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Phenotype Correction of Fanconi Anemia Group A Hematopoietic Stem Cells Using Lentiviral Vector

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Fanconi anemia (FA) is an autosomal recessive disease characterized by progressive bone marrow failure due to defective stem cell function. FA patients' cells are hypersensitive to DNA cross-linking agents such as mitomycin C (MMC), exposure to which results in cytogenetic aberrations and cell death. To date Moloney murine leukemia virus vectors have been used in clinical gene therapy. Recently, third-generation lentiviral vectors based on the HIV-1 genome have been developed for efficient gene transfer to hematopoietic stem cells. We generated a self-inactivating lentiviral vector expressing the FA group A cDNA driven by the murine stem cell virus U3 LTR promoter and used the vector to transduce side-population (SP) cells isolated from bone marrow of Fanconi anemia group A (Fanca) knockout mice. One thousand transduced SP cells reconstituted the bone marrow of sublethally irradiated Fanca recipient mice. Phenotype correction was demonstrated by stable hematopoiesis following MMC challenge. Using real-time PCR, one proviral vector DNA copy per cell was detected in all lineage-committed cells in the peripheral blood of both primary and secondary recipients. Our results suggest that the lentiviral vector transduces stem cells capable of self-renewal and long-term hematopoiesis *in vivo* and is potentially useful for clinical gene therapy of FA hematopoietic cells.

Key Words: Fanca knockout mice, lentiviral gene transfer, SP cell, bone marrow reconstitution

INTRODUCTION

Fanconi anemia (FA) is an autosomal recessive disease characterized by progressive bone marrow failure, developmental anomalies, and predisposition to cancer. FA patients' cells are hypersensitive to DNA cross-linking agents such as mitomycin C (MMC), which result in cytogenetic aberrations, G_2–M cell cycle arrest, decreased cell growth, and apoptosis. To date, seven FA complementation groups (A to G including B/D1 and D2) have been identified [1,2]. FA protein function has been implicated in the BRCA1/BRCA2 DNA repair pathway [3,4]. FA gene (*FANCA*) complementation group A (FA-A) patients are the most prevalent (70%) and FANCA mutations are variably distributed throughout the entire *FANCA* genome sequence [5]. Clinically, hematological abnormalities including bone marrow failure, myelodysplastic syndrome, and leukemia are predominant. Allogeneic bone marrow transplantation (BMT) from a histocompatible donor leads to hematologic cure but is accompanied by risk of graft-versus-host disease, graft rejection, and procedure-related morbidity [6,7]. New curative treatments are needed for the majority of FA-A patients lacking suitable donors.

Gene transfer to mutant cells is now being studied in the clinical setting. Autologous bone marrow reconstitution with gene-transduced hematopoietic stem cells has been attempted in a number of diseases, including FA. To date, Moloney murine leukemia retrovirus (MoMLV)-based vectors have been utilized as gene transfer vehicles because of their ability to integrate transgenes into chromosomes of hematopoietic cells [8–10]. In a trial of clinical gene therapy, including FA group C (FA-C) gene transfer of $CD34^+$ cells isolated from FA-C patients, persistent transgene expression and hematological improvement have not been achieved [8]. This suggests that the MoMLV vector used inefficiently integrated in slowly di-

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viding stem/progenitor cells and/or transgene expression was down-regulated by cellular factors in descendent cells derived from transduced cells. Apart from vector limitations of gene transfer, FA poses an additional dilemma. The observation of somatic reversion in some FA patients [11] demonstrates that gene-corrected FA cells may have an inherent selective growth advantage compared with mutant counterparts. Gene correction at the stem cell level should induce growth and proliferation of such corrected cells. However, our experience indicates that because of the marrow failure itself, the numbers of $CD34^+$ cells harvested from patients (1–10% of normal levels) may not be adequate for clinical gene therapy. This suggests that different stem cell sources may be needed for effective phenotype correction *in vivo*. Therefore improvements in gene transfer efficiency and available sources of stem cell populations are required for effective FA gene transfer.

Recently, lentiviral vectors that overcome the disadvantage of MoMLV vectors have been developed. Unlike retroviral vectors, lentiviral vectors are capable of gene transfer into nondividing cells [12,13]. Human immunodeficiency virus type-1 (HIV-1)-based lentiviral vectors transduced human $CD34^+$ cells without cytokine stimulation and transgene expression in multilineage cells was observed in the reconstituted bone marrow of nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice [14–17]. Both murine and human hematopoietic stem cells are susceptible to lentiviral gene transfer [18–21] and multilineage reconstitution of bone marrow originated from transduced cells has been achieved in nonhuman primates [22]. These results suggest that lentiviral vectors are suitable as gene transfer vehicles for hematopoietic stem cells from humans. For persistent gene expression in blood cells, the identification and isolation of self-renewing hematopoietic stem cells is important.

Currently, species-specific antibodies against cell surface antigens have been used to identify and isolate a rare stem cell population in bone marrow. The CD34, c-kit, and Thy-1 antigens are recognized as distinguishable hematopoietic stem cell markers for humans [23] and nonhuman primates [22,24]. The expression of c-kit and Sca-1 antigens also defines mouse stem cells [25]. Recent studies have revealed the existence of stem cells in the CD34-negative compartment in both human and mouse bone marrow [26–28]. The expression of the CD34 antigen reflects activation of stem/progenitor cells in response to stimuli facilitating hematopoiesis [29]. Recently a simple procedure was established utilizing Hoechst dye to identify hematopoietic cells (0.01–0.05% of whole bone marrow cells) termed side-population (SP) cells [27]. SP cells are characterized by a low Hoechst dye staining profile based on the dye-exclusion function mediated by the ABCG2 transporter expressed on hematopoietic cells [30] and highly conserved among species (human, mouse, rhesus monkey, and swine). SP cells possess an immature immunophenotype ($Sca\text{-}1^+$, $c\text{-}kit^+$) and reside in the G_0/G_1 phase of the cell cycle. A limiting number of SP cells can reconstitute lethally irradiated mouse bone marrow, suggesting stem cell activity [27]. In light of these observations, we utilized SP cells isolated from Fanconi anemia group A gene knockout mice (Fanca knockout mice), in an attempt to establish and validate lentiviral vectors in gene therapy of FA. Fanca knockout mice do not develop bone marrow failure spontaneously but do share characteristic features with FA-A patients. Following exposure to a DNA cross-linking agent such as MMC, they exhibit pancytopenia, indicating suppression of hematopoiesis due to stem cell dysfunction [31]. Resistance to MMC can determine correction of the *FANCA* gene defect in FA-A cells. We report here the use of a lentiviral vector to transfer the *FANCA* gene to hematopoietic SP cells and phenotype correction at the stem cell level in Fanca knockout mice given serial bone marrow transplants.

