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CXCR4 Induction in Hematopoietic Progenitor Cells from Fanca$^{-/-}$, -c$^{-/-}$, and -d2$^{-/-}$ Mice

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Abstract

Objective—Bone marrow failure is a near-universal occurrence in patients with Fanconi Anemia (FA) and thought to result from exhaustion of the hematopoietic stem cell (HSC) pool. Retrovirus mediated expression of the deficient protein corrects this phenotype and makes FA a candidate disease for HSC-directed gene therapy. However, inherent repopulation deficits and stem cell attrition during conventional transduction culture prevent therapeutic chimerism.

Methods—We previously reported rapid transduction protocols to limit stem cell losses after *ex vivo* culture. Here we describe a complementary strategy intended to improve repopulation through upregulation of chemokine receptor (CXCR) 4, a principal factor in hematopoietic homing.

Results—Using murine models with transgenic disruption of *Fanca*, *-c*, and *-d2* we found that c-kit^{+} and sca-1^{+} progenitor cells express levels of CXCR4 comparable with their *wild-type* littermates. Lineage-depleted progenitor populations rapidly upregulated CXCR4 transcript and protein in response to cytokine stimulation or hypoxia, regardless of genotype. Hypoxia conditioning of lineage-depleted *Fancc*$^{-/-}$ progenitors also reduced oxidative stress, improved *in vitro* migration and led to improved chimerism in myeloablated recipients after transplantation.

Conclusion—These studies provide evidence that CXCR4 regulation in progenitor cells from transgenic mice representing multiple FA genotypes is intact and that modulation of homing offers a potential strategy to offset the FA HSC repopulation deficiency.

Keywords

CXCR4; Hematopoietic Stem Cells; Fanconi Anemia

Introduction

Fanconi anemia (FA) is a recessively inherited DNA repair disorder with a complex genetic basis and prominent manifestation in the hematopoietic system [1]. Progressive marrow aplasia in FA patients is widely believed to result from a pro-apoptotic cellular phenotype and stem cell loss. This idea is supported by the exaggerated *in vitro* sensitivity of cells to reactive oxygen

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species (ROS), reduced progenitor clonogenicity, and the involvement of FA proteins in maintaining DNA integrity [2–5]. Further, overexpression of FANCC protects FA phenotype human and murine cells from apoptosis [6,7]. This suggests a role of FA genes in proliferation and survival in the progenitor compartment and may explain the compromised quality and quantity of FA HSC, as well as the repopulation defects in murine models of *Fancc*, and hypomorphic *–d1* [4,8,9]. To what extent the observed repopulation deficits might involve a role for FA proteins in progenitor cell homing and migration has not been studied to date. Taken together, efficient gene transfer to FA HSC and their subsequent engraftment under such constraints have been a challenge and transduction culture itself can compromise repopulating ability and genomic stability [10–13]. We and others recently reported that lentivirus vectors enable the transduction of murine HSC in simplified *ex vivo* protocols, thereby providing one model for how to limit *in vitro* differentiation and stem cell loss [14–16]. The current studies were designed to investigate complementary strategies to improve the subsequent homing of hematopoietic target cells after *ex vivo* transduction culture.

Hematopoietic stem cells are capable of tissue-specific homing and redistribute to the stem cell niche within 15 hours after intravenous injection in mice [17,18]. Homing requires the coordinated interaction of cells, endothelium, and the supportive microenvironment in the marrow through cell surface molecules and their ligands [18,19]. Chemokine receptor (CXCR) 4 and its ligand, stromal derived factor (SDF) -1α, play a prominent role in this process [20, 21]. Stable overexpression, or transient upregulation, of CXCR4 in hematopoietic cells improve homing to the marrow and resultant chimerism in murine transplantation experiments [22,23]. Conversely, downregulation of CXCR4 activity, or disruption of CXCR4-SDF1α binding, diminishes homing to the marrow after intravenous injection [22,24]. Homing of intravenously injected stem/progenitor cells is enhanced after brief culture in the presence of cytokines including stem cell factor (SCF), or in response to tissue hypoxia, each involving CXCR4 signaling [25–33]. Homing in general, and the role of CXCR4 expression and regulation in particular, have not previously been investigated in FA.

Results presented here in mice with transgenic disruption of *Fanca, -c*, or *-d2* genes indicate that CXCR4 regulation of FA hematopoietic progenitors is intact and that hypoxia conditioning can upregulate CXCR4 and improve chemotactic migration while limiting oxidative stress during *ex vivo* culture. We propose that hypoxia-induced CXCR4 upregulation provides a potential strategy to improve the homing of FA phenotype HSC and offset the repopulation disadvantage, especially in the context of *ex vivo* gene transfer protocols.

Material and Methods

Cell culture and retroviral transduction

293T human kidney fibroblast cells were propagated in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/1% streptomycin (Pen/Strep). L1210 cells, a murine hematopoietic cell line, was kindly provided by R. Storms and cultured in RPMI supplemented with 10% FBS and 1% Pen/Strep. Murine whole bone marrow (WBM) and lineage-depleted (lin-) cells were grown in Iscove's media supplemented with 10% FBS, 10% horse serum 1% Pen/Strep, 50 ng/mL murine Stem Cell Factor (mSCF), and murine interleukin (IL)-3 (Peprotech, Rocky Hill, NJ). Experiments in low oxygen environment were conducted in a dedicated chamber at 1% O_2, 5% CO_2 at 37°C. All other cell culture occurred at ambient O_2 levels.

