shown in gray</strong></td>
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<tr>
<td>Fusiform/lingual gyrus (BA 18, 19)</td>
<td>L</td>
<td>8,046</td>
<td>−34</td>
<td>−81</td>
<td>−9</td>
<td>4.91</td>
<td>0.00</td>
<td>NS</td>
</tr>
<tr>
<td>Fusiform/lingual gyrus (BA 18, 19)</td>
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<td>11,367</td>
<td>36</td>
<td>−78</td>
<td>−11</td>
<td>5.14</td>
<td>0.00</td>
<td>NS</td>
</tr>
</tbody>
</table>

For each activity cluster, listed are Brodmann areas (BA), hemisphere (left (L), right (R), or bilateral (B)), the volume (mm³), 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).
Connexin-43 prevents hematopoietic stem cell senescence through transfer of reactive oxygen species to bone marrow stromal cells

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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 recovery after myeloablation with 5-fluorouracil (5-FU). 5-FU-treated H-Cx43-deficient HSCs and progenitors (HSC/P) 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, p21waf1, 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 HM 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.

Gja1 | stem cell niche

Blood cell formation in the bone marrow (BM) is dependent on the close association of developing hematopoietic cells with supporting stromal cells. These hematopoietic demands are filled by a pool of hematopoietic stem cells (HSCs) with self-renewal and multipotential differentiation ability. The BM hematopoietic microenvironment (HM) has been shown to have a crucial role that influences the proliferative activity and differentiation process of HSCs and progenitors (HSC/P) by positive and negative humoral and paracrine signals.

Gap junctions (GJs) represent a system of direct cell-to-cell intercellular communication (IC) (1). Although the existence of GJs in BM has been known for over 30 y, their function remains unclear (2, 3). GJ channels are formed by dodecamers of protein subunits called connexins (Cx). Cx43 is the predominant Cx expressed in the BM, thymus, spleen, and other lymphoid tissues (4–6). Cx43 has been shown to be part of the signature of HSCs (7–9), being down-regulated during differentiation to progenitors (7) and up-regulated in the endosteal space of the BM following cytotoxic ablative therapy (6). Its deficiency in the BM results in deficient hematopoietic regeneration after in vivo challenge with 5-fluorouracil (5-FU) (10), which is an established stressor of quiescent HSCs (11). However, a mechanistic analysis of the role of Cx43 in hematopoietic regeneration has not been addressed. In this report, we present compelling data unveiling a mechanism of ROS detoxification in HSCs through Cx43-dependent transfer to the HM that prevents HSC quiescence/senescence after myeloablation.

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/Cx43lox/lox, hematopoietic specific (H)-Cx43-deficient]. As reported by others (12–15), we confirmed that Vav1-Cre expression is extremely efficient, inducing recombination of either a reporter gene (Fig. L4) or the floxed Gja1 (Cx43lox/lox) gene (Fig. 1 B and C). The mRNA expression of Cx43 in HSCs from Vav1-Cre;Cx43lox/lox mice was practically abolished in BM HSCs [defined as lineage−/c-kit+/Sca-1+/CD34−/LSK CD34−; Fig. 1D] and multipotent progenitors (MPPs) (defined as LSK/CD34−; Fig. 1E). Furthermore, we analyzed the protein expression of Cx43 in wild-type (WT) and Vav1-Cre;Cx43lox/lox HSCs. Confocal microscopy detected Cx43 protein expression in the membrane of isolated HSCs from WT but not from Vav1-Cre;Cx43lox/lox mice (Fig. 1F). Expression of Cx43 (Gja1), another connexin family protein involved in hematopoiesis, was detected in both WT and Vav1-Cre;Cx43lox/lox HSCs (Fig. S1) and found not to be significantly up-regulated in H-Cx43-deficient HSCs (Table S1). Cx37 (Gja8) and Cx50 (Gja4) showed marginal 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 no significant differences in blood counts or the content of Lin-/c-kit+ (LK), LSK, MPP, or HSC populations in H-Cx43-deficient mice (Fig. S2B). Second, the HSC content and function were assessed in vivo, using serial competitive repopulation assays. HSCs from H-Cx43-deficient mice exhibited no significant defects in their competitive engraftment and lineage hematopoiesis regeneration (Fig. S3) in the primary (Fig. 1G), secondary (Fig. 1H), and tertiary recipient mice (Fig. 1I).

