Overnight transduction with foamy viral vectors restores the long-term repopulating activity of FancG−/− stem cells

*Yue Si,1,2 *Anna C. Pulliam,1,2 Yvonne Linka,3 Samantha Ciccone,1,2 Cordula Leurs,3 Jin Yuan,1,2 Olaf Eckermann,3 Stefan Fruehauf,4 Sean Mooney,5,6 Helmut Hanenberg,2,3 and D. Wade Clapp1,2

Departments of 1Microbiology and Immunology and 2Pediatrics, Herman B Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis; 3Department of Pediatric Oncology, Hematology and Clinical Immunology, Children’s Hospital, Heinrich Heine University, Duesseldorf, Germany; 4Department of Internal Medicine III, University of Heidelberg, Heidelberg, Germany; and 5Center for Computational Biology and Bioinformatics and 6Department of Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis

Fanconi anemia (FA) is a complex genetic disorder characterized by congenital abnormalities, bone marrow failure, and myeloid malignancies. Identification of 13 FA genes has been instrumental to explore gene transfer technologies aimed at correction of autologous FA-deficient stem cells. To date, 3 human FA stem cell gene therapy trials with standard 4-day transduction protocols using gammaretroviral vectors failed to provide clinical benefit. In addition, 2- to 4 day ex vivo manipulation of bone marrow from mice containing a disruption of the homologue of human FANCC (Fancc) results in a time-dependent increase in apoptosis and a risk for malignant transformation of hematopoietic cells. Here, we show that a 14-hour transduction period allows a foamyviral vector construct expressing the human FANCC cDNA to efficiently transduce murine FA stem cells with 1 to 2 proviral integrations per genome. Functionally, the repopulating activity of FancG−/− stem cells from reconstituted mice expressing the recombinant FANCC transgene was comparable with wild-type controls. Collectively, these data provide evidence that short-term transduction of c-kit+ cells with a foamyviral vector is sufficient for functional correction of a stem cell phenotype in a murine FA model. These data could have implications for future gene therapy trials for FA patients.

(Blood. 2008;112:4458-4465)

Introduction

Fanconi anemia (FA) is a complex recessive inherited disorder that is clinically characterized by variable congenital abnormalities, progressive bone marrow (BM) failure, and a high propensity to develop myeloid and epithelial malignancies.1-5 On a cellular level, FA is characterized by a profound hypersensitivity upon exposure to DNA cross-linking agents such as mitomycin-C (MMC) or diepoxybutane (DEB).6,9 Genetically, germ-line mutations in 13 genes (FANCA/B/C/D1/D2/E/F/G/I/J/L/M/N) result in the clinical phenotype of FA.2-8,10,14

Spontaneous genetic correction of a germ-line mutation leading to repopulation of the entire hematopoietic system with normal progeny has been identified in a few FA patients.15-19 These observations, in combination with the fact that the hematopoietic system can be functionally corrected in mice with targeted disruptions of FA genes by retroviral vectors expressing human analogous of the targeted mouse genes in stem cells,20-22 have led to 3 clinical stem cell gene therapy phase 1 studies in FA-A and FA-C patients. So far, neither long-term marking/correction of cells nor clinical benefits for the patients were observed.23,24 Due to the biologic characteristics of the gammaretroviral vectors used for transduction of stem cells,25,26 optimal gene transfer protocols for delivery of genes to mammalian stem cells require a prestimulation period of 1 to 2 days with cytokines that promote the proliferation and survival of stem/progenitor cells. This is followed by a 2- to 3-day exposure of the target cells to vector containing supernatant on the recombinant fibronectin fragment CH-296.27,28 This gene transfer protocol was successful in transducing hematopoietic stem cells in humans, primates/monkeys, and mice.29-31 However, in murine FA models, prolonged in vitro culture of FancG−/− BM results in a length-of-culture–dependent reduction in myeloid progenitors and repopulating ability,32,33 and the surviving untransduced FancG−/− repopulating cells have an increased risk of developing cytogenetic abnormalities and myeloid malignancies.22 Therefore, limiting the in vitro culture would be predicted to enhance both the efficacy and safety for genetic therapies of FA stem cells.