Flow-cytometry

Cellular CXCR4 expression was analyzed at serial time points using a FACS-Calibur instrument (BD Biosciences) and data was processed using FlowJo software (Tree Star,

Ashland, OR. USA). Samples were stained with murine antibodies directed against surface epitopes: CXCR4 (FITC), sca-1 (PE), c-kit (APC), and IgG (FITC, as background-staining control) (all BD Biosciences, San Jose, CA). Following antibody staining according to manufacturer's recommendation, samples were washed in PBS containing 2% FBS and resuspended in 2% FBS-PBS containing 1 μg/ml propidium iodide solution to exclude dead cells from analysis. For clarity, IgG background values are not shown on all histogram overlays, but were subtracted from each sample to calculate numeric gains. For follow-up studies after transplantation, harvested WBM and peripheral blood underwent hemolysis, leukocytes were stained with anti-CD45.2 (PE) antibody at 4°C for 30-minutes, washed twice in 2%FBS-PBS, and analyzed. Leukocyte subset analysis was based on forward- versus side-scatter gates to distinguish lymphocytes, monocytes and granulocytes, as previously described [14,34,35]

Quantitative real-time rt-PCR assay

Total RNA was extracted from samples using an RNeasy Mini Kit, according to the manufacturer's protocol (Qiagen Inc., Valencia, CA. USA). Reverse transcription was performed with $Oligo(dT)_{12–18}$ and SuperScript™ II RT (Invitrogen), according to manufacturer's protocol. Complementary DNA expression was assayed via quantitative real time PCR, using CXCR4 primer-probe mix (sense: 5′-GAC CGC CTT TAC CCC GAT AG -3′) and (anti-sense: 5′-GTC CAC CCC GCT TTC CTT TG -3′), purchased from ABI (Perkin-Elmer Applied Biosystems, Foster City, CA. USA) [36] and 18S endogenous control 20x primer-probe set, according to the manufacturer's instructions (Perkin-Elmer Applied Biosystems, Foster City, CA. USA). DNA from L1210 murine cells was used as a positive control, as this cell line has relatively high, stable endogenous CXCR4 expression. All PCR reactions were set up in a MicroAmp Optical 96-well Reaction Plate (Applied Biosystems), and samples were run in duplicate. All threshold cycle (Ct) values of CXCR4 were normalized to Ct values of an 18S ribosomal RNA internal control. Reactions were run using the ABI Taqman Universal PCR Mastermix (Applied Biosystems) on the ABI prism 7300 sequence detection system (Applied Biosystems) using the following thermal cycling conditions: 50°C for 2min, 95°C for 10min, followed by 40 cycles of 95°C for 15s and 60°C for 1min. The spectrum was then analyzed using ABI Sequence detector v.1.3.

Amplex Red Assay

To compare relative ROS released from *Fancc*$^{-/-}$ or *wt* lineage-depleted bone marrow cells and as a general measure of oxidative stress during culture, the Amplex Red Assay (Invitrogen, Carlsbad, CA) was performed according to manufacturer's protocol. The Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) reacts with H_2O_2 in a 1:1 stoichiometry, emitting a red-fluorescent oxidation product. Aliquots were normalized for cell number and cultured in media containing Iscove's Modified Dulbecco's Media (IMDM), 10% horse serum (Gibco), 10% fetal bovine serum (Gibco), 1% Penicillin/Streptomycin, 50 ng/ml mSCF (PeproTech, Inc.), and 50 ng/ml IL-3 (PeproTech, Inc.) under hypoxic (3.5% O_2) or normoxic conditions for 18 hours. Triplicate reactions were carried out in a 96 well microplate (in additional experiments cells were pelleted and 50 μl cell-free media aliquots were also analyzed, yielding similar results). Fluorescence was measured with a fluorescence microplate reader using excitation and emission detection at 530 nm 590 nm, respectively. A hydrogen peroxide standard curve was generated by diluting known concentrations of H_2O_2 into IMDM.

In vitro transwell migration assay

Progenitor cells were isolated from wild type, *Fancc*$^{+/-}$, and *Fancc*$^{-/-}$ mice, and were cultured in the presence of IL-3 and mSCF, under normoxic or hypoxic conditions, respectively. Aliquots (2.5×10^5 cells) were sampled every 24 hours for ability to migrate across the 8 μm transwell insert (Corning) toward lower chamber containing media with 30 ng/ml SDF-1α

(Peprotech). To ascertain CXCR4 specific migration, a control well contained 1 nM CXCR4 antagonist AMD3100 (Sigma). Cells migrated for 2.5 hours, followed by enumerating migrated cells in lower chamber on a hemacytometer. Each sample was counted 5 times. To calculate statistical differences a 2-tailed, unpaired homoscedastic student t-test was performed. Results were confirmed with a one way ANOVA and Kruskal-Wallis Test (to accommodate small sample size). Because all genotypes were statistically comparable at each time point based on 3 statistical analyses, values were grouped into normoxia- versus hypoxia-conditioned samples for further statistical analysis.

Animal husbandry and transplantation

Mice (C57BL/6 and Boy J -B6.SJL-) were group-housed and allowed *ad libitum* access to standard chow pellets (Purina Laboratory Rodent Diet 5001, Ralston Purina Co., St. Louis, MO). Mice with transgenic disruption of *Fancc* (C57BL/6 strain, CD45.2 genotype) were kindly provided by M. Buchwald [37]. Mice with transgenic disruption of *Fanca* and *Fancd2* were described previously [38,39]. Whole bone marrow (WBM) cells were collected by flushing femurs and tibias from 8 to 12 week-old Boy J mice (CD45.1) and CD45.2/Fancc$^{-/-}$ mice with Iscove's Modified Dulbecco's Media. Samples were depleted of red cells by hemolysis and lineage-depleted using an Easy Sep® Mouse Hematopoietic Progenitor Cell Enrichment kit according to the manufacturer's instructions (StemCell Technologies Inc., Vancouver, Canada). Following overnight culture in hypoxic or normoxic conditions, 2.5 x10^5 lineage-depleted CD45.2/Fancc$^{-/-}$ cells were washed twice in PBS, mixed with 2.5 x 10^5 CD45.1 lineage-depleted competitor cells, resuspended in 200 μl Hanks Balanced Salt Solution (HBSS) and injected intravenously into myeloablated (950cGy) recipients (CD45.2/Fancc$^{+/-}$ or CD45.2/Fancc$^{+/+}$). Additional cohorts were transplanted using a ratio of 1 x 10^4 CD45.2 lineage depleted/2.5 x 10^5 CD45.1 whole bone marrow competitor cells. Following transplantation, retro-orbital eye bleeds were performed at intervals, and white blood cells were analyzed for CD45.2 expression by flow-cytometry.

