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PRINCIPAL INVESTIGATOR: Jose Cancelas, M.D., Ph.D.

CONTRACTING ORGANIZATION: Children's Hospital, Cincinnati
Cincinnati, OH 45229

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14. ABSTRACT

The mechanisms responsible for both acquired and inherited bone marrow failure (BMF) are not yet understood. Although most inherited BMF syndromes can be linked to specific genetic defects, these defects do not fully explain the range of physical characteristics observed in affected individuals. In addition, animal models for observed BMF genetic defects have not been able to fully recreate the associated syndromes. With support from a Fiscal Year 2010 Bone Marrow Failure Research Program Exploration–Hypothesis Development Award, Dr. Cancelas is investigating whether BMF syndromes are related to a defect in cell-to-cell communication between mesenchymal stem cells and progenitors (MSC/P) and hematopoietic stem cells (HSC). Blood cell formation in the bone marrow (hematopoiesis) is dependent on the close association of HSC and the surrounding microenvironment, of which MSC/P are a major component. Natural causes such as aging, or external insults, such as radiation, toxin exposure, or chemotherapy, are known to cause DNA damage and affect HSC activity, which may in part be due to hematopoietic stress from increasing cellular levels of reactive oxygen species (ROS). Fanconi anemia and Schwachman-Diamond syndrome are two BMF syndromes with defects in DNA repair enzyme systems, making individuals with these syndromes highly sensitive to DNA-damaging events. However, researchers suspect that modifier genes or traits are responsible for the differences in the severity of these diseases and their relation to skeletal malformations in BMF syndromes. Using a model of DNA damage induced by chemotherapy-induced ROS production, Dr. Cancelas tested the ability of HSC to resume blood formation after hematopoietic stress. His results indicated that hematopoietic recovery is delayed when HSC are deficient in connexin-43 (Cx43), a gap junction protein involved in cell-to-cell communication and highly expressed in HSC and MSC/P. Furthermore, the results showed that Cx43 mediates the transfer of ROS to the MSC/P in the bone marrow microenvironment. This transfer of ROS to the MSC/P is a mechanism by which hematopoietic recovery is achieved after chemotherapy. This work was recently published in the Proceedings of the National Academy of Sciences USA (Taniguchi-Ishikawa et.al, 2012). In a second step, we investigated the relationship between the loss of cell-to-cell communication between HSC and MSC/P as a modifier trait and the genetic defects of Fanconi anemia-A. A humanized animal model of FANC-A silenced hematopoiesis and Cx43-deficient hematopoietic microenvironment shows that the deficiency of either of them results in diminished human engraftment in human, but not murine, MSC microenvironment. A possible synergism between the deficiency of HSC/P FA-A and MSC Cx43 may result in decreased engraftment of human lymphoid progenitors.

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Introduction

Bone marrow failure (BMF) syndromes are characterized by loss of the hematopoietic capacities of the BM HSC. In BMF syndromes, BM aplasia/hypoplasia is commonly associated with skeletal defects, cardiac and neurological abnormalities. These non-hematopoietic defects have been associated to defects in mesenchymal cell migration and tissue colonization during fetal development. Most inherited BMF syndromes have already been associated with a single gene defect. A major example of the progress in this area is Fanconi Anemia (FA), where mutations in up to 15 different proteins have been associated to this disease, being FA-A the most frequent (1, 2). Single mutated genes in the DNA repair or ribosome biogenesis of HSC are necessary in the pathogenesis of these BMF disorders. Allogeneic stem cell transplantation has demonstrated ability to cure these disorders but frequently results in bone marrow engraftment failure requiring a large number of donor cells to succeed (3). However, these mutations inducing intrinsic HSC defects may not suffice. Advance in the area of BMF research has been hampered by inability to reproduce BMF in most available knock-out murine models, and in general, the potential interactions of defective genes with the environment and modifier genes is believed to be responsible for the poor correlation between genotype and phenotype, such as in Schwachman-Bodian-Diamond syndrome (SBDS) (4), Blackfan-Diamond anemia (5) and thrombocytopenia-absent radius syndrome (6).

Mesenchymal cells contribute to the MSC/P of most normal and malignant tissues, with specific mesenchymal participating in the regulatory niches of HSC. These mesenchymal cells are major constituents of the hematopoietic microenvironment (HM), which maintains HSC functions. Different cell populations have been claimed to be in charge of the support of long-term HSC (LT-HSC) in the HM, but there is a general agreement that a significant fraction of them are of osteoprogenitor mesenchymal lineage, including N-cadherin⁺ pre-osteoblastic cells (7), CXCL12-secreting adventitial reticular (CAR) cells, (8) and MSC/P (9). The overall group of these osteoprogenitor cells has been called mesenchymal stem cells and progenitors (MSC/P) (10). Adhesion, communication and synchronized activity between MSC/P and HSC has been shown to be critical in hematopoiesis (11). Growing evidence suggests that BMF syndromes may be pathogenetically related to a previously underappreciated mesenchymal defect (12, 13), normal MSC/P can improve the adhesion, homing and engraftment of FA HSC(10) and

osteolineage mesenchymal dysfunction induces BM aplasia and pre-leukemia associated with downregulation of SBDS protein expression (14). The *in vivo* role of MSC/P within the HM and the putative mechanisms responsible for their loss-of-function in BMF syndromes have not been specifically addressed yet.

Multiple factors are secreted by MSC/P and all of them may be responsible for a defective HM that is conducive towards BM aplasia(15-18). However, very few of them are strictly necessary for osteogenesis, osteoblast function, or mesenchymal-derived hematopoietic support. This project postulates that the loss of gap junction (GJ)-mediated intercellular communication (IC) in the osteogenic HM is one of the mechanisms involved in dysfunctional mesenchymal hematopoietic support.

GJ are cell-to-cell adhesion and communication systems that have been shown to be pivotal in cell migration during neurogenesis, cardiogenesis and in epithelial/mesenchymal transition (19-29), and are crucial in the establishment of electrical synapses in the central nervous system, heart, immune system and bone (30-39). GJ are formed by hexamers (hemichannels) of a family of proteins called connexins, with cell specific expression and functions(40, 41). Adhesion and/or direct communication depend on channel-to-channel docking to connect/communicate cytoplasm-to-cytoplasm, creating a functional syncytium. Bone MSC/P express high levels of connexin-43 (Cx43) and, in lesser quantities, Cx45 (42, 43). A deficiency of Cx43 in BM mesenchymal osteolineage cells induces complete failure to transfer calcein, and loss of function of Cx43 in osteoblasts induces bone loss and skeletal defects (35). Reintroduction of Cx43 in fetal liver MSC/P restores hematopoietic support (42). Cx43 is also highly expressed in the HSC population, and is downregulated in expression during differentiation (<http://franklin.imgen.bcm.tmc.edu/loligag>)(44). Deficient Cx43 expression in BM HSC and MSC/P induces BMF (43). In humans, most of 30 different mutations in Cx43 cause oculodentodigital dysplasia (ODDD) syndrome, an inherited disorder characterized by developmental defective osteogenesis resulting in craniofacial and skeletal anomalies and associated with heart defects and neurological defects. ODDD phenocopies some of the skeletal defects of BMF syndromes and some ODDD patients (31, 45-47) and ODDD animal models (31, 48-52) show BMF, specifically anemia and thrombocytopenia. It is unknown whether the hematopoietic response of ODDD patients is altered upon hematopoietic stressors like anemia, chemotherapy or others conditions.