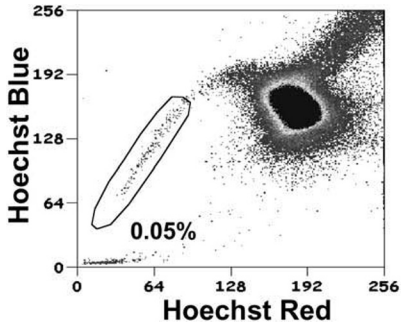

FIG. 1. Hoechst 33342 staining profile of murine bone marrow. The fluorescence with two emission wavelengths (red and blue) was displayed to identify the cell population that possessed low Hoechst fluorescence. Freshly isolated bone marrow was stained with Hoechst 33342 and subjected to flow cytometry. The Hoechst dye was excited at 350-nm wavelength when the emission intensity was measured at both 405 and 670 nm. A distinct population became apparent sloping away from most cells. The circled region indicates the side population, SP. It consists of 0.05% whole bone marrow.

RESULTS

Primary Bone Marrow Transplantation with Gene-Transduced Hematopoietic SP Cells Isolated from Fanca Knockout Mouse Bone Marrow

Using the Hoechst dye staining method, we collected SP cells amounting to 0.01–0.05% of total bone marrow (Fig. 1). The Hoechst staining procedure is based on the dye exclusion function mediated by the ABCG2 transporter in hematopoietic stem/progenitor cells [32,33]. We confirmed that SP cells lacked detectable CD34 antigen and that most SP cells were positive for c-kit and Sca-1 antigens [27] (data not shown). These markers are expressed on immature hematopoietic cells.

In this study, we set out to determine whether SP cells can reconstitute defective FA hematopoiesis as in

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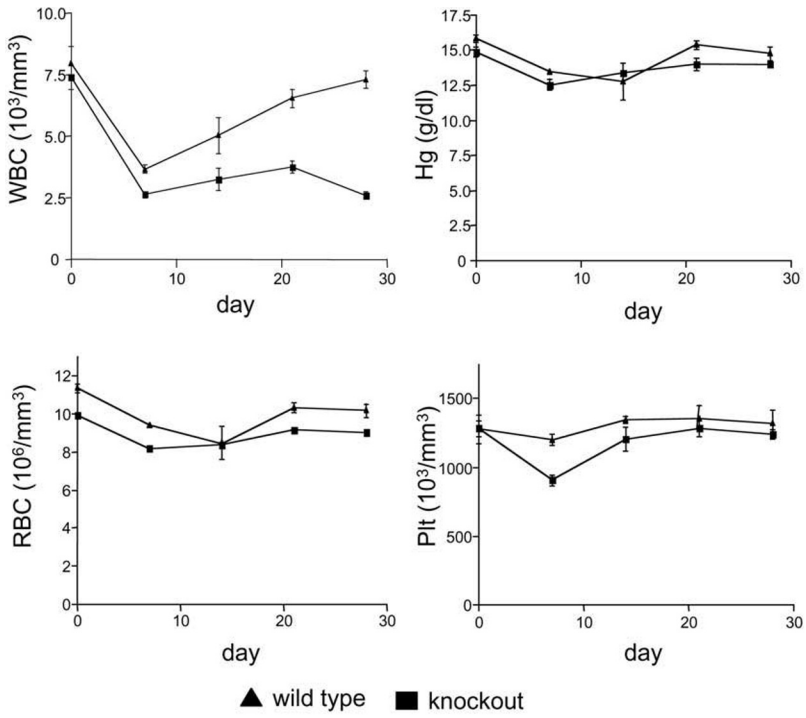

FIG. 2. Blood cell counts of 4-Gy-irradiated knockout and wild-type mice. Fanca knockout mice ($n = 5$) and wild-type mice ($n = 5$) were irradiated with 4 Gy and blood cell counts were examined weekly.

lineage$^-$c-kit$^+$Sca-1$^+$ cells following gene transduction using recombinant lentiviral vectors. SP or lineage$^-$c-kit$^+$Sca-1$^+$ cells isolated from Fanca knockout mouse bone marrow were transduced with a lentiviral vector expressing the *FANCA* gene under conditions facilitating cell division in the presence or absence of cytokines. We irradiated the recipient Fanca knockout mice with a 4-Gy dose to suppress endogenous hematopoiesis. The growth inhibition of irradiated fibroblast cell lines derived from Fanca knockout mice [34] suggests irradiation susceptibility of bone marrow in these mice. After 4 Gy irradiation, the white blood cell counts of Fanca knockout mice did not recover to the preirradiation level by day 28 (Fig. 2) or normalize without BMT. The irradiated mice received 1000 SP cells transduced with or without cytokine stimulation. The blood cell counts of irradiated mice that received transduced SP cells reached the normal range at 4–5 weeks, like those of wild-type mice (data not shown). We observed similar reconstitution kinetics in Fanca mice that received transplants of lineage$^-$c-kit$^+$Sca-1$^+$ cells transduced with or without cytokines (data not shown).

Phenotype Correction of Fanca Hematopoietic Cells

Fanca knockout mice are hypersensitive to DNA cross-linking agents such as MMC. A dose of MMC that is ineffective in wild-type mice leads to pancytopenia and bone marrow aplasia in those mice [31,35]. First, we injected MMC (0.3 mg/kg/week for 4 weeks, intraperitoneally) into Fanca knockout and wild-type mice to detect aplastic effects on hematopoiesis in the mice of both groups. We monitored peripheral blood indices weekly to verify the MMC effects. As expected, blood cell counts of knockout mice markedly decreased approximately in proportion to the cumulative MMC dose. After four MMC administrations, all knockout mice died with severe pancytopenia (Fig. 3). In contrast, the blood counts of wild-type animals remained at pre-MMC levels (Fig. 3). We concluded that four 0.3 mg/kg MMC injections suffice for evaluation of *FANCA* gene expression. Three months after BMT, we tested primary recipients by MMC challenge. The blood counts of animals that received transduced SP cells decreased transiently in proportion to the degree of endogenous hematopoiesis suppression but then returned to normal in the fourth week after MMC initiation (Fig. 4B). This reconstitution pattern was similar in mice that received wild-type SP cells (Fig. 4A). No significant difference was observed between the groups in which SP or lineage$^-$c-kit$^+$Sca-1$^+$ cells were transplanted (Fig. 4C). These results suggest that the gene-complemented hematopoietic cells expanded with growth advantage under selective pressure. Cytokine stimulation of target cells at vector transduction did not affect hematopoietic reconstitution kinetics.