Statistical analysis

Numerical results are expressed as average plus or minus standard deviation (SD), except in Figure 6, where median values were used. Data were analyzed using the paired 2-tailed Student t test. P values of less than 0.05 were considered significant. For migration assays, results were confirmed with a one way ANOVA and Kruskal-Wallis Test (to accommodate small sample size).

Results

Mice with transgenic disruption of FA genes present instructive models for the study of qualitative and quantitative defects in FA hematopoietic stem cells [4,8]. We investigated the modulation of CXCR4 cell surface expression in *Fanca*$^{-/-}$, *Fancc*$^{-/-}$, *and Fancd2*$^{-/-}$ mice as part of a strategy to improve homing and mitigate FA HSC phenotype repopulation deficits incurred during *ex vivo* culture necessary for retroviral correction.

Fanca$^{-/-}$, Fancc$^{-/-}$ and Fancd2$^{-/-}$ mice express similar cell surface levels of CXCR4

Whole bone marrow cells were recovered from animals of all genotypes, *Fanca*$^{-/-}$, *Fancc*$^{-/-}$, *Fancd2*$^{-/-}$ *and wild-type (wt)*, and progenitor cells were isolated by immunomagnetic lineage-depletion. Cells were cultured in the presence of cytokines (mSCF and IL-3) under normoxic conditions. We relied on a flow-cytometric assay to detect surface binding of a FITC labeled, anti-murine CXCR4 antibody and determine changes in cell surface protein expression. FITC labeled, anti-murine IgG antibody was used for gating and background staining was subtracted of each sample. Samples were analyzed immediately after harvest and twice more over the subsequent 2-day period. Progenitor cells from each of the

FA genotypes demonstrated systematic gains in fluorescence (1.5 to 2-fold, after correction for IgG background for each sample) compared to equivalent baseline levels (Fig. 1A). The increase in CXCR4 expression observed in *Fanca*$^{-/-}$, *Fancc*$^{-/-}$, or *Fancd2*$^{-/-}$ cells was not statistically different from *wt* cells (student 2 tailed, paired t-test). Upregulation was similar for heterozygous *Fancc*$^{+/-}$ animals (not shown) and they are used interchangeably with *wt* as control genotype. L1210 cells, a murine T-cell line, served as a positive control and demonstrated stable CXCR4 expression throughout (data not shown). We next confirmed that these gains in CXCR4 antibody binding (i.e fluorescence shifts) by murine progenitors reflect increased CXCR4 message by real-time rt-PCR determination. Results show that cytokine induction (mSCF + mIL-3) led to substantial increases of mRNA species (predominantly by 24 hours with minor additional gains by 48 hours) in samples from *Fanca*$^{-/-}$, *Fancc*$^{-/-}$ *and Fancd2*$^{-/-}$ progenitor cells (Fig. 1B). With concordant *in vitro* results for all three murine FA genotypes ($p > 0.05$, student 2 tailed t-test), we focused further experiments on mice with biallelic *Fancc* disruption.

Leukocyte subset composition and CXCR4 expression among defined progenitor sets

Murine stem and immature progenitor subsets expressing cell surface epitopes *sca*-1 and *c-kit* contribute to long-term hematopoietic reconstitution after transplantation. The progenitor yield after depletion of lineage-committed cells was 4.5 +/− 2.6% with similar recovery from *Fancc*$^{+/-}$ and *Fancc*$^{-/-}$ animals (data not shown). In order to test CXCR4 expression on leukocyte subsets relevant to hematopoietic repopulation in animals, we next ascertained similar subset distribution among leukocyte progenitors after immunomagnetic depletion of lineage-committed cells with slightly higher percentages of stem and progenitor cells in heterozygous compared to knock-out mice. Changes in leukocyte subset composition during *in vitro* culture demonstrated similar kinetics in both genotypes (Fig. 2AB). Cell proliferation based on serial cell counts over 72 hours from culture initiation showed comparable contraction in whole bone marrow cells of both genotypes and subsequent modest expansion of lineage depleted progenitor cells in mSCF/mIL-3 supplemented culture of both genotypes (Fig. 2C). In evaluating the differential expression of CXCR4 on progenitor subsets, we found consistently increased levels of cell surface protein by sca-1 and c-kit -positive cells as compared to sca-1, c-kit -negative events (Fig. 3).

Hypoxia culture induces increased CXCR4 expression

Culture of FA cells in hypoxia appears to improve their survival and engraftment in xenogenic murine models [10]. At the same time, hypoxia inducible factor (HIF) –1α upregulates CXCR4 expression in hematopoietic cells [30,33]. With CXCR4 regulation unaffected by germline murine FA gene disruption we hypothesized that transient culture in hypoxia can be exploited to upregulate CXCR4 and improve hematopoietic homing while simultaneously limiting oxidative stress-mediated stem and progenitor cell losses *in vitro*. Our initial studies confirmed upregulation of CXCR4 cell surface expression and mRNA levels in whole bone marrow cells after overnight hypoxia culture (not shown). Further analysis confirmed that CXCR4 was also upregulated on lineage-depleted bone marrow progenitor subsets and double positive sca-1^{+}/c-kit^{+} cells following overnight hypoxia (1% O_2) in mSCF/mIL-3 supplemented culture, without substantial differences among *Fancc*$^{+/-}$ and *Fancc*$^{-/-}$ genotypes (Fig. 4AB).

Generation of reactive oxygen species by murine progenitors after overnight hypoxia culture

Responses to oxidative challenge play a prominent role in the pathophysiology of FA hematopoiesis and are presumably involved in the pro apoptotic stem cell phenotype [3,40]. Having demonstrated hypoxia-induction of CXCR4, we next compared the *in vitro* generation of reactive oxygen species (ROS) by progenitors from *Fancc*$^{+/+}$ and *Fancc*$^{-/-}$ animals. Results from multiple repeat experiments demonstrate that overnight hypoxia conditions produce a

concomitant significant ($p< 0.05$) decrease in the generation of (ROS) in both genotypes (Fig. 4CD). These hypoxia conditions do not adversely affect lentiviral gene transfer to target cells (data not shown).