Body

Two questions were proposed:

#1. Do FA-A and/or SBDS deficiency require loss-of-function of intercellular communication in the human BM MSC/P to induce hematopoietic failure?

#2: Do FA-A and/or SBDS loss-of-function induce loss of cell-to-cell communication in human MSC/P?.

FA-A and SBDS are two BMF syndromes that affect DNA repair enzyme systems. In order to determine whether the replicative potential of HSC depends on intercellular communication between HSC and MSC/P, we first analyzed whether intercellular communication in BM was crucial in the control of HSC function in conditions that induced DNA damage. For that purpose, we chose the use of 5-fluorouracil-mediated DNA damage induced by ROS production. To clarify whether Cx43 plays a crucial role in HSC 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/Cx43^{flox/flox}, H-Cx43-deficient). In these mice, Cx43 expression is severely downregulated in BM HSC (Figure 1A) and multipotential progenitors (MPP, Figure 1B) To examine whether the loss of Cx43 expression in HSC impairs their self-renewal, the HSC content and function were assessed *in vivo*, using serial competitive repopulation assays. H-Cx43-deficient mice showed no significant hematopoietic abnormalities in their peripheral blood counts or BM content, and HSC from these mice exhibited normal competitive engraftment and hematopoiesis in the primary (Figure 1C), secondary (Figure 1D), and tertiary recipient mice (Figure 1E) without a lineage bias (data not

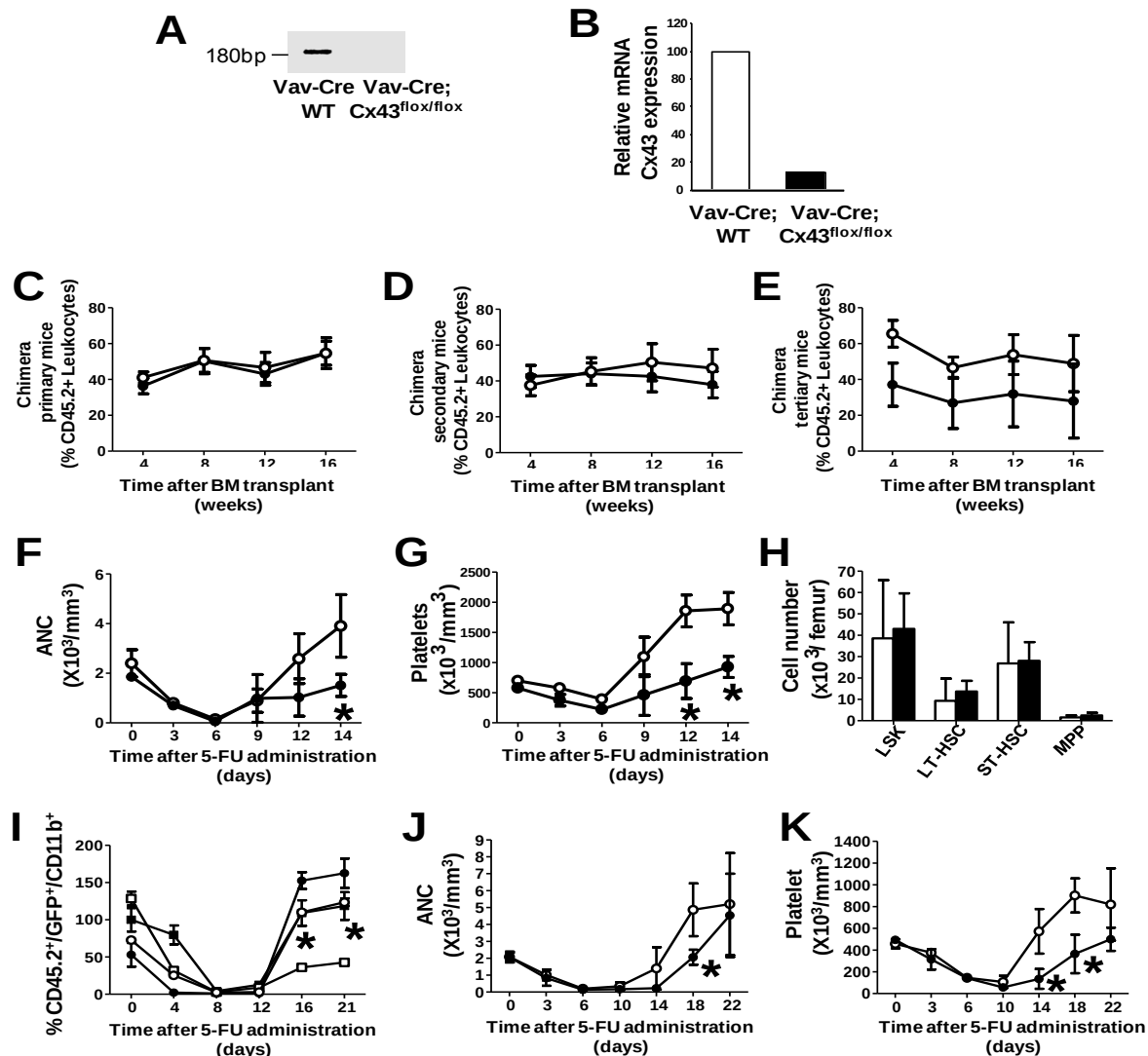


Figure 1. Hematopoietic-Cx43-deficient does not impair serial competitive repopulation but impairs the hematopoietic recovery after 5-FU administration. (A and B) Semi-quantitative RT-PCR for Cx43 expression in HSC (A) and quantitative RT-PCR for Cx43 expression in MPP (B), isolated from control and H-Cx43-deficient mice (n=pool of 3 mice per group). (C-E) Serial competitive repopulation assays. Lethally irradiated primary recipient CD45.1⁺ mice were transplanted with a mixture of Vav1-Cre; 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). (C) Peripheral blood chimera analysis was performed at 1, 2, 3 and 4 months after transplant in primary recipients. (D) Peripheral blood chimera of secondary recipients transplanted with 10×10^6 BM cells obtained from primary mice. (E) Peripheral blood chimera of tertiary recipients transplanted with 10×10^6 BM cells obtained from secondary mice. For competitive repopulation assay, 8-16 mice per group were transplanted and analyzed from two independent experiments. (F-G) Peripheral blood count of WT (open circle) or H-Cx43-deficient mice (solid circles) after 5-FU administration. Counts were performed at the indicated days post 5-FU administration. (F) Absolute neutrophil counts (ANC). (G) Platelet counts. (* $p < 0.05$, n=3 mice per group in each of two independent experiments) (H) HSC and MPP content of BM on day 14 after 5-FU treatment. (I) Transplant of HSC/P transduced with a Cx43-expressing lentiviral vector rescues peripheral blood recovery after 5-FU administration in H-Cx43-deficient mice. Recipient mice were transplanted with Lin⁻/c-kit⁺/Sca-1⁺ BM cells transduced with an empty vector or a Cx43-expressing lentiviral vector. After 4 weeks post-transplantation, recipient mice were administered 5-FU and peripheral blood cell counts and flow cytometry analysis of myeloid (CD11b) recovery were performed at the indicated days post 5-FU administrations. ■, WT with empty vector transduction, □, H-Cx43-deficient with empty vector transduction, ●, WT with Cx43 transduction, ○, H-Cx43-deficient with Cx43 transduction. * $p < 0.05$ (n=4 mice/group). Values represent mean $\pm$ SD. (J-K) 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⁺). Peripheral blood count of WT (open circle) or H-Cx43-deficient mice (solid circle) after 5-FU administration were analyzed after 4 weeks of transplant. (I) Absolute neutrophil count (ANC). (J) Platelet count. (* $p < 0.05$, n=5 mice per group). Values represent mean $\pm$ SD.