Transgene Expression in Progenitor Cells Isolated from the Primary Recipient Bone Marrow

We performed a clonogenic assay to verify transgene expression in hematopoietic progenitor cells from the transplanted animals. We harvested the bone marrow from the primary recipients, then plated it in methylcellulose cul-

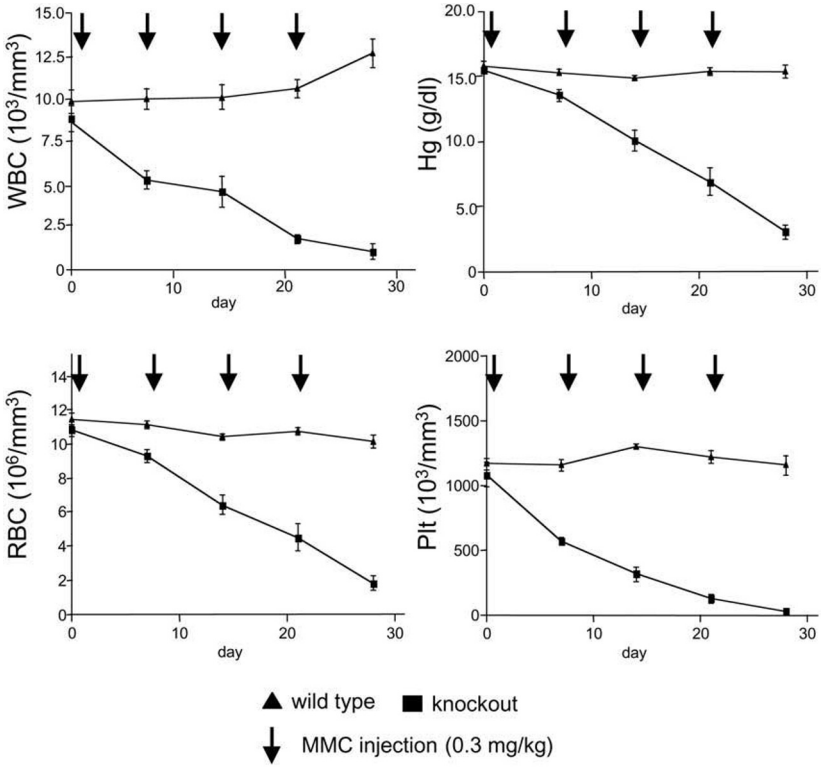

FIG. 3. Blood cell counts of knockout and wild-type mice that received four doses of MMC. Fanca knockout mice ($n = 5$) and wild-type mice ($n = 5$) were injected with MMC (0.3 mg/kg, ip). Blood cell counts were monitored weekly.

ture media in increasing concentrations of MMC. No significant colony growth from bone marrow of nontreated knockout mice was observed at the higher concentration of 10 nM MMC. On the other hand, clonogenic growth of wild-type and heterozygote cells was not impaired in the presence of MMC. In animals that received *FANCA*-transduced hematopoietic cells of either type, SP or lineage$^-$c-kit$^+$Sca-1$^+$ cells, colony growth was comparable to that of wild-type mice at all MMC concentrations and was dramatically relative to that of control knockout mice (Fig. 5) ($P < 0.0001$, two-tailed t test). Cytokine stimulation of target cells at vector transduction did not affect the number of MMC-resistant colonies.

Phenotype Correction of Long-Term Bone Marrow-Reconstituting Hematopoietic Stem Cells

We performed secondary bone marrow transplantation to determine whether phenotype correction had occurred at the level of the hematopoietic stem cells capable of self-renewal and reconstitution of bone marrow. We harvested bone marrow from the primary recipient animals and isolated SP cells. The percentage of SP cells in the primary recipient bone marrow was similar to that in the donor mice (0.01 to 0.05% for SP cells in whole bone marrow). More than 5000 SP cells were harvested from each primary recipient. The results suggested that the transduced and transplanted 1000 SP cells expanded in the recipient bone marrow up to normal levels under MMC selective pressure. We injected 1000 SP cells harvested from the primary recipients into the irradiated secondary recipient Fanca knockout mice to determine whether the gene-corrected SP cells derived from the primary recipient still maintained the capacity of self-renewal and repopulation required for long-term hematopoiesis. Simultaneously we injected 1×10^6 nonfractionated bone marrow cells harvested from the same primary recipients into the secondary recipients as a control. Three months posttransplantation, blood indices were maintained in the normal range in all recipients. Then the animals received weekly doses of MMC (0.3 mg/kg, intraperitoneally) to assess the phenotype correction in cells derived from the primary recipient cells. The blood count was monitored every week. In contrast with the primary recipients, changes in the blood counts in secondary recipients after MMC administration were smaller. All blood indices returned to normal after four MMC doses (Fig. 6).

We examined functional transgene expression in bone marrow-derived progenitor cells. We harvested marrow from the secondary recipients and performed a clonogenic assay. The MMC-resistant colony numbers from both SP cells and nonfractionated bone marrow recipients were similar. The results in Fig. 7 are comparable to those of the primary recipient shown in Fig. 5. These results suggested that the gene-corrected hematopoietic cells

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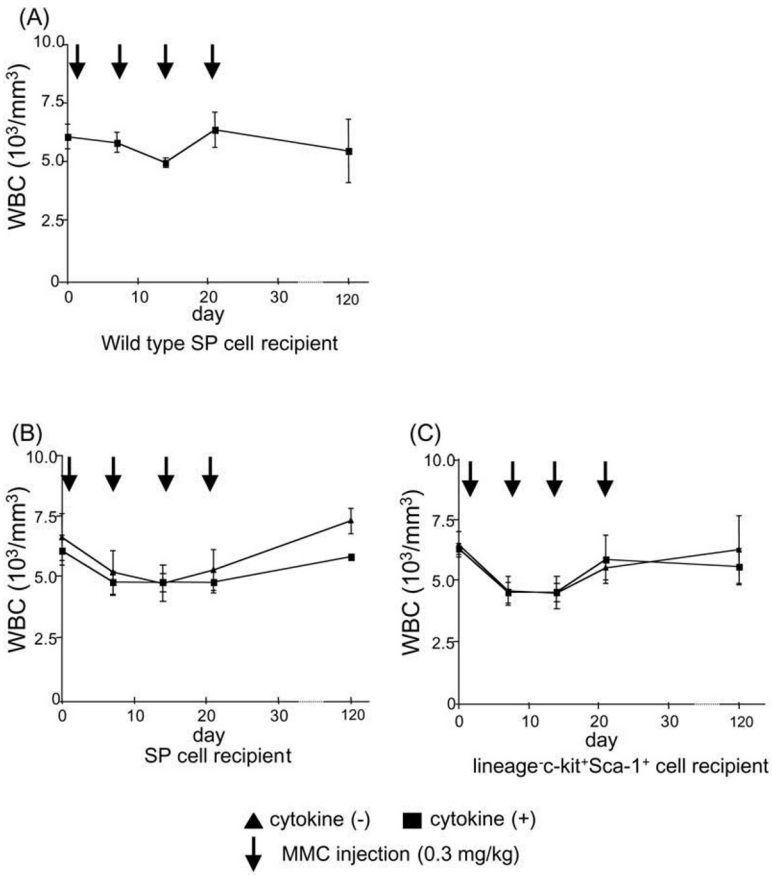