Wild-type foamy viruses are the only retroviruses that are not associated with any disease in their natural hosts or in accidentally infected human beings.34-38 It has been shown that vectors based on the prototype (formerly human) foamy virus (FV) can efficiently transduce hematopoietic stem cells from mice,39 dogs,40 and nonobese diabetic/severe combined immunodeficiency (NOD/SCID) repopulating human cells.39,41 Further, FV vectors are at least equally efficient at transduction of CD34+ umbilical cord blood cells engrafing in NOD/SCID mice as lentiviral vectors based on HIV-1.40 In the present study, we demonstrated for the first time the ability of FV vectors encoding the human FANCC transgene to completely correct FancG−/− myeloid progenitors and repopulating hematopoietic stem cells in a 14-hour transduction protocol without prestimulation. This short gene transfer protocol resulted in 1 to 2 proviral integrations in the reconstituting stem cells and was not associated with the development of myelodysplastic syndromes (MDSs)/acute myeloid leukemia (AML) in FancG−/− stem cells transduced with the reporter construct. These characteristics support the hypothesis that FV vectors are a viable strategy for stem cell gene transfer strategies in FA.
Methods

Mice

FANc<sup>−/−</sup> and FANc<sup>+/+</sup> mice (C57Bl/6 × SV129) were backcrossed 10 generations into a C57Bl/6 strain (CD45.2<sup>+</sup>). Congenic C57Bl/6 strain (CD45.2<sup>+</sup>) and B6.SJL-Ptprc<sup>p<sup>3<sup>b</sup></sup>Boya<sup>j</sup></sup> (Boya<sup>j</sup>) mice (CD45.1<sup>+</sup>) were originally purchased from The Jackson Laboratory (Bar Harbor, ME) and are maintained in our animal facility. All studies were approved by the institutional animal care and use committee of Indiana University.

Foamyviral vectors and virus production

The foamyviral constructs used in our studies were derivatives of the MD9 construct, a kind gift of Axel Rethwilm (Würzburg, Germany). In the MD9 vector, all foamyviral genes and also the enhancer elements in the 3' U3 region (Figure 1) have been functionally inactivated by partial deletions. The remaining noncoding 5' region of GAG and the 3' region of POL are harboring the packaging signals (CAS I and II) and are therefore essential for the production of recombinant foamyviral particles. A linker was cloned into the NorI site 3' of MD9 and then an expression cassette containing the encephalomyelocarditis virus (EMCV) internal ribosomal entry site (IRES) and the enhanced green fluorescence protein (EGFP) cDNAs introduced from S11IEG3 via BamHI and SpeI, thereby creating the MD9-EGFP construct. The human FANCC cDNA was cloned into the BamHI site resulting in the MD9-FANCC/EGFP vector.

FV-containing supernatant was generated in 293T cells with the FV helper plasmids pcp1 and the FV envelope plasmid EM02 as previously described. The titers of the viral supernatant were 1 to 5 × 10<sup>7</sup> viral particles/mL for MD9-EGFP and 4 to 10 × 10<sup>6</sup> viral particles/mL for MD9-FANCC/EGFP construct after concentration.

Foamyviral–mediated BM transduction and transplantation

BM cells were obtained from FANc<sup>−/−</sup> or FANc<sup>+/+</sup> wild-type (WT) mice and purified for c-kit<sup>+</sup>/CD117<sup>+</sup> cells, as described previously. CD117<sup>+</sup> FANc<sup>−/−</sup> cells were transduced for 14 hours with the foamyviral vectors (MOI 20) on non–tissue culture–treated plates treated with the recombinant human fibronectin (100 ng/mL; both from Peprotech, Rocky Hill, NJ). WT cells were transduced only. Cells were harvested following transduction and washed with the recombinant human fibronectin (100 ng/mL; Novus Biologicals, Littleton, CO) that recognizes the human FANCC and the EGFP cDNAs.

For detection of FV integration sites, BM MNCs from 2 primary recipients of 2 viral vectors were plated in triplicate 35-mm plates (Becton Dickinson, Franklin Lakes, NJ) with increasing concentration of MMC or tumor necrosis factor alpha (TNF-α) as described. To determine the phenotype correction or the gene transfer efficiency, the number of total colonies formed per plate was enumerated and the EGFP-expressing colonies were counted by the fluorescent microscopy.