shown). Hematopoietic stress induced by 5-FU has been used to test the ability of HSC to

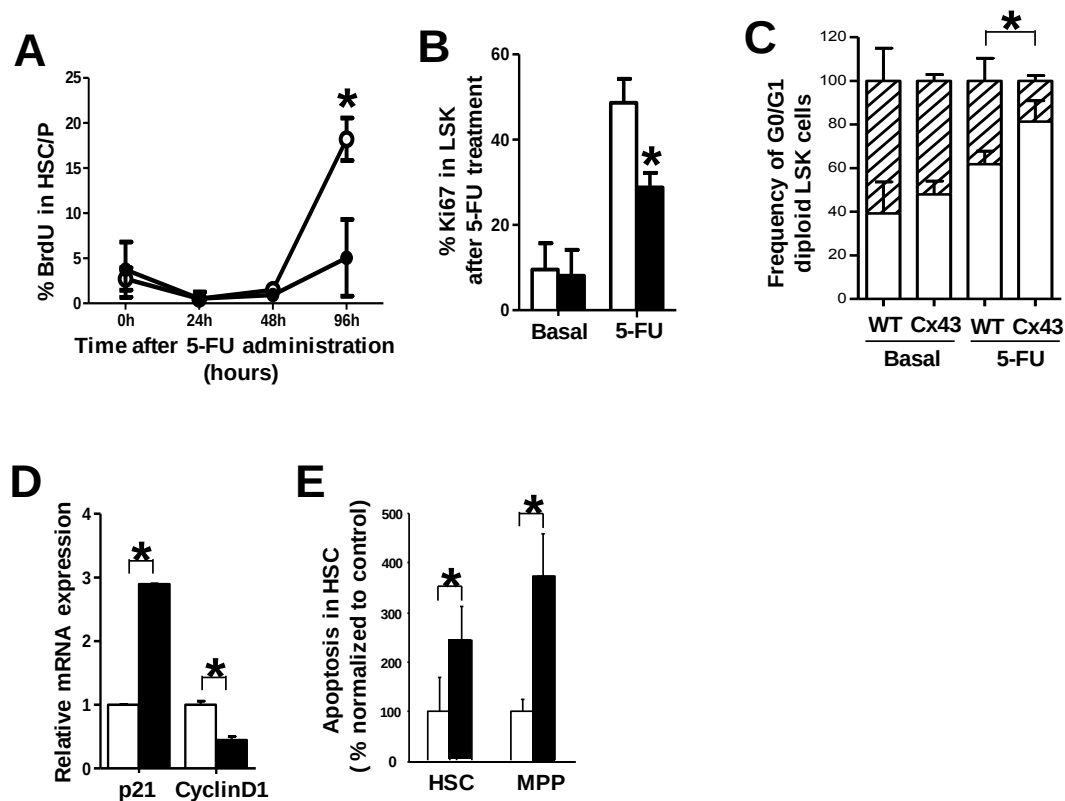


Figure 2. Cx43-deficient HSC are impaired to enter cell cycle to proliferate and have decreased cell survival after 5-FU administration (A) Kinetics of BrdU incorporation in HSC after 5-FU administration. Proliferation of BM-Lin⁻CD41⁺CD48⁺CD150⁺ cells in WT or H-Cx43-deficient mice were measured by analysis of BrdU incorporation at 24hr, 48hr and 96hr after 5-FU administration in vivo. Analysis in WT (open circles) or H-Cx43-deficient (solid circles) mice are presented. (* $p < 0.05$, a minimum of 3 mice per group in two independent experiments was analyzed). (B) Proliferation of BM-Lin⁻CD41⁺CD48⁺CD150⁺ cells after 5-FU administration in WT or H-Cx43-deficient mice was measured by analysis of Ki67 staining in vivo. Analysis in WT (open bars) or H-Cx43-deficient (solid bars) mice are presented. (* $p < 0.05$, $n = 3$ mice per group in each of two independent experiments) (C) 7-aminoactinomycin D (7-AAD)/Pyronin-Y cell-cycle analysis of WT or H-Cx43-deficient BM after in vivo 5-FU administration. □, G0 phase, ▨, G1 phase. (* $p < 0.05$, $n = 3$ mice per group in each of two independent experiments) (D) Quantitative RT-PCR for p21 and cyclinD1 expression in Lin⁻CD41⁺CD48⁺CD150⁺ BM cells. Analysis in WT (open bars) or H-Cx43-deficient (solid bars) mice are presented. * $p < 0.05$ (M) (E) Frequency of apoptotic (active caspase-3-expressing) cells on HSC and MPP cell populations after 5-FU administration in vivo. Data from WT (open bars) or H-Cx43-deficient mice (solid bars) are presented. * $p < 0.05$.

recover homeostatic blood formation (53). After a period of pancytopenia, the magnitude of the compensatory BM regeneration phase indicates the functional reserve of HSC (54). Repeated administration of 5-FU to mice induces exhaustion of the HSC pool and a progressive inability to recover (55) indicating HSC damage. Similarly to mice in which both HSC and the HM are Cx43-deficient (43), H-Cx43 deficient mice treated with 5-FU (150 mg/kg intravenously) lack a hyperregenerative response phase as demonstrated by an abrogated rebound in neutrophil (Figure 1F) and platelet (Figure 1G) counts in PB, indicating a loss of hematopoietic response activity after stress. This deficiency is not persistent because the content of HSC and multipotential progenitors (MPP) in the BM by Day 21 after 5-FU challenge is similar to wild-type (WT) levels (Figure 1H). 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 Lineage⁻/c-kit⁺/Sca1⁺ (LSK) cells (Figure 1I). Altogether, these results indicate that Cx43 expression is

critical in the maintenance of an adequate cell cycle activation response, and for the survival of HSC. Finally, the reduced hematopoietic regeneration of Cx43-deficient HSC was confirmed in full chimeras of WT or H-Cx43-deficient BM (Figure 1J-K), 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 HSC ($\text{Lin}^-/\text{CD41}^-/\text{CD48}^-/\text{CD150}^+$ cells) after 5-FU administration. We first analyzed the frequency of HSC in DNA synthesis phase in vivo at 0, 24, 48 and 96 hours after 5-FU administration. While WT BM HSC showed a ~4-fold increase in the frequency of HSC in S-phase between Days 2 and 4 after 5-FU administration (Figure 2A), Cx43-deficient HSC did not significantly cycle, as assessed by lack of increase in (bromodeoxyuridine) BrdU uptake (Figure 2A) or expression of Ki67 (Figure 2B), by 96 hours post-5-FU administration compared to WT HSC, which confirmed an impaired cell cycle entry in response to chemotherapeutic stress. Pyronin/7-aminoactinomycin D (7-AAD) staining showed accumulation of Cx43-deficient HSC in the G0 phase of the cell cycle (Figure 2C), indicating that stress Cx43-deficient HSC also failed to transit through the G0/G1 checkpoint. Pathway analysis of the differential transcriptional expression of 5-FU(96h)-treated Cx43-deficient HSC showed significant impairment of the G0/G1 and G2/M checkpoints (Table S1), and quantitative real-time polymerase chain reaction (Q-RT-PCR) confirmed the upregulation of the cyclin dependent kinase p21^{cip1} and downregulation of cyclin D1 mRNA levels in 5-FU-treated Cx43-deficient HSC (Figure 2D). In addition, HSC (and MPP) from H-Cx43-deficient mice showed increased apoptosis in vivo (Figure 2E) and activation of cell death genes (data not shown).