Hypoxia conditioning of lineage depleted progenitor cells improves in vitro transwell migration

To confirm that up-regulation of CXCR4 following hypoxia reflected chemotactic activity of cells toward a SDF-1α gradient, we performed an *in vitro* transwell migration assay, previously used by others [41,42]. Comparing SDF-1α-mediated migration of progenitor cells cultured under hypoxic *versus* normoxic conditions we found no statistically significant differences between *wild-type*, *Fancc*$^{+/-}$, *Fancc*$^{-/-}$ genotypes at any of the time points examined (Fig. 5). But, the percentage of cells that migrated across the transwell membrane increased steadily with time from culture initiation in a statistically significant manner ($p <0.01$ for non-conditioned samples compared to day 2 normoxia or hypoxia conditioned samples). Next, in pooling data from all genotypes (in the absence of statistically significant differences between them) we found a significant statistical difference in migration between normoxia and hypoxia-conditioned progenitor cells ($p < 0.001$, student 2-tailed t test). To demonstrate that the migration observed was specifically due to up-regulation of CXCR4, we tested the ability of AMD 3100 (a CXCR4 antagonist) to impede migration. This antagonist inhibited migration of cells cultured under normoxic or hypoxic conditions in all genotypes. The reduction in cell migration in the presence of AMD 3100 is statistically significant (p-value for each genotype is <0.01).

Transient hypoxia effects on chimerism after transplantation

Based on our *in vitro* data showing hypoxia-induced upregulation of CXCR4 and improved migration in hematopoietic progenitors, we decided to test the feasibility of this strategy in improving the *Fancc*$^{-/-}$ HSC repopulation in murine recipients. We studied cohorts of myeloablated recipients that received grafts of immunomagnetically lineage-depleted cells, composed of 2.5 x 10^5 CD45.2/*Fancc*$^{-/-}$ cells cultured over night at normal versus reduced (1%) O_2 concentration pooled with 2.5 x 10^5 CD45.1 competitor cells (also lineage-depleted and normoxia cultured), respectively. Following hematopoietic reconstitution, we evaluated the CD45.2 (i.e. *Fancc*$^{-/-}$) chimerism contributions in animals at serial time points. Results demonstrate increasing chimerism ($p= 0.055$ at 17 weeks) in animals that received hypoxia-conditioned CD45.2/*Fancc*$^{-/-}$ cells as compared to the cohort receiving normoxia-cultured cells (Fig. 6). To amplify the potential hypoxia on *Fancc*$^{-/-}$ progenitor repopulation we next transplanted additional cohorts of animals with reduced numbers of conditioned cells (1 x10^4) and unselected whole bone marrow (2.5 x10^5) competitor populations. In these animals leukocyte specific chimerism differences at 4 and 12 weeks was improved similarly in lymphocyte, granulocyte and monocyte subsets for animals that received hypoxia conditioned cells, but did not reach statistical significance (Table 1). While we did not observe a substantial difference in cell survival during the brief culture period in hypoxia versus normoxia, this does not preclude more subtle changes in viability among the different genotypes. Taken together, this data complements our *in vitro* studies on hypoxia upregulation of CXCR4, but falls short of demonstrating significant advantages for the hypoxia conditions explored. Precise conditions for graft composition, oxygen concentration and hypoxia duration may yield more distinct results.

Discussion

Hematopoietic stem and progenitor cells from FA patients and murine models of FA display a complex phenotype characterized in part by the impaired *in vitro* clonogenicity and poor stem cell mobilization [1,43,44]. However, attempts to rescue the FA phenotype by retroviral

transduction of HSC entail their *ex vivo* culture and result in engraftment and repopulation defects, in sum undermining the therapeutic efficacy of gene therapy in FA [13,45–47]. Studies in *Fancc*$^{-/-}$ mice illustrate the FA HSC repopulating defect which can be overcome by increasing the cell inoculum to increase the number of engrafting cells [4,48,49]. This is not an option for FA patients who mobilize autologous HSC targets poorly, but it suggests that inherent FA phenotype repopulation deficits could be overcome by enhancing homing mechanisms, thereby increasing numbers of HSC and progenitor cells relocating to the microenvironment [44,49]. The current study is the first to investigate CXCR4 expression and homing mechanisms in the FA HSC and progenitor compartment.

Initial experiments investigated CXCR4 expression on progenitor cells from three murine models of FA, representing complementation groups A, -C, and -D2. Flow-cytometric and confirmatory real-time rt-PCR studies demonstrated that cytokine mediated regulation of CXCR4 was intact in lineage-depleted progenitor cells from all three genotypes. These experiments show systematic upregulation of CXCR4 over 48 hours of culture and levels comparable with those on *wild-type* cells. Similar starting percentages of c-kit^{+} and sca-1^{+} progenitor subsets and proliferation kinetics between the different genotypes minimized any confounding bias from differential expansion during the observation period and allowed the direct comparison of CXCR4 expression on progenitor subsets. The subsequent direct comparison of CXCR4 levels on lineage-depleted progenitor cells from *Fancc*$^{-/-}$ and *Fancc*$^{+/-}$ animals revealed increased cell surface protein levels on c-kit^{+} and sca-1^{+} cells, again without substantial differences between genotypes.

CXCR4 is known to be upregulated in response to HIF-1α induction [31]. We hypothesized that CXCR4 surface expression after transient hypoxia might not only improve stem and progenitor cell homing following injection, but simultaneously minimize exaggerated FA-phenotype apoptosis by reducing ROS. Results from our experiments confirm that CXCR4 can be efficiently upregulated during incubation in hypoxia culture with comparable resulting CXCR4 levels in lineage-depleted progenitors from *Fancc*$^{-/-}$ and *Fancc*$^{+/-}$ animals. Further, our studies specifically show that sca-1^{+}/c-kit^{+} double-positive cells (which encompass murine hematopoietic repopulating cells) express higher levels of CXCR4 after overnight hypoxia culture than those negative for these markers, regardless of *Fancc* genotype. Importantly, we demonstrate that the *de novo* generation of ROS, H_2O_2 specifically, is significantly reduced under hypoxia conditions.