Amplification of genomic and proviral DNA

Colonies of FV-transduced cells plated in progenitor assays were individually collected and suspended in PBS. The genomic DNA was isolated and polymerase chain reaction (PCR) for EGFP was performed: forward 5'-ATGGTGACAGGCGGAGGAGG-3', reverse 5'-AAGTGCGTCT-GCTTCTATGTG-3', with the following program: 95°C, 5 minutes; 95°C, 40 seconds; 55°C, 30 seconds; 72°C, 1 minute; cycled to step 2 for 31 cycles; 72°C, 10 minutes and then stored at 4°C and analyzed on a 1% agarose gel. The amplified product has a size of approximately 250 bp. In addition, PCR for the genotype of the progenitor cells was performed. Three primers were used: 5'-GAGCGAACAAATGGAATGG-3', 5'-CCTGCGCGTCCCAGAATGG-3', and 5'-TTGATGAGAATGGATGGGACG-3' with the following program: 95°C, 5 minutes; 95°C, 30 seconds; 55°C, 2 minutes; 72°C, 1.5 minutes; cycled to step 2 for 31 cycles; 72°C, 10 minutes, and then stored at 4°C and analyzed on a 1% agarose gel. The amplified product of the WT copy of the FANc gene is approximately 800 bp, whereas the knockout gene PCR product is approximately 600 bp.

Ligation-mediated polymerase chain reaction

For detection of FV integration sites, BM MNCs from 2 primary recipients of FANc<sup>−/−</sup> mice transduced with MD9-FANCC/EGFP from the first cohort and 2 primary recipients from the second cohort were enriched for CD45.2<sup>+</sup> cells by fluorescence-activated cell sorting (FACS) and then plated in standard progenitor assay. From each of the 4 mice, 20 progenitor colonies were picked and then

From www.bloodjournal.org by guest on April 9, 2017. For personal use only.
subjected to ligation-mediated (LM)–PCR as described previously\(^4\) with minor variations. The restriction enzyme was \textit{HaeIII} (New England Biolabs, Frankfurt, Germany). The biotinylated primer \textit{5’-biotin-GTACATCTAGGAC-CACCTTC-3’} (407) was used in a one-step extension at 94°C, 15 minutes; 58°C, 2 minutes; 72°C, 10 minutes; 2 cycles. The 2 internal primers for the nested PCR were \textit{5’TCTCATCAGGATCGTCATATGA-3’} (404) and AP2 as previously described.\(^5\) The DNA from excised bands was cloned into pCR2.1 using the TOPO cloning kit (Invitrogen, Frederick, MD) and then sequenced on an ABI Gene Amp 3770 System (Applied Biosystems, Foster City, CA). As described previously,\(^6\) SeqMap (http://seqmap.compbio.iupui.edu, Indiana University School of Medicine) was used to map the sequences against the mouse genome. This was then confirmed by mapping the positions using the Ensembl website (http://www.ensembl.org) and mouse musculus database release 42, December 2006,\(^7\) and the UCSC Genome Browser (http://genome.ucsc.edu).\(^8\)

**Southern blot for junctional fragment analysis**

Genomic DNA from BM and spleen specimens was isolated using phenol-chloroform extraction and digested with \textit{XhoI} (New England Biolabs, Ipswich, MA). Fragments were isolated via ethanol precipitation and run on a 1% agarose gel. The DNA was transferred to a nylon membrane using the TurboBlotter system (Schleicher & Schuell, Keene, NH). To generate the hybridization probe, the MD9 plasmid was digested with \textit{PstI} (New England Biolabs, Ipswich, MA) and the 1655-bp fragment was isolated using QAQuick gel extraction kit (QIAGEN, Valencia, CA), labeled using the Prime-It II Random Primer labeling kit (Stratagene, La Jolla, CA) and purified using a microspin 30 column (Bio-Rad, Hercules, CA). The membrane was prehybridized for 2 hours at 42°C with the hybridization solution (6 × SSC, 50% formamide, 5 × Denhardt, 0.5% SDS in water) supplemented with 100 μg/mL denatured salmon sperm DNA (Stratagene). After prehybridization, the membrane was hybridized for 16 hours at 42°C with the hybridization solution supplemented with 100 μg/mL denatured salmon sperm DNA (Stratagene) and denatured labeled probe. The next day, the membrane was washed 4 times for 15 minutes at 42°C with the wash solution (2 × SSC, 0.1% SDS in water) and exposed to film (BioMax MS film; Kodak, Rochester, NY) at −80°C with a Cronex Lightning Plus intensifying screen (DuPont, Wilmington, DE).