FIG. 4. White blood cell counts of the primary recipients. The lentiviral-transduced SP cells or lineage$^-$c-kit$^+$Sca-1$^+$ cells were transplanted into the sublethally irradiated Fanca knockout mice. Three months after BMT, four doses of MMC (0.3 mg/kg) were administered intraperitoneally. The blood cell count was monitored every week. (A) The recipients transplanted with SP cells harvested from wild-type mice. (B) The recipients transplanted with SP cells transduced with or without cytokine stimulation. (C) The recipients transplanted with lineage$^-$c-kit$^+$Sca-1$^+$ cells transduced with or without cytokine stimulation. Representative data are shown (n = 5 for each group).

probably including self-renewing stem cells expanded in number and were capable of sustaining normal hematopoiesis after the serial BMT.

Verification of Gene Transfer with Real-Time PCR

We performed further verification of gene transfer and expression using the real-time PCR technique. Real-time PCR allows quantification of rare genes with a strict linear amplification process [36]. Primers and probes were designed to detect the vector-specific sequence. We isolated DNA from peripheral blood samples of the primary recipient animals transplanted with SP cells or lineage$^-$c-kit$^+$Sca-1$^+$ cells and the secondary recipients that received SP cells or whole bone marrow from the primary recipients before and after MMC administration. As shown in Table 1, the presence of transgenes was detected in the DNA samples prior to MMC dosing at a level of 0.1–0.2 copies per cell. However, following MMC treatment, the copy number increased significantly up to 1 copy per cell in all primary recipients. In contrast to the primary recipients, the PCR signal of all secondary recipient animals was 0.5 copies per cell prior to MMC injection but subsequently increased to 1 copy per cell after completion of MMC dosing. These data are consistent with the blood counts in the secondary recipient animals injected with MMC. No significance was observed between each group transduced with or without cytokines in both primary and secondary recipients ($P > 0.05$). The transgene copy number of recipients that received whole bone marrow from the primary recipients was 0.8 copies per cell before MMC, representing the number of administered transgene-expressing cells, and increased to 1 copy after MMC. Clearly, MMC administration induced expansion of transgene-expressing cells. The similar transgene copy numbers in SP and whole bone marrow recipients after MMC indicate that the few gene-corrected SP cells were sufficient to repopulate bone marrow to the same extent as in animals given whole bone marrow. We also examined transgene expression in the lineage-committed cells. Such expression was detected in lymphocyte, myelomonocyte, and B cells isolated from the primary and secondary recipients (Table 2), indicating that gene-corrected stem cells produced descendent cells carrying the transgene.

Our results demonstrate that gene transfer of Fanca SP cells with a lentiviral vector caused phenotype correction at presumably stem cell level capable of long-term bone

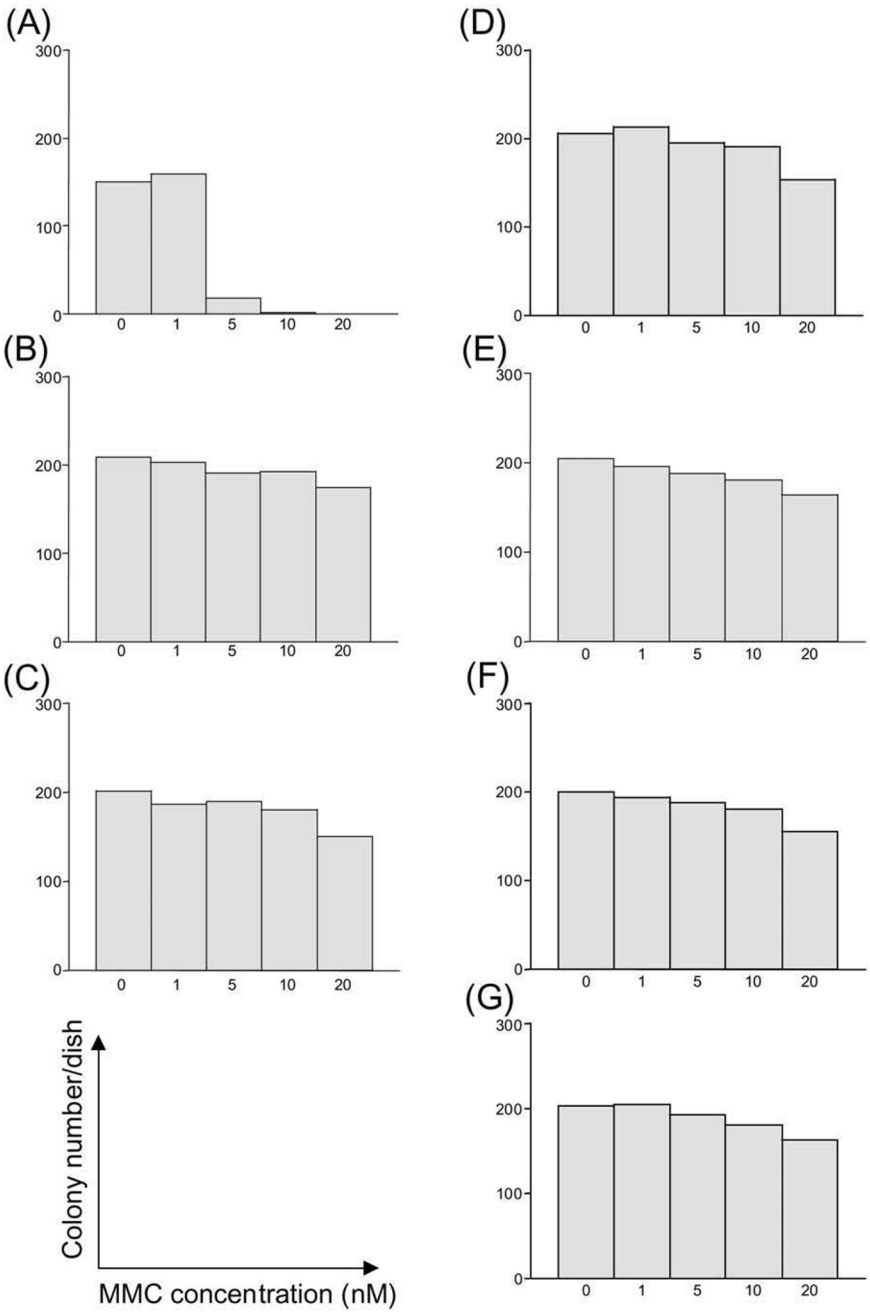

FIG. 5. Clonogenic assay of the primary recipient bone marrow. Bone marrow was harvested from three primary recipients after four doses of MMC injection (0.3 mg/kg) and 1×10^5 cells per dish were plated in methylcellulose culture media with increasing concentrations of MMC. Duplicate cultures were established from each animal. Colonies were enumerated at day 14. The mean data of the duplicate culture for two animals from each group are shown. (A) Fanca knockout, (B) wild type, (C) heterozygote, (D) recipients that received SP cells transduced with cytokine stimulation, (E) recipients that received SP cells transduced without cytokine stimulation, (F) recipients that received lineage$^-$c-kit$^+$Sca-1$^+$ cells transduced with cytokine stimulation, (G) recipients that received lineage$^-$c-kit$^+$Sca-1$^+$ cells transduced without cytokine stimulation.

marrow reconstitution in knockout mice. The transgene was fully functional under the influence of a selective agent such as MMC, providing a growth advantage to gene-corrected cells.