Others have shown that hypoxia improves the survival and replicative potential of hematopoietic progenitor cells [10,50]. To demonstrate that hypoxia conditioning of progenitor cells directly improves progenitor cell homing we conducted an *in vitro* transwell migration assay. SDF-1α-directed migration of cells was significantly improved when cells were cultured under hypoxic *versus* normoxic conditions. The increase in cellular migration peaked at 48 hours and followed the kinetics of CXCR4 upregulation in our experiments. Migration was abrogated in the presence of AMD 3100 confirming the direct role of CXCR4 in cell migration. These results demonstrate that hypoxia-mediated CXCR4-upregulation leads to increased progenitor cell homing. This is also the first demonstration that CXCR4 regulation and migration capacity are conserved in FA phenotype murine progenitor cells.

With intact CXCR4 regulation and migratory responses, our subsequent transplantation experiments focused on exploiting CXCR4 upregulation as part of a homing based strategy to offset repopulation deficits in this model of FA. They also highlight the unanticipated complexity of our strategy with changes in graft phenotype, cell number and degree of hypoxia all likely to contribute to the varying outcomes. In the initial cohorts, we performed a transplantation study using hypoxia-, or normoxia conditioned CD45.2 isotype hematopoietic progenitors competed against identical numbers of CD45.1 competitors for injection in CD45.2

lethally irradiated recipients. Remarkably, the increasing chimerism in the *Fancc*$^{-/-}$ hypoxia cohort developed in the face of substantial numbers of lineage-depleted *Fancc*^{wt} CD45.1 competitor cells. Additional cohorts of animals received grafts with decreased numbers of hypoxia-conditioned cells and unseparated whole bone marrow competitors, to reduce competitive engraftment. In these animals we analyzed engraftment in different leukocyte subsets and showed non-significant advantages resulting from hypoxia conditioning. Others have described similar hypoxia culture conditions (5% O_2) as conducive to engraftment of genetically modified human FA HSC in a xenogenic FA transplantation model [10]. We suggest that our studies, in directly demonstrating hypoxia effects on ROS generation, homing receptor expression in murine cells and cell migration, may provide the mechanistic basis for the improvements in cell survival and engraftment reported by these investigators.

In sum, we show that a principal homing mechanism for hematopoietic cells is conserved after genetic disruption of murine loci for FA complementation groups A, C, and D2. This finding would argue that the previously reported repopulation deficiency in *Fancc*$^{-/-}$ animals is based on a replicative deficiency, rather than an engraftment defect, at least one based on CXCR4 expression [4]. With a prior report that extended culture of *Fancc*$^{-/-}$ progenitors *in vitro* amplifies their myeloproliferative potential (precluding *ex vivo* cell expansion strategies) and repopulation disadvantage, as well as the growing implication of ROS in the pathophysiology of the FA HSC phenotype [13,40], CXCR4 upregulation and hypoxia culture should be further explored in preclinical models of FA.

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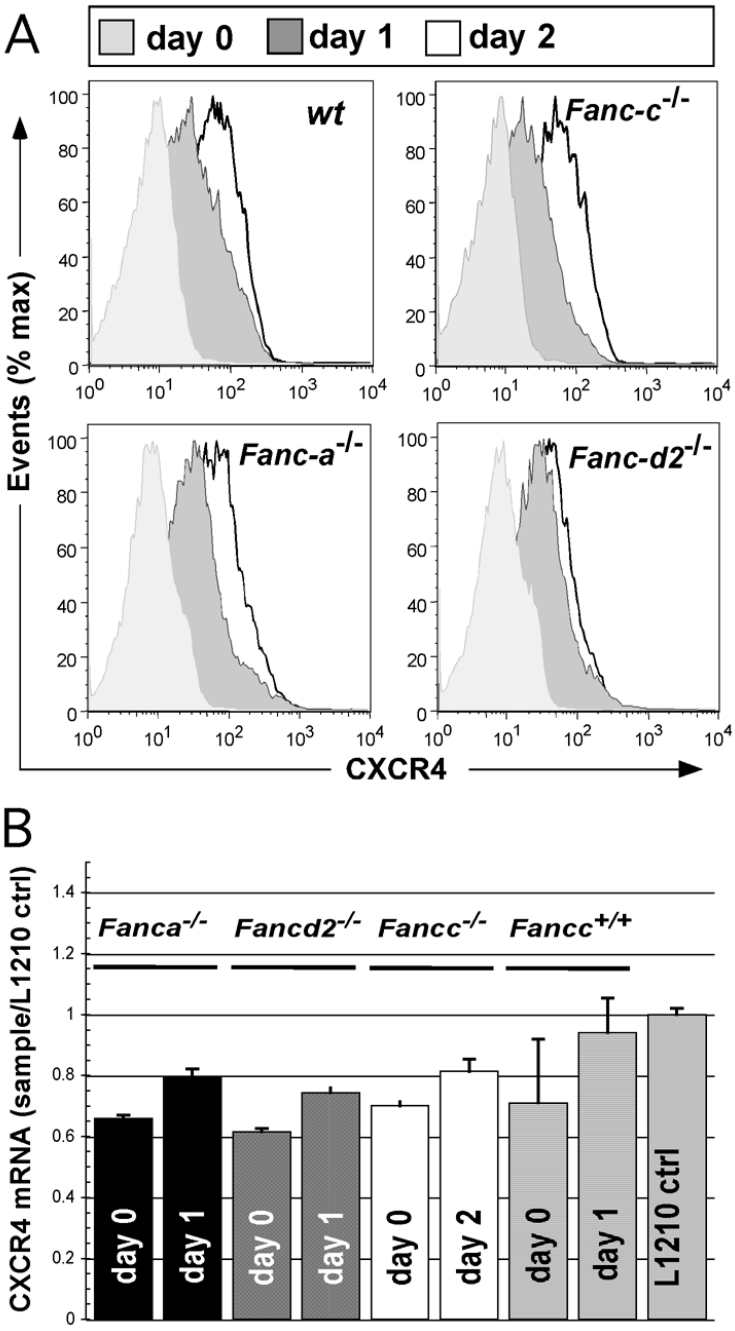

Figure 1.