**Microscopy**

Colony pictures (Figure 2A,B) were taken on a Zeiss Axiovert 25 (Zeiss, Ontario, NY) inverted microscope with an Epiplan 5×/0.13 numeric aperture (NA) objective using an X-cite 120 lamp (EXFO, Mississauga, ON). Images were captured using a SPOT RT Color camera, model 2.2.1 (Diagnostic Instruments, McHenry, IL) and edited using SPOT advanced software version 4.1.2 (Diagnostic Instruments).

The remaining pictures (Figure 5B) were taken on a Zeiss Axioscope (Zeiss) with the Plan Neofluar 40×/1.30 NA oil objective (Zeiss). Images were captured using a SPOT RT Color camera, model 2.2.1 (Diagnostic Instruments) and edited using SPOT Advanced software version 4.1.2 (Diagnostic Instruments). Slides were stained with hematoxylin and eosin (H&E) stain.

**Results**

**Efficient transduction of hematopoietic progenitors from \textit{Fancc}−/− mice by short-term exposure to FV vectors in the absence of prestimulation**

The recombinant MD9 FV vector\(^2\) was used (Figure 1) to express the human \textit{FANCC} and the \textit{EGFP} cDNA linked via an EMCV IRES element. This expression cassette was under the transcriptional control of the spleen focus-forming virus (SFFV) promoter element, which had been sufficient to mediate expression of transgenes in NOD/SCID repopulating human CD34+ umbilical cord blood cells.\(^9\) An MD9 vector that expresses the \textit{EGFP} transgene only was used as a control (Figure 1).

CD117+ cells from WT and \textit{Fancc}−/− mice were transduced with foamy viral vectors in the presence of growth factors and viral supernatants on CH296-coated plates for 14 hours. Recovery of CD117+ cells after the overnight transduction protocol was comparable in all experimental groups and was consistently 70% to 80% of input cells (data not shown). The next day, cells were cultured in semisolid medium to determine the gene transfer efficiency into clonogenic myeloid progenitors. After 7 days, the proportion of EGFP-positive myeloid progenitors was scored (Figure 2). Collectively, short-term exposure of both WT and \textit{Fancc}−/− CD117+ cells to FV supernatants on recombinant fibronectin resulted in a gene transfer efficiency of greater than 50% of clonogenic progeny based on EGFP expression in 4 independent experiments.

**Foamy virus-mediated expression of \textit{FANCC} corrects the DNA damage and inflammatory cytokine hypersensitivity of \textit{Fancc}−/− myeloid progenitors**

Since FANCC is a component of the FA core nuclear complex and is required for FANCD2 monoubiquitination in response to DNA damage and during S-phase,\(^9,\) the detection of monoubiquitinated FANCD2 can be used as a measure of functional FANCC protein. Unaffected human cutaneous and \textit{Fancc}-deficient fibroblast lines were transduced and 48 hours later the cells were treated with 10 Gy ionizing radiation. Three hours subsequently, cells were harvested and protein extracts were isolated. As expected, the \textit{Fancc}-deficient fibroblasts transduced with the MD9-\textit{EGFP} reporter construct do not express the monoubiquitinated form of FANCD2 (Figure 3A). In contrast, \textit{Fancc}-deficient fibroblasts transduced with MD9-\textit{FANCC/EGFP} can restore the assembly of the FA nuclear complex, leading to efficient monoubiquitination of FANCD2 (Figure 3A). These data provide biochemical evidence indicating that the FV vector encoding human \textit{FANCC} expresses a functional recombinant protein that is sufficient to allow activation of the downstream effector FANCD2.