Discussion

In the context of clinical gene therapy of FA patients, it is essential to understand FA stem cell biology. However, it is difficult to obtain sufficient numbers of hematopoietic stem/progenitor cells from FA-A patients for analysis and treatment. Generally the number of CD34$^+$ cells in patient bone marrow is significantly less than in normal individuals (1–10% of normal CD34$^+$ content) and CD34$^+$ cells often cannot be mobilized in peripheral blood of FA patients after G-CSF administration [37]. The Fanca knockout mouse provides a model system to study hematopoiesis and therapeutic strategies. Although they

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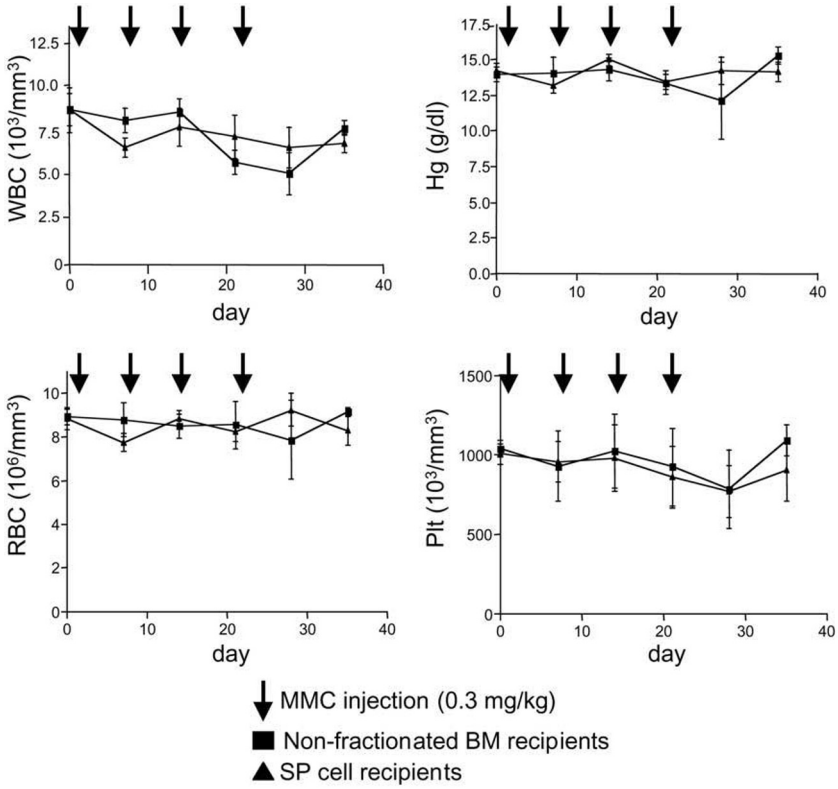

FIG. 6. Blood cell counts of the secondary recipients. One thousand SP or 1×10^6 bone marrow cells harvested from the primary recipients were transplanted into the sublethally irradiated secondary recipients. After blood indices returned to the normal range, four doses of MMC (0.3 mg/kg) were administered. The number of blood cells of each animal was monitored weekly ($n = 4$ for SP cell recipients, $n = 5$ for nonfractionated bone marrow recipients).

do not develop bone marrow failure spontaneously, they exhibit marrow aplasia following exposure to MMC [35] or cyclophosphamide [38]. The bone marrow-repopulating ability of Fancc knockout mice is inferior to that of wild-type cells, as assessed by competitive repopulation assays [39] and serial BMT [40]. In rare FA patients, spontaneous intragenic reversion has been observed, resulting in self-correction of the hematologic defect [11,41]. Clonal analysis of peripheral blood cells suggests the growth advantage resulted from a single reverted stem cell. Taken together, these data indicate that gene complementation may lead to a growth advantage of corrected cells, a consideration that provides the rationale for gene transfer.

We previously developed an amphotropic retrovirus vector carrying the human cDNA of FANCC [35]. We used a murine model of Fancc deficiency to determine whether our vector would transduce long-term marrow-reconstituting cells and asked whether gene-corrected cells would restore normal hematopoietic function to aplastic animals. We transduced nonfractionated bone marrow and observed long-term functional gene expression after administration of MMC. Bone marrow-repopulating cells were transduced from these animals and expanded after MMC administration. However, in clinical FA-C gene therapy using the same retrovirus vector [8], gene-transduced cells failed to compete with mutant cells in the absence of selective pressure *in vivo*. Results of that trial suggested that a more effective vector was needed, and in addition alternative stem cell populations other than $CD34^+$ cells were needed for gene transfer.

Among the gene transfer vehicles for quiescent cells, the lentiviral vector has exhibited high efficiency on integration into nondividing cells such as stem cells [42]. The *cis* sequence of the lentiviral vector facilitates nuclear translocation and efficient integration of the transgene in hematopoietic cells [43]. We reported that the first-generation lentivirus vector based on HIV-1 carrying the *FANCC* gene transduced the B cell lines established from FA-C patients and led to phenotype correction comparable with those of the retrovirus and equine infectious anemia virus vectors [44]. Recently, lentivirus gene transfer using nonfractionated bone marrow of Fanca and Fancc knockout mice was reported. After the secondary bone marrow transplantation followed by MMC injection, the platelet counts of recipients were in the normal range, reflecting sustained transgene expression [45]. However, data supporting gene transduction at the stem cell level were not provided. Transgene integration into self-renewing stem cells should produce persistent gene expression in all hematopoietic lineages. To address this issue, we attempted to isolate the definitive hematopoietic stem cell population from Fanca knockout mice for lentiviral gene transfer.