CXCR4 upregulation on the cell surface of whole bone marrow cells from *wt*, *Fancc*$^{-/-}$, *Fanca*$^{-/-}$ and *Fancd2*$^{-/-}$ mice. **(A)** Cells were harvested from one animal per genotype, immunomagnetically depleted of lineage-committed cells and cultured in the presence mIL-3 and mSCF under normoxic conditions. Aliquots were removed from culture at indicated intervals (0h, 24h, 48h) and stained with anti-IgG and anti-CXCR4-FITC labeled antibody. During flow-cytometric analysis, events (>10,000) were gated to exclude dead cells and analyzed for CXCR4 expression. The experiment was performed twice with similar trends observed. **(B)** CXCR4 *mRNA* levels during cytokine supplemented culture. Lineage-depleted *wt*, *Fancc*$^{-/-}$, *Fanca*$^{-/-}$ and *Fancd2*$^{-/-}$ whole bone marrow cells were cultured in the presence

mIL-3 and mSCF. Aliquots were removed from culture at indicated intervals (0h, 24h, or 48h) and total RNA was extracted. Following RNA extraction and subsequent cDNA production, quantitative real-time (Q)-PCR analysis was used to determine the changes in the level of CXCR4 mRNA transcripts over the incubation period. Complementary DNA generated from the L1210 cell line, maintained in expansion culture, was used as a positive control for CXCR4 expression. Real-time-PCR samples were prepared in triplicate. Bars depict averages and standard errors.

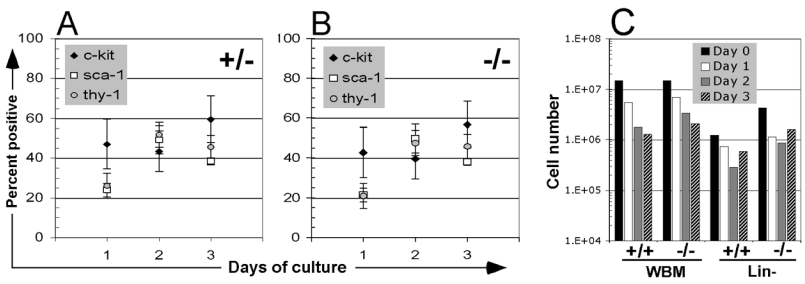

Figure 2.

Bone marrow leukocyte progenitor composition and kinetics during *in vitro* culture. **(A)** Bone marrow cells from *Fancc*$^{+/-}$ and **(B)** *Fancc*$^{-/-}$ littermates were immunomagnetically depleted of lineage committed cells and cultured in the presence mIL-3 and mSCF. Aliquots were removed from culture at indicated intervals (24h, 48h, 72h) and stained with antibodies against c-kit (open diamond), sca-1 (open square). Events (>10,000) were gated to exclude dead cells and analyzed for epitope expression. Results represent averages from 3 independent experiments and error bars depict the resultant standard deviation. **(C)** The absolute number of *Fancc*$^{+/+}$ and *Fancc*$^{-/-}$ cells in WBM or lineage-depleted populations over time (day 0 to day 3) is also shown.

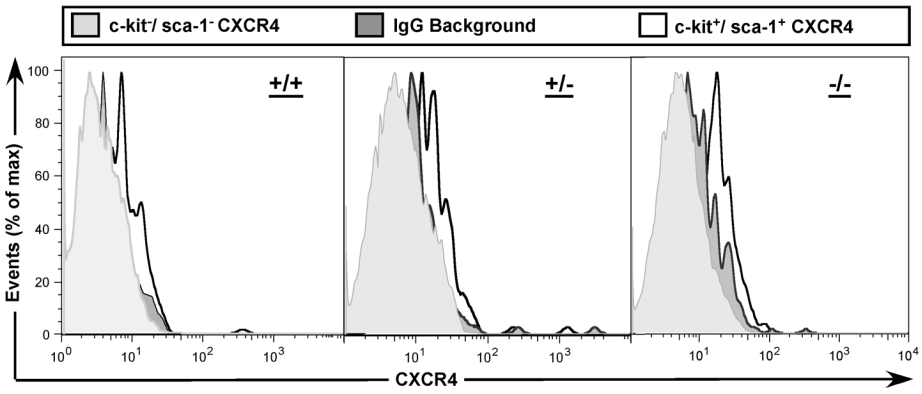

Figure 3.

Increased CXCR4 expression on progenitors expressing c-kit and sca-1 epitopes. Bone marrow cells from *Fancc*$^{-/-}$ (left hand panels) and *Fancc*$^{+/-}$ (right hand panels) littermates were lineage-depleted, cultured for 24 hours and stained separately with antibodies against c-kit, sca-1 and CXCR4, respectively. Events were collected and analyzed to exclude dead cells and analyze CXCR4 expression on c-kit and sca-1 positive or negative cells.