To determine whether the SFFV promoter–mediated expression of \textit{FANCC} was sufficient to phenotypically correct \textit{Fancc}−/− primitive clonogenic cells, CD117+ cells were transduced with MD9-\textit{FANCC/EGFP} or the reporter construct, and clonogenic assays of myeloid progenitors were established in the presence of a range of concentrations of MMC, a bifunctional alkylating agent, or the inhibitory cytokine TNF-α. Consistent with established work,\(^9,44\) \textit{Fancc}−/− progenitors...
transduced with the reporter construct were hypersensitive to both MMC and TNF-α. In contrast, progenitors transduced with the construct expressing FANCC were corrected to WT levels (Figure 3B,C). As an initial assessment of in vivo function of the expressed FANCC protein, transduced CD117+ cells were transplanted into lethally irradiated syngeneic recipients to allow long-term reconstitution of the hematopoietic system by genetically modified stem cells. Six months after transplantation, BM low-density MNCs from the reconstituted mice were isolated and clonogenic cells from the respective experimental groups were analyzed in triplicate cultures (WT-MD9-EGFP, n = 3; Fancc−/−-MD9-EGFP, n = 3; Fancc−/−-MD9-FANCC/EGFP, n = 5). Data reflect the mean and standard error of the mean (SEM) of all recipients examined in each experimental group. *P < .05 comparing Fancc−/−-EGFP to the other experimental groups.

Competitive repopulation is an established quantitative measure of stem cell repopulating activity that allows a direct comparison of the proliferative activity of reconstituted stem cells of different genotypes via their relative proliferation of myeloid and lymphoid lineages to a common pool of competitor cells. Since murine Fancc−/− BM cells have reduced repopulating ability compared with WT cells, a prevalent phenotype in FA patients is BM failure, we used this methodology to assess the potential of the foamyviral vector MD9-FANCC/EGFP to correct the repopulating ability of Fancc−/− stem cells to that of syngeneic WT stem cells. CD117+ WT and Fancc−/− BM cells were isolated, transduced with either the MD9-FANCC/EGFP or the control vector, and then cotransplanted with a common pool of CD45.1+ competitors into lethally irradiated recipients using previously established methods. As shown in Figure 4A, the test cell chimerism of individual recipients from 1 of 2 independent experiments with 6 to 8 primary recipients per experimental group was followed on serial measurements over the course of one year. Consistent with previous results, recipients reconstituted with mock-transduced Fancc−/− cells have a reduced repopulating ability compared with recipients reconstituted with WT cells. In contrast, mice reconstituted with Fancc−/− cells expressing the FANCC cDNA after foamyviral gene
To confirm that the correction in repopulating ability is associated with transduction of the transgene, it would have been ideal to evaluate expression of the EGFP transgene directly in CD45.2+ cells. Unfortunately, we found that EGFP expression was down-modulated in the bicistronic vector containing both EGFP and FANCC, consistent with previous work by us and others.46,56 Therefore, to confirm that the CD45.2+ test cells were of the predicted genotype and contained the transgene, CD45.2+ BM cells were sorted by FACS and plated in standard progenitor assays. DNA from individual progenitors was isolated and amplified to assess the genotype and the presence of the transgene. The PCR analysis of 70 colonies from 7 recipients containing Fancc-/- MD9-FANCC/EGFP test cells demonstrated that 67 (96%) of 70 colonies were positive for the Fancc-/- genotype. A representative analysis is shown in Figure 4C. A high proportion of those same Fancc-/- test progenitors (90%) also contained the provirus. A representative blot demonstrating this is shown in Figure 4D. The majority of progenitors (25/29) analyzed from CD45.2+ bone marrow cells of 3 representative recipients that received a transplant of Fancc-/- cells transduced with MD9-EGFP also were Fancc-/-.

To assess that the transgene was transduced into a stem cell with repopulating ability as well as the long-term proliferative ability assessed in primary recipients, low-density MNCs from selected primary recipients were transplanted into lethally irradiated recipients. The chimerism 6 months following transplantation into secondary recipients did not change, and the test cell chimerism of mice reconstituted with corrected Fancc-/- cells remained comparable to that of recipients of transplanted WT cells (Figure 4B), indicating that the transgene was functional in repopulating stem cells. In that same experiment, recipients reconstituted with Fancc-/- cells transduced with the FANCC transgene had comparable numbers of “test” colony-forming units/femur (2.8 ± 0.1 × 10⁴) as WT recipients (2.4 ± 0.2 × 10⁴), whereas recipients reconstituted with Fancc-/- test cells encoding EGFP only had significantly reduced test progenitors (1.5 ± 0.3 × 10⁴).