We next determined the molecular mechanisms associated with HSC impaired cell cycle entry in stressed Cx43-deficient HSC. We analyzed the expression and activation through Ser-10 phosphorylation of p53 in Cx43-deficient HSC after 5-FU administration which is associated with HSC quiescence. We found that Cx43-deficient HSC from unchallenged mice expressed a ~2.5-fold higher level of activated p53. In 5-FU-treated Cx43-deficient HSC/P the activation of p53 (Figure 3A) or its downstream targets Gadd45a, Pim1 and Bmi1 (Figure 3B) were similar to WT HSC. In contrast to p53-dependent HSC quiescence, HSC senescence depends on upregulated expression of the cyclin-dependent kinase inhibitor, $\text{p16}^{\text{INK4a}}$, a hallmark of stem cell aging (56). There was a ~2-fold increase in the expression of nuclear $\text{p16}^{\text{INK4a}}$, which is upregulated during cell senescence (Figure 3C)(56), in both unchallenged and in vivo 5-FU-challenged Cx43-deficient HSC (Figure 3C). In addition, there was a ~5-fold upregulation of the expression of Rb1, a central regulator of the G1 phase of the cell-cycle and a regulator of interactions between HSC and the HM (data not shown) (57). These results indicate that the Cx43-deficient HSC are prone to senescence under stress..

A major pathway of p16 upregulation in HSC senescence is ROS- dependent activation of p38 (58). Pathway analysis of the top signaling pathways differentially expressed by 5-FU-treated Cx43-deficient HSC showed a statistically significant activation of oxidative damage in stressed HSC from H-Cx43-deficient mice (data not shown). Analysis of the intracellular levels of ROS (H_2O_2 and O_2^-) in WT and Cx43-deficient HSC after 5-FU administration, showed that stressed Cx43-deficient HSC had an ~1.8- to 2.1-fold increase in intracellular ROS content compared with WT HSC (Figures 3D and E). The production of ROS is one of the by-products of mitochondrial respiration, and mitochondrias have frequently been considered as the main source of cellular-derived ROS (59). 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 (60). Analysis of O_2^- generated by mitochondrial activity showed that, similarly to overall intracellular ROS levels, mitochondrial-derived superoxide levels were increased in 5-FU treated Cx43-deficient HSC compared with WT HSC (Figure 3F). However, the mitochondrial mass was not affected and the mitochondrial membrane potential only marginally increased in unchallenged Cx43-deficient HSC but not after 5-FU administration (Figure 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 (Figure 3I), but not Erk/MAPK activation (Figure 3J), and Foxo1 expression (Figure 3K), that in addition to $\text{p16}^{\text{INK4a}}$ upregulation, have been shown to

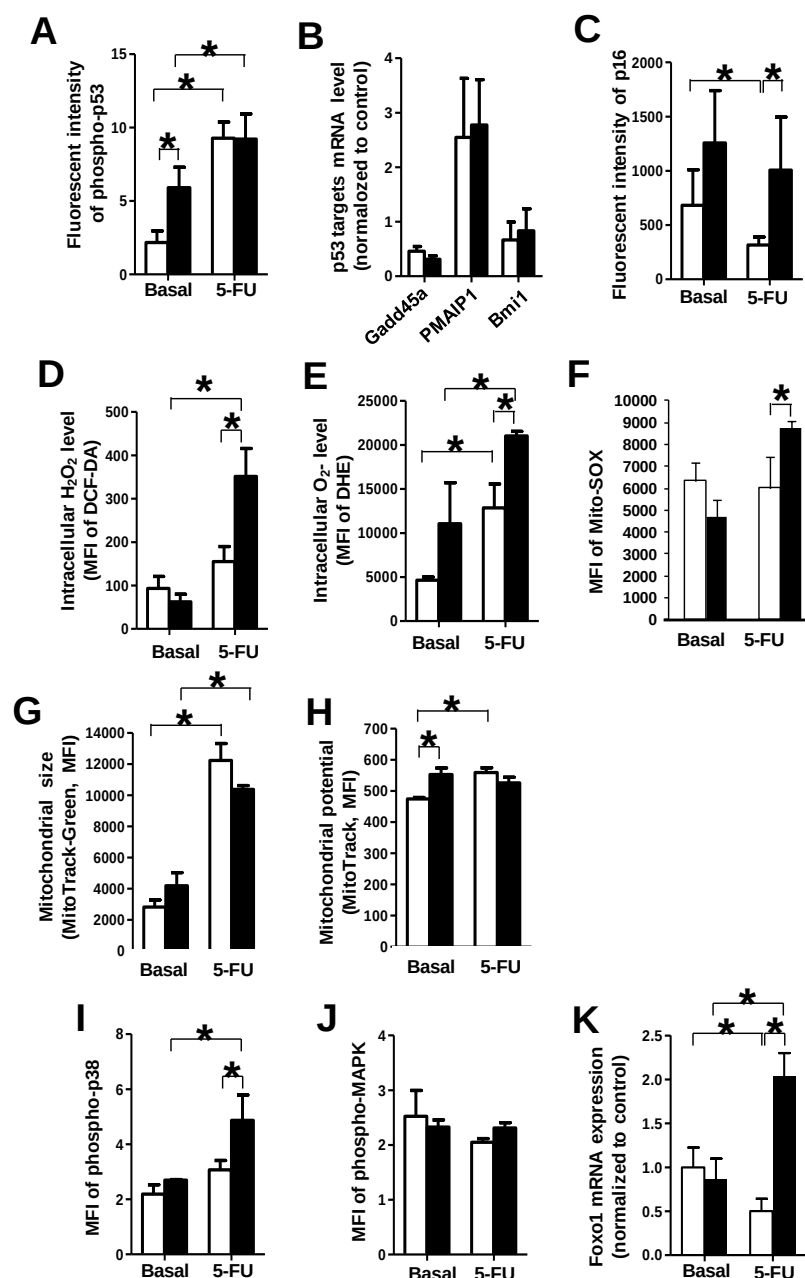


Figure 3. Cell cycle arrest in H-Cx43-deficient after 5-FU treatment is associated with increased levels of intracellular ROS and activation of quiescence markers p16 and p38. (A) Immunofluorescence intensity of anti-phospho-p53 immunostaining of BM-Lin⁺CD41⁺CD48⁺CD150⁺ cells from WT (open bars) or H-Cx43-deficient mice (solid bars) after 5-FU administration was measured by using computer-imaging software (Axovision, Zeiss) (**p*<0.05, *n*=20-35 cells per group were measured from a minimum of 2 mice per group). (B) Quantitative RT-PCR of p53 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 2 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-H) 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 bars) or H-Cx43-deficient mice (solid bars) were measured in vivo. **p*<0.05 (*n*=3 mice per group). (I-J) Mean fluorescent intensity of phospho-p38 (I) 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) Quantitative 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 3 mice per group). Values represent mean ± SD.

be hallmarks of ROS dependent HSC repopulation loss-of-function (58) and HSC resistance to physiologic oxidative stress (61), respectively.