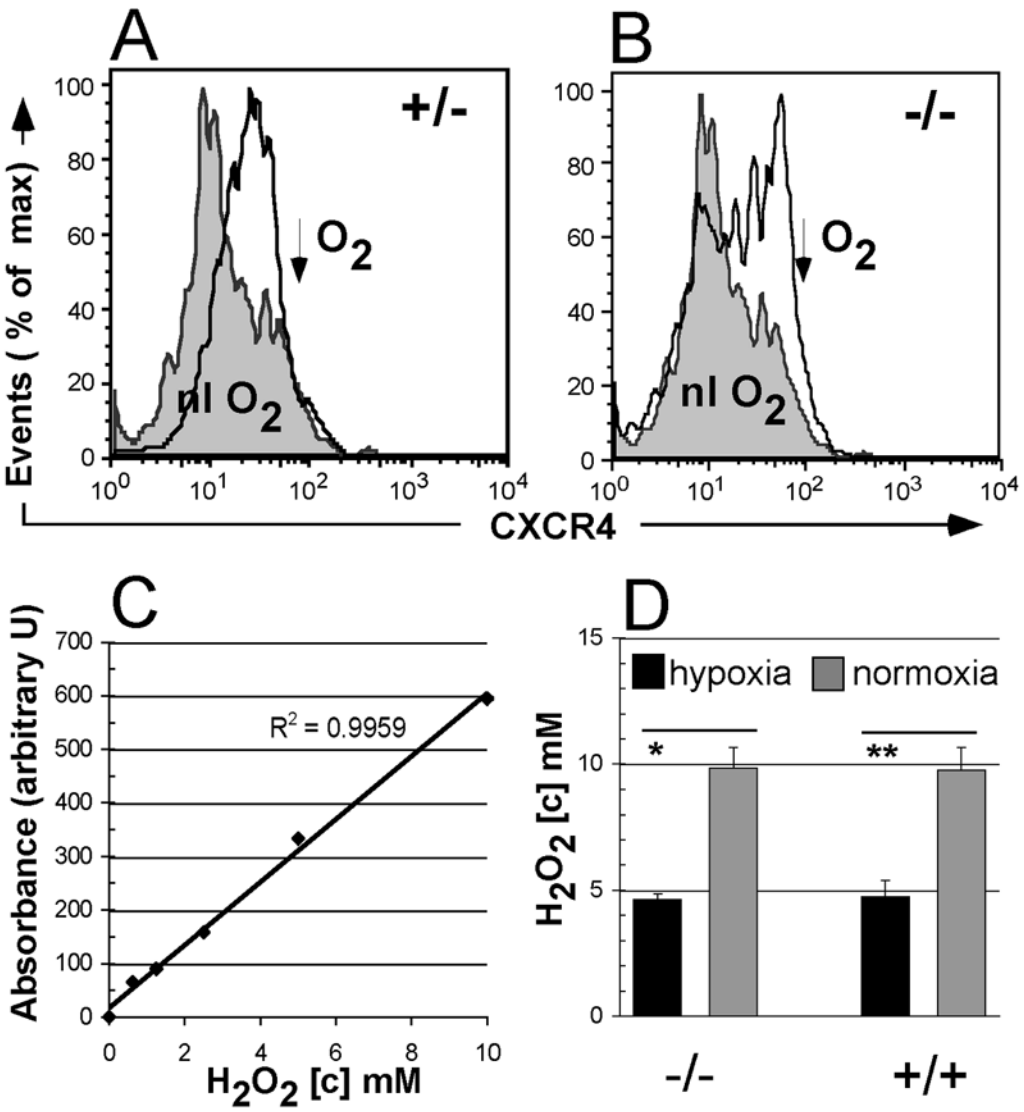

Figure 4.

CXCR4 expression and ROS production in murine hematopoietic progenitors. Increased CXCR4 expression on hypoxia-conditioned sca-1^+/c-kit^+ double-positive cells from *Fancc*$^{+/-}$ **(A)** and *Fancc*$^{-/-}$ **(B)** animals. Lineage-depleted marrow progenitor cells were cultured for 24 hours under normoxic or hypoxic conditions and analyzed by flow-cytometry for CXCR4 expression. Grey histogram, normoxia; open line histogram, hypoxia. **(C)** Standard curve from Amplex Red assay used to determine H_2O_2 concentrations. **(D)** H_2O_2 generation by lineage-depleted progenitor cells from *Fancc*$^{-/-}$ and *Fancc*$^{+/+}$ animals. Reactions were performed in triplicate. Error bars denote standard deviation. Asterisk denotes significant (*, $p<0.05$) or highly significant (**, $p<0.01$) difference between hypoxia- and normoxia- cell populations (2 tailed, paired T-test).

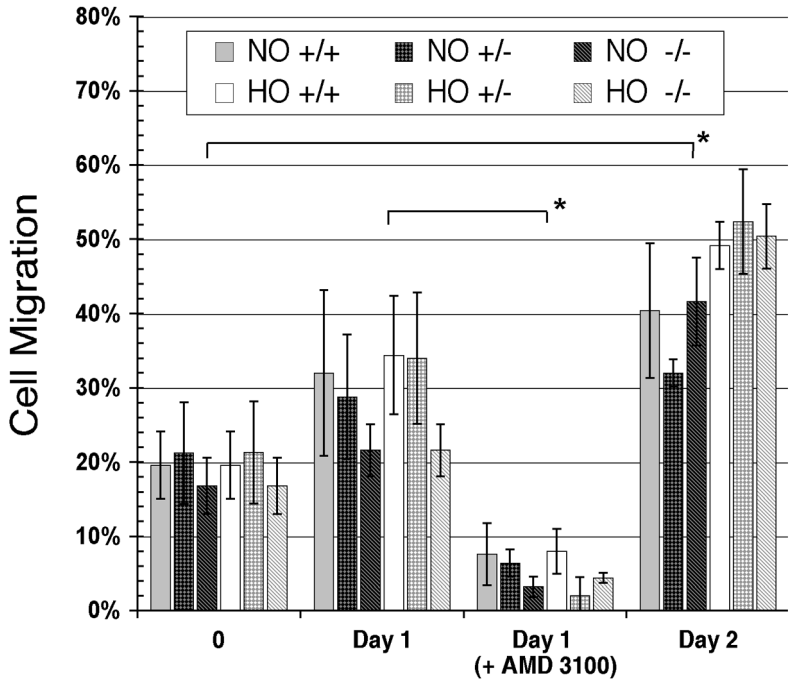

Figure 5.