Evaluation for evidence of myelodysplasia in mice reconstituted with Fancc-/- cells

We have previously found in 2 separate studies involving 5 cohorts of mice that in vitro culture of uncorrected Fancc-/- BM for 2 to 4 days prior to transplantation predisposes the recipients of those cells to MDS.2,33 A characteristic of the MDS phenotype observed in Fancc-/- mice is that myeloid progenitors are resistant to the apoptotic signals induced by TNF-α in progenitor assays.33 To determine whether this indicator of malignant transformation was

<table>
<thead>
<tr>
<th>Fancc genotype</th>
<th>Vector</th>
<th>No. of mice</th>
<th>Repopulating units</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/-</td>
<td>MD9-EGFP</td>
<td>7</td>
<td>6.11 ± 0.96</td>
</tr>
<tr>
<td>+/-</td>
<td>MD9-FANCC/EGFP</td>
<td>5</td>
<td>1.03 ± 0.11*</td>
</tr>
<tr>
<td>+/-</td>
<td>MD9-EGFP</td>
<td>8</td>
<td>7.01 ± 0.95</td>
</tr>
</tbody>
</table>

Quantities shown are mean plus or minus SEM.
*P < .05 compared with either WT MD9-EGFP or Fancc-/- MD9-FANCC/EGFP.
transduced mononuclear cells from Fancc–transduced found in primary and secondary recipients receiving MD9-EGFP ing the respective sensitivity to TNF-α/H9251.

EGFP progeny of the transduced Fancc were cultured. Analyses revealed that the Fancc−/− progenitors retained the characteristic hypersensitivity to TNF-α (Figure 5A). In addition, the BM and spleen of 7 primary recipients and 4 secondary recipients reconstituted with Fancc−/− cells expressing the EGFP transgene only were examined at postmortem and had normal architecture (Figure 5B). Similar histologic data were found in primary and secondary recipients receiving MD9-FANCC/EGFP–transduced Fancc−/− cells (data not shown). Collectively, all functional and histologic data fail to detect evidence of transformation of uncorrected Fancc−/− stem cells in primary or secondary recipients.

Evaluation of proviral integrants

We next wanted to assess the number of integrations that are present in progeny of the transduced Fancc−/− stem cells. BM low-density MNCs from 4 primary recipients were harvested at the time of secondary transplantations, sorted for CD45.2 expression, and then plated in methylcellulose assays. After one week, 20 colonies were picked for each mouse and subjected to standard LM-PCR analysis for identifying the location of the provirus in the genome. As described previously,65 the position of the transgene from each colony was evaluated using the SeqMap website (http://seqmap.compbio.iupui.edu) and confirmed using the http://genome.ucsc.edu and http://www.ensembl.org websites. From each mouse, one proviral integration was detected (Table 2). Each integration site detected was independent of the others and was contained either in an intron or outside of a gene, consistent with other studies of FV integration patterns.37,38,46,57,60 Because provectors progenitors provide limited amounts of DNA for LM-PCR analysis and proviral integrants may not always be detected, integrations were also evaluated from whole BM and spleen samples by Southern analysis in a replicate set of experiments using similar transduction conditions. Results by Southern blot revealed one junctional fragment per recipient for 9 of 12 mice and 2 junctional fragments per recipient for 3 of 12 mice evaluated. These data are summarized in Table 3.