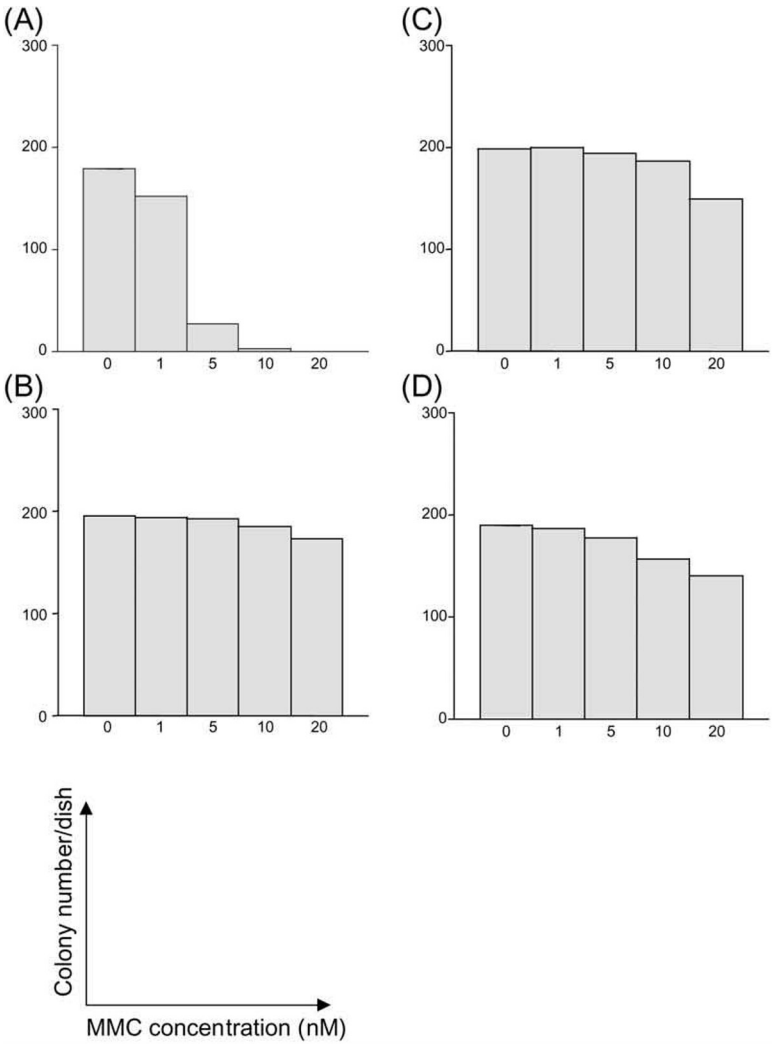

FIG. 7. Clonogenic assay of the secondary recipient bone marrow. Bone marrow was harvested from the secondary recipients after four doses of MMC and 1×10^5 cells were plated in methylcellulose culture media with increasing concentrations of MMC. Colonies were enumerated at day 14. The mean data of the duplicate culture for two animals from each group (A and D) are shown: (A) Fanca knockout, (B) wild type, (C) recipients that received SP cells isolated from the primary recipients, (D) recipients that received 1×10^6 nonfractionated bone marrow cells isolated from the primary recipients.

As hematopoietic stem cells of mice, lineage$^-$c-kit$^+$Sca-1$^+$ cells have been accepted because of their ability to reconstitute hematopoiesis in mice. Using a lentiviral vector carrying the *EGFP* transgene and transduction of murine lineage$^-$c-kit$^+$Sca-1$^+$ cells without cytokine stimulation, transgene expression was detected in the pe-

TABLE 1: Transgene copy number in peripheral blood of recipient mice

	Cytokine	Pre MMC	After MMC
Primary recipient			
SP	+	0.21 ± 0.05 (n = 5)	0.9 ± 0.09 (n = 4)
SP	−	0.12 ± 0.05 (n = 10)	1.1 ± 0.01 (n = 10)
Lineage$^-$c-kit$^+$Sca-1$^+$ cells	+	0.13 ± 0.12 (n = 5)	1.0 ± 0.04 (n = 5)
Lineage$^-$c-kit$^+$Sca-1$^+$ cells	−	0.10 ± 0.02 (n = 5)	0.9 ± 0.04 (n = 5)
Secondary recipient			
SP cell recipient		0.50 ± 0.08 (n = 4)	1.04 ± 0.10 (n = 4)
BM recipient		0.83 ± 0.05 (n = 5)	1.09 ± 0.03 (n = 5)

The primary recipients received either SP cells or Lineage$^-$c-kit$^+$Sca-1$^+$ cells transduced in the presence or absence of cytokines. The secondary recipients received either SP cells or non-fractionated bone marrow isolated from the primary recipients in which SP cells transduced with cytokine stimulation had been transplanted.

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TABLE 2: Transgene copy number in lineage committed cells of bone marrow transplanted mice

	Mac1 + Gr1	CD3
Primary SP cell recipient	0.95 ± 0.06 (n=4)	1.02 ± 0.11 (n=4)
Secondary recipient		
SP cell recipient	0.98 (n = 2)	0.85 (n = 2)
BM recipient	1.0 (n = 2)	0.99 (n = 2)

Lineage committed cells were sorted from peripheral blood of transplanted mice with flow cytometry and the transgene copy number was examined by real-time PCR.

ripheral blood of both primary and secondary recipients [19]. In this study, we utilized both lineage$^-$c-kit$^+$Sca-1$^+$ and SP cells as target cells for gene transfer. Because SP cell isolation is based on dye efflux, SP cells are harvested from many species, including mouse, dog, primate, and human [27]. One thousand SP cells can fully reconstitute hematopoiesis in mice and express cell surface Sca-1 and c-kit antigens and may differentiate into other nonhematopoietic tissues [46,47]. We confirmed that more than 95% of SP cells resided in the quiescent phase (G_0/G_1) of the cell cycle (data not shown). These features suggest that SP populations includes cells that fulfill the criteria for stem cells.

We developed a self-inactivating lentiviral vector carrying the human *FANCA* gene and transduced SP cells isolated from Fanca knockout mouse bone marrow and examined the efficiency of gene transfer before and after MMC selection. To optimize the gene transfer of SP cells, we transduced SP cells isolated from Fanca knockout mice with or without cytokine stimulation ($n = 15$ and $n = 13$, respectively). We observed that the gene transfer results were comparable between SP and lineage$^-$c-kit$^+$Sca-1$^+$ cells and that cytokine preincubation had no significant effect on the efficiency of gene transfer. The transgene copy number was equivalent in all lineage cells (lymphocytic, myelomonocytic, B cell). These data confirm that cell cycle induction via cytokine stimulation is not required for optimal gene transfer of SP cells capable of bone marrow reconstitution using the lentiviral vector.