In vitro transwell murine protenitor cell migration assay. Lineage depleted progenitor cells from *Fancc*$^{+/+}$, *Fancc*$^{+/-}$, *Fancc*$^{-/-}$ were divided for normoxia (NO) and hypoxia (HO) conditioning in the presence of mSCF/IL-3. Samples at the 0-hour time point are duplicates preceeding conditioning. Every 24 hours an aliquot of 2.5 x 10^5 cells was added to a transwell and tested for the ability to migrate toward SDF-1 containing media. Error bars are standard deviations of 5 counts taken per sample. AMD 3100 (1 nM) was added to the lower chamber of transwells on day 1 to block CXCR4 from binding SDF-1α. Asterisks denote significant ($p<0.01$) differences between day 0 and day 2 populations; and between day 1 and day 1 + AMD 3100 populations.

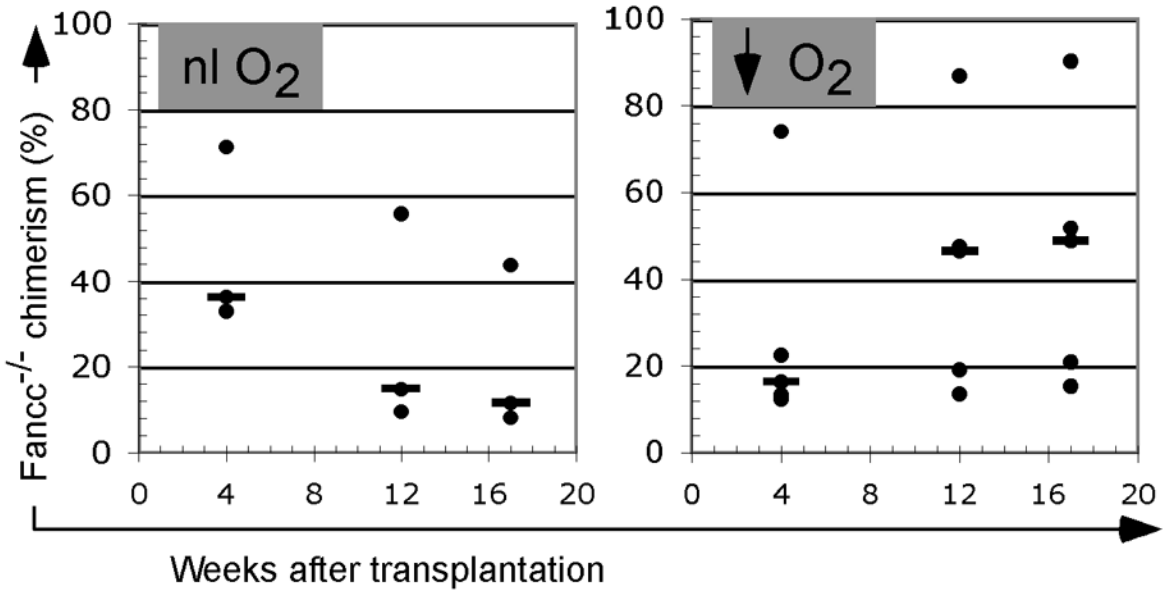

Figure 6.

Chimerism from CD45.2/*Fancc*$^{-/-}$ donor cells after transplantation. **(A)** Myeloablated recipients received 2.5 x10^5 CD45.2/*Fancc*$^{-/-}$ cells cultured over night in normal O_2 environment. **(B)** Comparison cohort received the identical number of *Fancc*$^{-/-}$ cells cultured a 1% O_2. Animals in both cohorts (n=3–5/cohort) also received 2.5 x10^5 CD45.1/*Fancc*$^{+/+}$ competitor cells (normoxia cultured) at the same time. Blood was obtained at indicated time-points and the percentage donor isotype was determined by staining with antibody against CD45.2. Bars illustrate median values.

Table 1

Post transplantation chimerism at 4 and 12 weeks in leukocyte subsets from animals receiving hypoxia- or normoxia-conditioned, lineage-depleted *Fancc*$^{-/-}$ progenitor cells. CD45.2 chimerism in leukocyte subsets of myeloablated recipients of cells conditioned over night in hypoxia (1% O_2) vs. normoxia. Animals received 950 cGy irradiation followed 24 hours later by 1 x104 lineagedepleted *Fancc*$^{-/-}$, conditioned progenitors mixed with 5 x10^5 CD45.1 (normoxia-cultured) whole bone marrow cells. The table shows the percentage of CD 45.2 (*Fancc*$^{-/-}$) events in leukocyte subsets at 4 and 12 weeks after transplantation. Subpopulations were gated based on forward and side-scatter (FSC – SSC) profile.

Leukocyte subset	**Granulocytes**		**Lymphocytes**		**Monocytes**		**Total Leukocytes**	
Weeks after transplantation	**4**	**12**	**4**	**12**	**4**	**12**	**4**	**12**
	13.5	10.8	46.4	10.9	28.9	4.4	25.6	11.0
	33.5	4.4	39.3	21.7	38.6	5.7	40.4	20.8
Hypoxia	17.0	58.7	19.6	34.4	18.7	13.2	20.2	35.5
	2.6	5.2	27.4	5.9	3.2	20.0	14.5	6.73
	17.3	21.5	27.4	11.8	5.1	7.3	26.6	12.5
	4.6	84.8	88.7	97.0	ND	98.4	82.6	96.8
Average	***14.8***	***30.9***	***40.2***	***30.3***	***18.9***	***24.8***	***35.0***	***30.6***
	13.3	12.3	24.8	34.7	21.2	12.4	21.3	33.0
	2.7	43.0	13.7	26.4	ND	10.1	12.5	27.6
Normoxia	15.6	13.5	37.8	51.1	15.3	26.5	29.6	47.8
	26.3	0.65	41.0	28.1	34.8	10.3	35.4	25.2
	14.9	29.6	34.8	35.1	39.6	14.6	27.7	19.8
	3.8	15.2	36.7	7.3	24.4	5.9	17.0	8.9
Average	***12.8***	***19.0***	***31.5***	***30.4***	***27.1***	***13.3***	***23.9***	***27.1***