Transfer of small molecules is arguably a well recognized function of Cx43-dependent channels (62). We hypothesized that Cx43-deficiency would lead to accumulated levels of intracellular ROS in HSC, resulting in cell cycle arrest, apoptosis, and senescence. To test this hypothesis, we performed a set of mechanistic experiments aiming 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 which 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

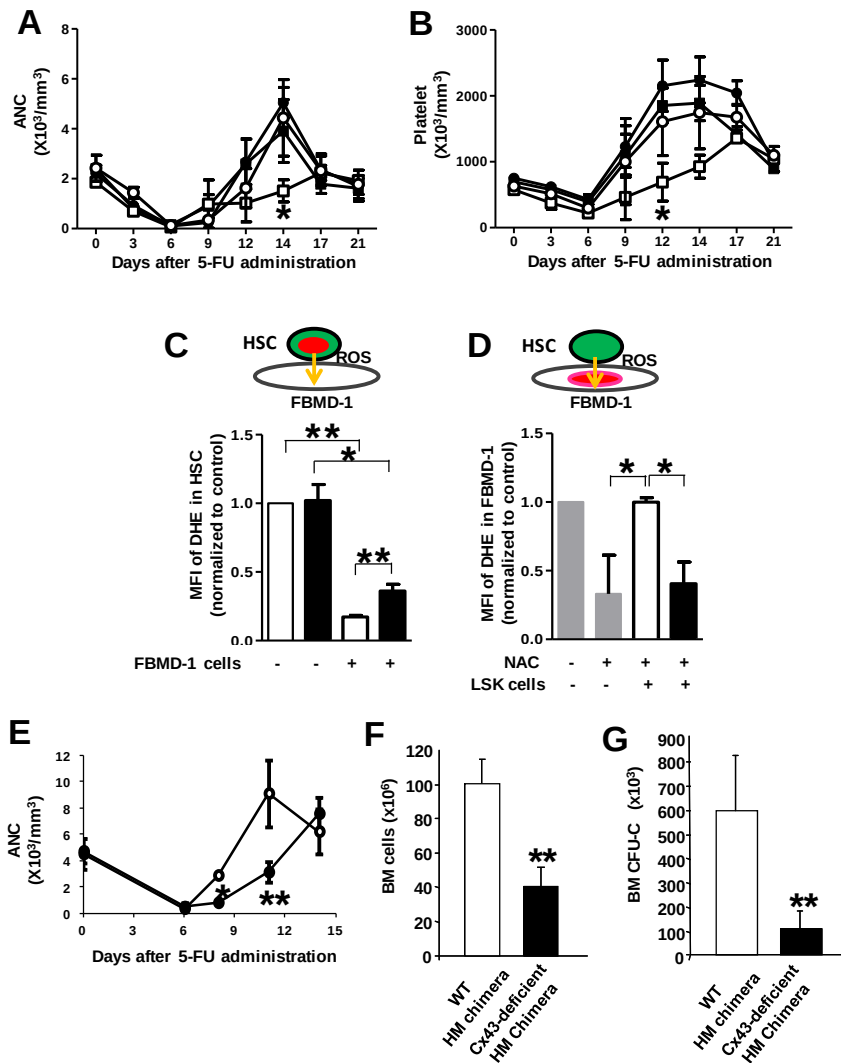


Figure 4. Antioxidant therapy reverses the impaired hematopoietic regeneration after 5-FU in H-Cx43-deficient mice and HSC/P Cx43 is required for ROS transfer to BM stromal cells. (A-B) In vivo NAC treatment restored peripheral blood recovery after 5-FU administration in H-Cx43-deficient mice. (A) Absolute neutrophil count (ANC). (B) Platelet count. $\circ$; control, $\bullet$; control + NAC, $\square$; H-Cx43-deficient, $\blacksquare$; H-Cx43-deficient + NAC. (* $p < 0.05$, $n = 3$ mice per group). (C-D) ROS transfer from HSC/P into FBMD-1 stromal cells. Diagram of the co-culture method (upper panels). Green cells represent sorted LSK cells. Red areas represent nuclei containing the ROS reporter DHE. Orange arrows represent expected directional transfer of ROS. (C) CFSE/DHE double-labeled HSC/P cells isolated from WT or H-Cx43-deficient mice were seeded onto FBMD-1 stromal cells for 3h. Before coculture, sorted HSC/P were treated with LY83583 to induce ROS production, and FBMD-1 were treated with NAC for 16h. Median fluorescent intensity of DHE in CFSE⁺ HSC/P was measured by flow cytometry. (D) CFSE-labeled, sorted HSC/P cells from WT or H-Cx43-deficient mice were cultured with preformed DHE-labeled FBMD-1 stroma for 3h. Before loading, HSC/P were treated with LY83583 to induce ROS and FBMD-1 cells had been treated with NAC for 16h. MFI of DHE in FBMD-1 was measured by FACS. MFI of FBMD-1 cells preincubated or not with NAC was also analyzed. * $p < 0.05$, ** $p < 0.01$. Data represent three independent experiments. (E) Neutrophil count of WT HM chimeric mice (open circles) Cx43-deficient HM chimeric mice (solid circles) after 5-FU administration. (* $p < 0.05$, $n = 3$ mice per group). (F and G) BM cell and progenitor (CFU-C) content in 2 femurs, 2 tibia and pelvis of WT HM and Cx43-deficient HM chimeric mice on day 11 post-administration of 5-fluorouracil (* $p < 0.05$, $n = 3$ mice per group). Value represent mean $\pm$ SD.

with NAC or control vehicle starting one day 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) (Figures 4A-B). These data proof that oxidative stress is causal in the hematopoietic recovery delay of Cx43-deficient HSC after 5-FU administration. Second, it has been shown that Cx43 mediates BM MSC/P cell adhesion to HSC (63). In order to address whether the contact of HSC with BM MSC/P cells was causal in the control of HSC ROS levels, we co-cultured HSC/P derived from WT or H-Cx43-deficient mice with pre-plated BM MSC/P (FBMD-1), a well recognized model of hematopoiesis-supporting BM MSC/P (64), in which long-term cultures of Cx43-deficient HSC had previously been shown to fail to produce hematopoiesis in vitro (42). We then analyzed whether ROS could be efficiently transferred from WT-HSC to BM MSC/P. Before culture, primary sorted HSC/P were treated with LY83583 (6-anilino-5,8-quinolinequinone), a generator of superoxide anions (65), to model increased ROS production as seen in vivo after 5-FU administration; and BM MSC/P cells were treated with NAC. The intracellular concentration of

ROS in HSC/P was measured in sorted HSC/P by flow cytometry analysis of fluorescence intensity of dihydroethidium (DHE), a DNA-binding reporter that cannot be transferred through GJ. Using the absence of FBMD-1 as a control, LY83583-treated WT or Cx43-deficient HSC/P showed high intracellular ROS levels (Figure 4C). When WT HSC/P were cultured onto FBMD-1 cells, however, the intracellular ROS levels in HSC/P diminished significantly (~83% reduction, $p < 0.001$) compared with Cx43-deficient HSC/P (Figure 4C). Although FBMD-1 cells maintained their ability to reduce ROS content of Cx43-deficient HSC/P (~64% reduction, $p = 0.02$), they did it 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 is lessened by the Cx43-deficiency in HSC/P.