Hematopoietic stress induced by 5-FU has been used to test the ability of HSCs to recover homeostatic blood formation (16). After a period of pancytopenia, the magnitude of the compensatory BM regeneration phase indicates the functional reserve of HSCs (17). Repeated administration of 5-FU to mice induces exhaustion of the normal HSC pool and a progressive inability to recover (18), indicating HSC damage. Similarly to mice in which both HSCs and the HM are H-Cx43-deficient (10), H-Cx43-deficient mice treated with 5-FU (150 mg/kg i.v.) lack a hyperregenerative response phase, as demonstrated by an aborted rebound in neutrophil (Fig. 1J) and platelet (Fig. 1K) counts in PB, indicating a loss of hematopoietic response activity after stress. This deficiency is not persistent because the content of HSCs and MPPs in the BM by day 21 after 5-FU challenge is similar to WT levels (Fig. 1L). The myeloid regeneration of H-Cx43-deficient animals after 5-FU administration is completely rescued in hematopoietic chimeric mice, where hematopoietic Cx43 expression has been reintroduced through transplantation of lentivirally transduced LSK cells (Fig. 1M). Altogether, these results indicate that hematopoietic Cx43 expression is critical for hematopoietic regeneration after 5-FU administration. Finally, the reduced hematopoietic regeneration of H-Cx43-deficient HSCs was confirmed in full chimeras of WT or H-Cx43-deficient BM (Fig. 1N and O), supporting a cell autonomous role of Cx43.

To examine the underlying cellular mechanism responsible for the impaired hematopoietic recovery after 5-FU administration in H-Cx43-deficient mice, we analyzed the proliferation status of H-Cx43-deficient BM Lin-CD41-/CD48-/CD150+ cells, which also define phenotypically the HSC population (19, 20) and is not affected by down-regulation of c-kit expression during hematopoietic regeneration (21) after 5-FU administration. We
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. Pyronin/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 transit 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 cell cycle checkpoints (Table S2), and Q-RT-PCR confirmed the up-regulation of the cyclin dependent kinase p21cip1 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 p38 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 p38. In 5-FU-treated H-Cx43-deficient HSCs, the activation of p38 (Fig. 3A and Fig. S5) or its downstream targets Gadd45a, Pimp1, and Bmi1 (Fig. 3B) were similar to WT HSCs. In contrast to p33-dependent HSC quiescence, HSC senescence depends on up-regulated expression of the cyclin-dependent kinase inhibitor p16INK4a, a hallmark of stem cell aging (22). There was a ~two-fold increase in the expression of nuclear p16INK4a, 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 ~two-fold 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 mitochondrias have frequently been considered as the main source of cellular-
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 \( \cdot O_2^- \) 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. 3G 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 \( p16^{INK4a} \) 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.

Transfer of small molecules is arguably a well-recognized 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. 4A 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-high and ROS-low cell populations (Fig. 5). 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 \( \cdot O_2^- \) 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. 4C). When WT HSC/P cells were cultured onto FBMD-1 cells, however, the intracellular ROS levels in WT HSC/P cells diminished significantly (~83% reduction; \( P < 0.001 \)) compared with H-Cx43-deficient HSC/P cells (Fig. 4C). Although FBMD-1 cells maintained their ability to reduce ROS content of H-Cx43-deficient HSC/P cells (~64% reduction; \( P = 0.02 \)), they did so to a lesser degree (\( P = 0.03 \)) than in WT HSC/P, indicating that the ability of FBMD-1 cells to diminish ROS concentration in HSC/P cells is lessened by the Cx43 deficiency in HSC/P cells.

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
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 myelosuppression in response to cytotoxic chemotherapy drugs suggests a reduced marrow 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). Although 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 HM function 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. 1 J 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.

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In this report, we demonstrate a function of the HM as a scavenger of ROS from stressed HSC/P 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 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 reverted by NAC administration in vivo or by the reintroduction of Cx43, indicating that Cx43 is a crucial molecule in the maintenance of HSC fitness. The reintroduction of Cx43 also rescues the ROS transfer from ROS-stressed HSC/P cells to BM stromal cells, 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 hematopoietic (>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 mechanism of BM microenvironmental control on HSC activity through indispensable expression of Cx43 in both HSCs and the cellular HM.

Materials and Methods

Information on generation of H-HM-Cx43-deficient and chimeric mice, repopulation experiments, drug administration and cell sorting, may be found in SI Materials and Methods. For HSC/P 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.

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