We designed the experiments to mimic our clinical gene transfer trial. A low dose of irradiation was used to allow for engraftment and to maintain a reduced number of endogenous hematopoietic cells. We observed reconstitution of gene-marked cells at 0.2 copy/cell at 3 months posttransplantation. Following application of MMC, the copy number increased significantly to 1.0 copy/cell, indicating that all cells were gene-marked. More significantly, we found that the serial transplantation of 1000 gene-marked SP cells into the secondary recipient yields a copy number at 0.5 copy/cell. This suggests that the transduced SP cells exhibited growth kinetics equivalent to wild-type cells and a greater growth advantage. The transduced SP cells used in this experiment exhibited growth kinetics equivalent to those of wild-type cells. These results confirm that the *FANCA* transgene expression in the SP population is fully functional.

Phenotype correction of FA-A hematopoiesis is achievable with lentiviral gene transfer. Furthermore, the CD34-negative phenotype of SP cells suggests a possibility of another source of stem cells in FA patients. Our results serve as the basis for proposing FA gene therapy using lentiviral vectors.

MATERIALS AND METHODS

FANCA lentiviral vector plasmids. The self-inactivating lentiviral vector plasmid SINF-MU3-FANCAΔUTR-W was derived from SINF-MU3-EGFP-W [48] by removing the *EGFP* sequences with *Eco*RI–*Bam*HI digestion and inserting the human *FANCA* cDNA minus the 3′ UTR region (4.5 kb). The vector utilizes the U3 promoter region of the murine stem cell virus long terminal repeat (MSCV LTR; abbreviated MU3). The vector also contains the central polypurine tract and central termination sequence of HIV-1, which specify the creation of a plus-strand overlap during reverse transcription referred to as the central DNA flap (abbreviated F) [43,49], and the woodchuck hepatitis virus posttranscriptional regulatory element (abbreviated W). In the conventional MoMLV vector system, diminished transgene expression reflecting the degree of promoter silencing is often observed. The lentiviral vector carrying the MSCV LTR promoter driving the *EGFP* transgene transduced into human CD34$^+$ cord blood cells demonstrated persistent transgene expression in NOD/SCID mice [17,50].

Lentiviral vector generation and titering. The plasmids expressing the lentiviral vector particle proteins (gag, pol, and rev; pMDLg/pRRE and pRSV-Rev) and the VSV-G envelope protein (pMD.G) were provided by Dr. Thomas Dull (Cell Genesys, Foster City, CA) [51]. Ten micrograms of SINF-MU3-FANCA-Δ3′UTR-W, pMDLg/pRRE, and pMD.G plasmids was cotransfected with 2.5 μg of pRSV-Rev plasmid using calcium phosphate into 2.5×10^6 human 293T cells plated onto poly-L-lysine-coated 10-cm tissue culture dishes to generate lentviral vector. Two days after transfection, supernatant was harvested, filtered through 0.45-μm filters, and concentrated about 100-fold by ultracentrifugation at 50,000*g* for 2 h at 4°C. The vector particles were resuspended in PBS and frozen at −80°C until use.

Vector particle number was estimated by RNA dot blot as described previously [44]. Briefly, vector particles were precipitated with 6% polyethylene glycol (MW 8000; Sigma, St. Louis, MO) and 0.3 M NaCl. Vector genome RNA was extracted from the virions with phenol–chloroform and digested with DNase I to eliminate contamination by plasmid DNA and then reextracted. Vector RNA was dot-blotted onto nylon membrane and then hybridized using a ^{32}P-labeled randomly primed 1.8-kb *FANCA* cDNA fragment digested with *Bam*HI and *Nde*I enzymes as a probe. A standard curve was generated by the serial dilution of vector plasmid. The phosphoimage value was captured and calculated with a phosphoimager (Storm 860; Molecular Dynamics, Amersham Biosciences, Sunnyvale, CA). The functional titer of FANCA vector was determined by comparing the vector genome particle numbers with the vectors possessing the same backbone and known infectivity estimated by EGFP marker gene expression.

Animals. Fanca knockout mice [31] were maintained in the animal facility at the University of North Carolina at Chapel Hill, in accordance with Institutional Animal Care and Use Committee standards. To obtain homozygous null Fanca knockout mice, each heterozygous female and male or homozygous male and heterozygous female were inbred by brother–sister mating. Bone marrow-transplanted mice were housed in autoclaved microisolator cages and given sterile food and water and pathogen-free room. Mice were used between 2 and 4 months of age.

SP cell sorting. SP cells were isolated following the protocol established by Goodell *et al.* [27]. Bone marrow was flushed from the femurs and tibiae of Fanca knockout mice with HBSS with 2% FBS and 10 mM Hepes buffer. Cells were enumerated using 3% acetic acid. Erythrocytes were lysed with ACK lysing solution (Bio-Fluids, Rockville, MD). Cells were centrifuged and resuspended in prewarmed RPMI with 2% FBS, 10 mM Hepes buffer, and 5 mg/ml Hoechst 33342 (Sigma) at a concentration of 1×10^6 cells/ml and incubated for 90 min at 37°C in a water bath. Cells were washed twice with cold HBSS medium and then resuspended in the same medium containing 2 mg/ml propidium iodide (PI; Sigma). The stained cells were kept on ice and analyzed immediately with flow cytometry.

Analysis and sorting of SP cells were performed on a dual-laser flow cytometer (MoFlo; Cytomation, Fort Collins, CO). Hoechst dye fluorescence was measured with a 405/30 nm BP filter (Hoechst blue) and 670/40 nm EFLP filter (Hoechst red). Hoechst blue and red were split with a 610 nm LP dichroic mirror. The gate to exclude erythrocytes was established on forward vs side scatter parameters. The living cell gate was established to eliminate dead cells according to PI fluorescence measured through a 670/40 nm EFLP filter. Doublets were excluded using integrated FSC and FSC pulse width. Hoechst-stained cells were profiled in the dot plots using Hoechst blue and red fluorescence. The SP cells were identified in this plot and gated according to the low Hoechst dye staining profile.

Sorting of lineage$^-$c-kit$^+$Sca-1$^+$ cells. Bone marrow was harvested from Fanca knockout mice and then incubated with the cell surface antigen-specific antibody cocktail containing anti-mouse CD3, CD19, Mac-1, Gr-1 (all antibodies were conjugated with fluorescein isothiocyanate; FITC), Sca-1 antibody conjugated with phycoerythrin (PE), and biotinylated c-kit antibody for 30 min on ice. The isotype-matched immunoglobulins were served as negative controls. All antibodies were purchased from Pharmingen (San Diego, CA). After the primary antibody reaction, cells were washed with PBS buffer twice, and streptavidin-conjugated Cy-Chrome (Pharmingen) was added to the cell suspension and incubated for 30 min on ice. After cells were washed with PBS buffer, lineage$^-$c-kit$^+$Sca-1$^+$ cells were immediately analyzed and sorted on a dual-laser flow cytometer (MoFlo). First the lineage antigen-positive cells were depleted from the sorting gate according to their FITC fluorescence positivity, and then the lineage$^-$c-kit$^+$Sca-1$^+$ compartment was sorted on the basis of the fluorescence of PE and Cy-Chrome.