Third, if ROS transfer is the mechanism of ROS scavenging, culture of high ROS-containing HSC/P onto FBMD-1 cells should increase the intracellular levels of ROS in the MSC/P cells. As a control, we checked that overnight NAC treatment of FBMD-1 cells significantly reduces the intracellular ROS levels (Figure 4D). A three-hour co-culture of WT HSC/P onto FBMD-1 reversed the effect of NAC, returning intracellular ROS levels similar to those in untreated FBMD-1 cells (Figure 4D). However, Cx43-deficient HSC were unable to increase the transfer of ROS to FBMD-1 cells beyond the basal levels of NAC-treated FBMD-1 cells (Figure 4D). Together, these data indicate that Cx43 mediates the transfer of ROS from HSC/P to hematopoiesis-supporting BM MSC/P cells.

Finally, in order to address if Cx43 homotypic interactions between HSC and BM MSC/P 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 (43). Mx1-Cre;WT and Mx1-Cre;Cx43^{flox/flox} 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 chimera) were challenged with 5-FU in the same way as in primary Vav1-Cre;Cx43^{flox/flox} mice. The myeloid regeneration of HM Cx43-deficient mice phenocopied the defective regeneration observed in H-Cx43-deficient mice, as assessed by neutrophil counts in the PB (Figure 4E), and reduced BM cellularity and progenitor content (Figure 4F and G). This suggests that Cx43-expression in the HM is similarly required to prevent hematopoietic regeneration failure and Cx43-Cx43 heterocellular interactions between HSC and the cellular HM are required for an adequate regenerative response after chemotherapy.

Cx43 expression in BM stromal cells does control the content of HSC, but not overall haematological content, in a model of extramedullary hematopoiesis in humanized animals (Fig. 5). Sublethally irradiated NSG mice were transplanted intrafemorally with cord blood CD34⁺ cells. At 2 months post-transplantation, the levels of chimera in peripheral blood were analyzed and the mice were stratified to have similar levels in two groups. Ceramic porous cubes (~9 mm³, ossicles) composed of 65% calcium phosphate hydroxyapatite and 35% tricalcium phosphate (Ceraform) (14) were washed and filled with fibronectin and WT or Cx43-deficient murine BM stromal cells in each of the two groups of mice. Ossicles were implanted subcutaneously into NSG mice and allowed to engraft for another 2 months. After 4 months, the ossicles were retrieved and analyzed. We noticed that while the overall contribution and hematopoietic distribution in sections was similar between ossicles containing WT and Cx43-deficient BM stromal cells, the content of HSC/P (CD34⁺ cells and subpopulations) was significantly diminished in ossicles containing Cx43-deficient BM stromal cells (Fig. 5).

Once we demonstrated that gap junction intercellular communication through Cx43 is required for HSC function, we intend to analyze whether FA-A and Cx43-dependent intercellular communication in relevant models of disease including humanized animals. First, we validated the use of a FancA shRNA lentiviral vector to significantly decrease FA-A expression and hematopoietic function. The lentiviral vector was able to knockdown FA-A expression in primary human cord blood (CB) CD34⁺ cells (Fig. 6A), significantly impair colony formation of human HSC/P (Fig. 6B-C) which were significantly silenced in their expression of FA-A (Fig. 6D).

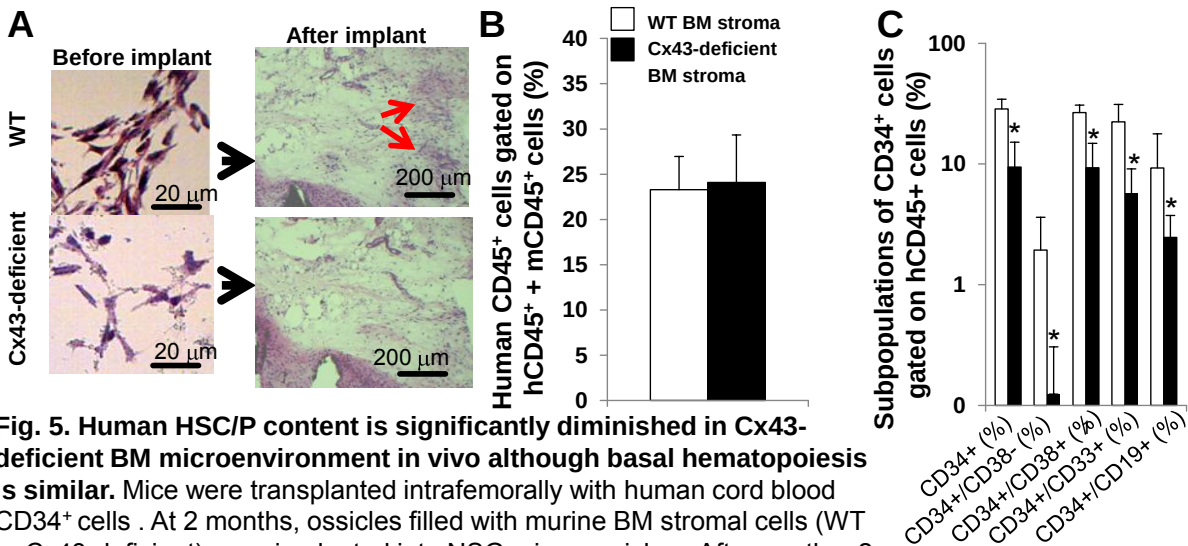


Fig. 5. Human HSC/P content is significantly diminished in Cx43-deficient BM microenvironment in vivo although basal hematopoiesis is similar. Mice were transplanted intrafemorally with human cord blood CD34⁺ cells. At 2 months, ossicles filled with murine BM stromal cells (WT or Cx43-deficient) were implanted into NSG mice. ossicles. After another 2 months (4 months), ossicles were retrieved and the hematopoiesis contribution analyzed in sections (A) and by flow cytometry (B-C). * $p < 0.01$. Red arrows denote large clusters of cells. These clusters are underdeveloped in ossicles containing Cx43-deficient BM stroma.

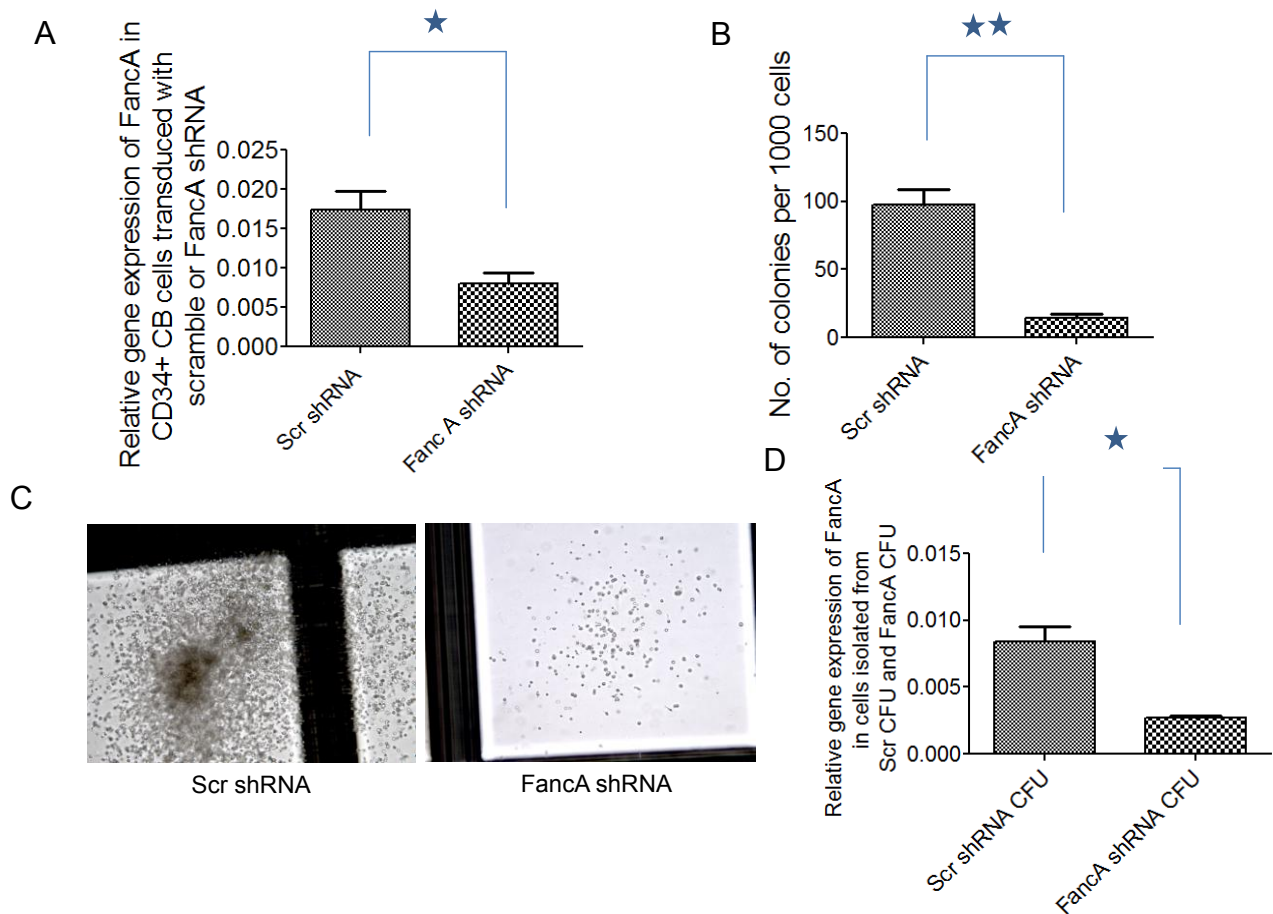
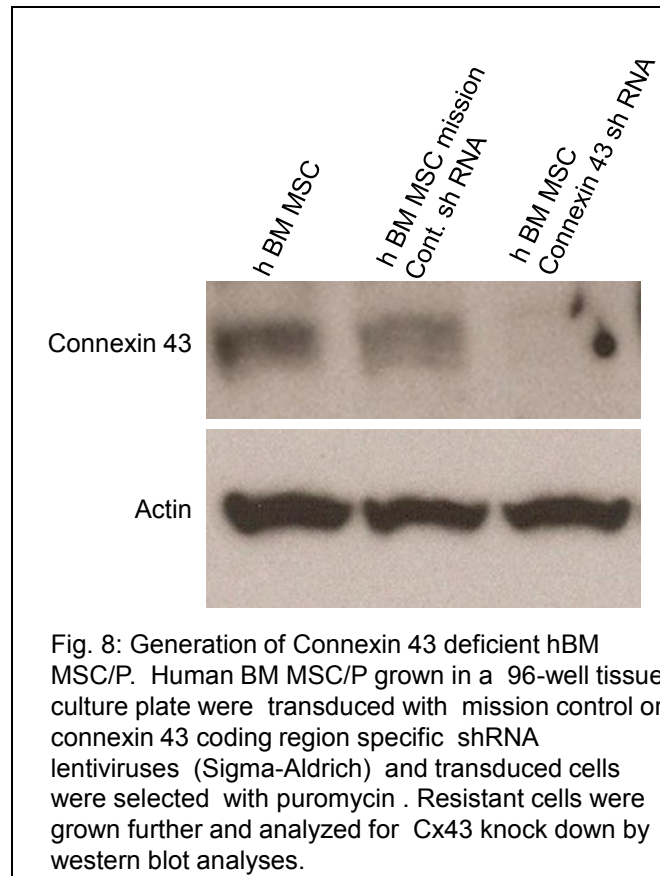
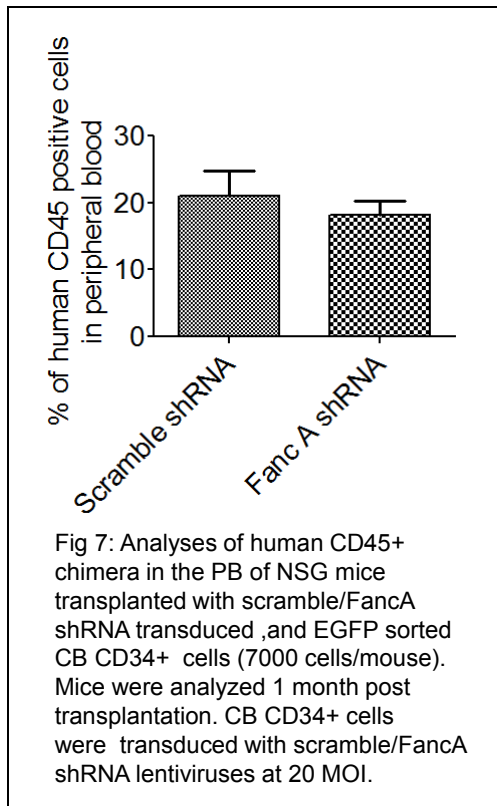


Fig. 6 CFU analyses of FancA depleted CB CD34⁺ cells. A, FancA mRNA level in CB CD34⁺ cells transduced with scramble/FancA shRNA lentiviruses at 100 MOI (2 rounds). B, CFU analyses of CB CD34⁺ cells transduced with either Scramble shRNA or FancA shRNA. C, Representative CFU of Scramble/FancA shRNA transduced CB CD34⁺ cells. D, FancA mRNA level in the cells isolated from the CFUs of scramble and FancA shRNA transduced CB CD34⁺ cells.



Transplantation of FA-A silenced CB CD34+ cells into NOD/SCID/gc^{-/-} (NSG) mice is feasible and induces a human chimera in control-transduced (Scramble shRNA) and FA-A silenced (FA-A shRNA) of ~20% (Fig. 7).

We have also successfully developed a way to silence Cx43 expression in human BM MSC/P by using shRNA technology (Fig. 8). These cells have been included in ossicle scaffolds and recently transplanted in humanized animal models of FA-A disease (Fig. 7).

Long-term engraftment of transduced (with Scrambled Sh-RNA or FA-A shRNA) cord blood CD34+ cells in NSG mice (n=3-5 mice per group) showed that while the deficiency of Cx43 and/or FA-A did not translate into a significant decreased engraftment on murine BM (Fig. 9A), the deficiency of either FA-A in HSC/P or Cx43 in BM MSC resulted in decreased engraftment of cord blood CD34+ onto human microenvironment (Fig. 9B). The combination of deficiency of FA-A and Cx43 resulted in further decreased engraftment of human CD34+/CD19+ cells (Fig. 9B). This experiment is being repeated for reproducibility analysis and increase of the sample size.

In summary, these studies indicate that a) HSC/P Cx43 deficiency induces senescence/apoptosis of HSC through loss of scavenging of ROS by MSC; b) Deficiency of human MSC Cx43 similar to FA-A deficiency of human HSC/P seems to result in decreased hematopoietic engraftment in ossicles xenografted in NSG mice. A possible synergism between both deficiency of FA-A in HSC/P and Cx43 in MSC is possible. Further analysis will be necessary to consolidate and establish the solidity of the second conclusion.