Gene transfer of Fanca knockout hematopoietic cells. SP or lineage$^-$c-kit$^+$Sca-1$^+$ cells sorted from bone marrow of Fanca knockout mice were incubated with RPMI containing 20% FCS and cytokines (mIL-3, 20 ng/ml; hIL-6, 100 ng/ml; mSCF, 100 ng/ml) for 12 h and then transduced with SINF-MU3-FANCAΔUTR-W vector in the presence of 5 μg/ml protamine sulfate at m.o.i. 100 (transducing vector particles per cell) for the following 12 h. In other experiments, cells were suspended in serum-free medium, X-VIVO 10 (BioWhittaker, Walkersville, MD), and incubated with vector particles for 12 h in the absence of cytokines.

Bone marrow transplantation with transduced SP or lineage$^-$c-kit$^+$Sca-1$^+$ cells. The primary recipient Fanca knockout mice were sublethally irradiated (4 Gy) from a ^{137}Cs source to reduce endogenous hematopoiesis and then anesthetized by intraperitoneal injection of 2,2,2,-tribromoethanol (Sigma). One thousand SP cells transduced with or without cytokine stimulation (five animals per cohort) or lineage$^-$c-kit$^+$Sca-1$^+$ cells transduced with or without cytokine stimulation (five animals per cohort) were administered into recipient mice by retro-orbital vein injection. The recipient mice were maintained with autoclaved water containing 1.1 mg/ml neomycin sulfate (Life Technologies, Inc., Rockville, MD). The number of peripheral blood cells was monitored every week. After 6 weeks, all blood cell indices reached the normal range in all recipients (data not shown). At 3 months posttransplantation, four doses of MMC (Calbiochem, San Diego, CA) (0.3 mg/kg) were administered intraperitoneally every week. Wild-type and untreated knockout mice were subjected to the same procedure as the controls to assess MMC effects.

Two months after the four doses of MMC, SP cells were harvested from the primary SP cell recipient bone marrow. Then 1000 SP cells or 1×10^6 nonfractionated bone marrow cells were injected into the secondary recipients (five animals per group) as described above. Two to three months after transplantation, all experimental mice maintained normal peripheral blood cell counts as observed in the primary recipients (data not shown). MMC challenge was then performed.

Hematological assay. Peripheral blood (50 μl) was collected from bone marrow-reconstituted mice by means of eye venipuncture into heparinized microcapillary tubes and flushed immediately into 1.5-ml tubes containing 5 μl of EDTA (25 μg/ml). The samples were analyzed at the University of North Carolina Animal Clinic Laboratory on an ABC animal blood counter (HESKA, Fort Collins, CO).

Clonogenic and MMC resistance assay of recipient mice bone marrow. Bone marrow was harvested from the primary and secondary recipient mice and 1×10^5 cells were plated into methylcellulose culture media containing cytokines for progenitor cell growth (mIL-3, hIL-6, and mSCF) (Methocult GF M3534; Stem Cell Technologies, Vancouver, Canada) with increasing concentrations of MMC (0, 1, 5, 10, and 20 nM). Cells were cultured in a humidified incubator flushing 5% CO_2 in the air at 37°C. Colonies were enumerated at day 14. Experiments were performed in duplicate.

Isolation of lineage-committed cells from recipient peripheral blood. Peripheral blood was harvested from bone marrow-transplanted recipient mice and red blood cells were lysed using ACK lysing buffer (Bio-Fluids). Mononuclear cells were incubated with each lineage-specific PE-labeled anti-mouse antibody (lymphocyte; CD3, B cell; CD19, or myelomonocyte; Mac-1 and Gr-1 (Pharmingen)) for 30 min on ice. Cells were washed with PBS buffer twice and analyzed and sorted on a dual-laser flow cytometer (MoFlo). DNA isolated from sorted cells was subjected to analysis of the transgene copy number with real-time PCR.

Transgene analysis by real-time PCR. Peripheral blood, before and after four doses of MMC, was collected from bone marrow-reconstituted animals as described above. DNA was isolated from nucleate cells with the use of the DNeasy tissue kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. DNA isolated from bone marrow and whole or sorted lineage compartments from peripheral blood was subjected to real-time PCR to determine the transgene frequency. The sequences of primers and probes for detection of lentiviral sequence were located in the R-U5 region of the LTR [52,53]. A neomycin-resistance gene inserted to generate the knockout strain was utilized as an internal control. The probes were fluorescence-labeled using the TaqMan system (Applied Biosystems, Foster City, CA). The sequences of primers and probe for lentiviral vector were as follows: forward primer, 5′-AGCTTGCCTTGAGTGCTTCA-3′; reverse primer, 5′-TGACTAAAAGGGTCTGAGGGA-3′; and probe, 5′-FAM (6-carboxyfluorescein)-TGCCCGTCTGTTGTGTGACTCTG-TAMRA (6-carboxyltetromethyl-rhodamine)-3′. The sequences of primers and probe for the neomycin-resistance gene designed using Primer Express software (Applied Biosystems) were as follows: forward primer, 5′-CCATTCGACCAC-CAAGCG-3′; reverse primer, 5′-CCGGCTCTCCATCCGA-3′; and probe, 5′-TET (tetrachloro-6-carboxyfluorescein)-AACATCGCATCGAGCGAG-CACG-TAMRA-3′. Real-time PCR was performed using an ABI Prism 7700 and a Gene Amp 5700 sequence detector and software version 1.6.3 (Applied Biosystems). The amplification mixture contained 1× TaqMan buffer, 200 μM dNTPs, 5.0 mM $MgCl_2$, 0.33 μM each primer, 333 nM TaqMan probe, 0.1 unit of uracil *N*-glycosylate, and AmpliTaq Gold (1.0 U) in a 30-μl volume. The cycling conditions were 50°C for 2 min, 94°C for 10 min, and 40 cycles of two-step PCR (at 94°C for 15 s and at 60°C for 60 s). A standard curve for transgene copy number was established by amplification of a series of genomic DNA mixtures with a vector-transduced B cell line (EUFA232 established from a FA-A patient by EB virus transformation, kindly provided by Dr. Joenje, Free University, Amsterdam, The Netherlands) that has one *FANCA* transgene per cell and mouse peripheral blood (data not shown). The neomycin-resistance gene was amplified simultaneously in a separate tube using the same amount of sample DNA. The copy number of the transgene was normalized using that of the neomycin-resistance gene.

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Statistical analysis. Two-tailed *t* tests were performed to compare the data on colony numbers and blood cell counts between bone marrow transplanted, wild-type, and untreated knockout mice. Statistical calculations were performed using Prism software (GraphPad Software, Inc., San Diego, CA).

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