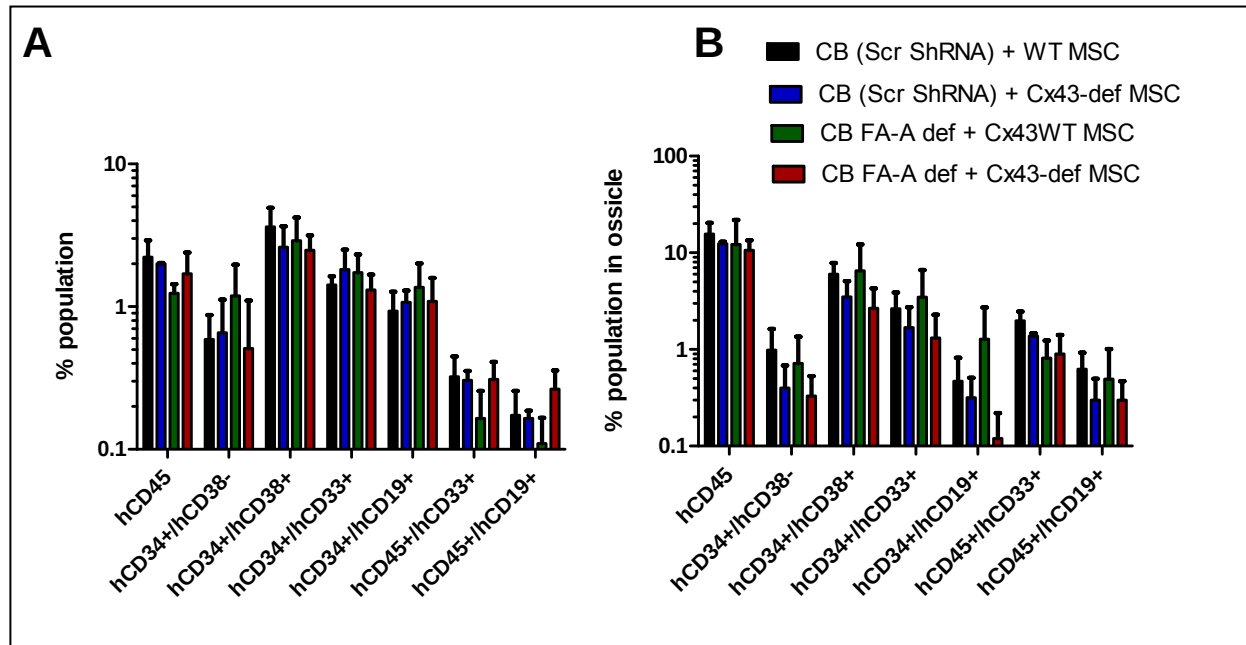


Figure 9. Deficiency of FA-A in human cord blood CD34+ cells or Cx43 in BM stromal cells results in decreased hematopoietic engraftment in xenografted ossicles containing human BM MSC. **A.** Femoral engraftment. **B.** Ossicle engraftment. NSG mice (3-5 per group) were transplanted serially with sorted, transduced (scrambled, Scr) ShRNA or FA-A shRNA transduced CB CD34+ cells. After 2 months, mice were implanted ossicles containing human BM MSC (WT or Cx43-deficient) obtained after transduction with specific shRNAs (WT=scrambled; Cx43-deficient: Cx43 shRNA). Analysis was performed 4 months after the ossicles were subcutaneously implanted. Femurs and ossicle content were analyzed by flow cytometry for overall human hematopoietic engraftment (hCD45), hematopoietic stem cell/progenitor populations (immature: hCD34+/hCD38-; mature: hCD34+/hCD38+; myeloid: hCD34+/hCD33+; lymphoid: hCD34+/hCD19+) and overall human myeloid (hCD45+/hCD33+) and lymphoid (hCD45+/hCD19+) engraftment.

Key research accomplishments

1. Connexin-43 is crucial to ameliorate chemotherapy induced ROS content in HSC through ROS transfer to BM MSC/P.
2. Deficiency of Cx43 in BM microenvironment phenocopies the deficiency of Cx43 in HSC.
3. A humanized animal model of FANC-A silenced hematopoiesis and Cx43-deficient hematopoietic microenvironment shows that the deficiency of either of them results in diminished human engraftment in human, but not murine, MSC microenvironment.
4. A possible synergism between the deficiency of HSC/P FA-A and MSC Cx43 may result in decreased engraftment of human lymphoid progenitors.

Reportable outcomes

Manuscripts:

1. Taniguchi Ishikawa E, Cancelas JA. Lack of communication rusts and ages stem cells. **Cell Cycle** 2012 Sep 1;11(17):3149-3150. PMID 22894903 PMC PMC3466507
2. Taniguchi Ishikawa E, Gonzalez-Nieto D, Ghiaur G, Dunn SK, Ficker AM, Murali B, Madhu M, Gutstein DE, Fishman GI, Barrio LC, Cancelas JA. Connexin-43 prevents hematopoietic stem cell senescence through transfer of reactive oxygen species to bone marrow stromal cells. **Proc Natl Acad Sci USA** 2012 Jun 5;109(23):9071-6. Epub May 18. PMID 22611193 PMC 3384185
3. Gonzalez-Nieto D, Li L, Köhler A, Ghiaur G, Ishikawa E, Sengupta A, Madhu M, Arnett J, Santho R, Dunn S, Fishman G, Gutstein D, Civitelli R, Barrio L, Gunzer M, Cancelas J. Connexin-43 in the osteogenic BM niche regulates its cellular composition and the bidirectional traffic of hematopoietic stem cells and progenitors. **Blood** 2012 May 31;119(22):5144-54. Epub Apr 12, 2012. PMID 22498741, PMC 3369607

Presentations in Meetings

1. Ishikawa E, Ghiaur G, Gonzalez Nieto D, Ficker A, Madhu M, Dunn S, Cancelas JA. Connexin 43 regulates intracellular level of reactive oxygen species in hematopoietic stem cells submitted to stress in vivo. Poster, ISEH Annual Meeting 2011, Vancouver, Canada. *Exp Hematol* 2011;39(Suppl 1):S98-S99(P1114762). *International*
2. Taniguchi Ishikawa E, Chang KH, Olsson HA, Nayak RC, Ficker A, Dunn SK, Sengupta A, Whitsett J, Grimes HL, Cancelas JA. Kruppel-Like-Factor 5 (Klf-5) controls hematopoietic stem cell/progenitor bone marrow homing and lodging through Rab5-mediated expression of active $\beta 1$ integrin. Oral (JAC), ASH, Oct 9, 2012, Atlanta GA. *Blood* 2012;120(21):Abs 113. *International*
3. Cancelas J.A. Connexin-43 controls the hematopoietic stem cell niche activity. American Society of Cell Biology, San Francisco (Dec. 15-18th), 2012.

Conclusion

We have shown that intercellular communication of HSC with BM MSC/P is crucial to protect HSC from ROS-dependent senescence and damage. In a novel humanized animal model of Cx43-deficient niche, we have demonstrated that the combination of FA-A hematopoiesis and niche Cx43 deficiency synergistically impair hematopoiesis development.

“So what?”

The results presented support a role of HSC-BM MSC/P communication in HSC function relevant in the protection of HSC against agents that induce ROS production and associated DNA damage. This study unveils a putative role of Cx43 as a modifier gene in BMF syndromes.

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