Discussion

The only long-term cure for the BM failure in FA patients is transplantation of normal hematopoietic stem cells, ideally from an HLA-matched sibling.4,61-64 Allogeneic BM or cord blood transplantation is not without subsequent risk as the conditioning regimens cause genotoxic stress that predispose patients to an increased incidence of squamous cell carcinomas especially when compounded by the presence of chronic graft-versus-host disease.65,66 Thus, the transduction of autologous, genetically corrected stem cells in the absence of genotoxic myelopreparation could provide a therapy that does not expose the patient to these ongoing potential sequelae.67

Natural reversions of inherited germ-line mutations seen in a small population of FA patients strongly suggest that an oligoclonal population or perhaps a single hematopoietic stem cell is sufficient to correct the hematopoietic system.15-19 Unfortunately, long-term multipotential cell transduction leading to hematologic improvement in the patient has not been observed with gammaretroviruses in FA clinical trials to date.7,24 One possible reason for this failure of genetic therapy is the low number of stem cell targets available for gene transfer.68 In addition, even in the presence of saturating concentrations of growth factors, FA cells have an increased propensity to undergo apoptosis.33 Therefore, the prolonged culture required to induce stem cells into cycle and allow gammaviruses to integrate into the target cell genome predisposes untransduced FA stem cells to undergo apoptosis.33

In studies here, we found that foamy virus vectors expressing the human FANCC cDNA can functionally correct multiple defects in Fancc−/− stem cells capable of repopulating primary and secondary recipients. Due to their specific biology, the use of FV vectors can theoretically address several problems associated with somatic stem cell gene therapy in FA. First, long-term serial evaluation of a cohort of patients strongly suggest that an oligoclonal population or perhaps a single hematopoietic stem cell is sufficient to correct the hematopoietic system.15-19

Table 2. Location of observed proviral integration sites on the mouse genome

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Chromosome</th>
<th>Location</th>
<th>Gene ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>91 (cohort 1)</td>
<td>16B4</td>
<td>Upstream of Zbtb20 and Ndufs5</td>
<td>NM 019778andBC002163</td>
</tr>
<tr>
<td>93 (cohort 1)</td>
<td>5E3</td>
<td>No nearby gene</td>
<td>NM 098063</td>
</tr>
<tr>
<td>1 (cohort 2)</td>
<td>10B3</td>
<td>Intron 2 of Pikb</td>
<td>NM 098063</td>
</tr>
<tr>
<td>2 (cohort 2)</td>
<td>13A3.1</td>
<td>No nearby gene</td>
<td>NM 098063</td>
</tr>
</tbody>
</table>

Table 3. Proviral integration number in the mouse genome

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Transgene</th>
<th>No. of mice</th>
<th>No. of mice with 1 integrant</th>
<th>No. of mice with 2 integrants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fancc−/−</td>
<td>FANCC/EGFP</td>
<td>6</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Fancc−/−</td>
<td>EGFP</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>WT</td>
<td>EGFP</td>
<td>3</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>
target cell genome is needed for the therapeutic effect. Second, in
contrast to gammaretroviruses that have a half-life of only 4 to 6 hours in
vivo, nonintegrated FV virions retain the potential for stable integra-
tion in quiescent cells for up to 30 days following transduction. Thus,
cells need to be exposed in vitro to virions for only a short time, allowing
cycling of the target cells and proviral integration to occur in vivo. Since
FA murine and human FA stem cells have increased in vitro time-
dependent induction of apoptosis and at least FANCE cells develop in
vitro dependent clonal transformation, this abbreviated transduction
protocol appears optimal for genetic correction of FA stem cells. Here,
we demonstrate that in vitro manipulation of FANCE/CD117 cells for 14
hours in the absence of prestimulation is sufficient for FV-mediated
delivery of FANCC to stem cells and correction of stem cell repopulating
activity in primary and secondary transplantations. In preliminary
studies, we have now found that efficient transduction can be observed
following in vitro culture in as few as 8 hours (data not shown).

We observed 1 to 2 proviral integrants in the repopulating cells of
reconstituted mice. There are at least 3 possibilities for this observation.
It is possible that analogous to rare cases of apparent spontaneous
correction of FA stem cells, correction of a subpopulation of
FANCE cells with a functional transgene leads to an in vivo selection
of the corrected cells. Future studies mixing ratios of transduced and
untransduced cells into irradiated recipients may allow formal testing of
this hypothesis. Alternatively, previous studies have shown that hematopo-
iesis in reconstituted lethally irradiated mice tends to be oligoclonal in
nature. This correlates with our data, which show a low number of
integrants, regardless of the transgene or stem cell genotype. In contrast
to our studies in mice, a recent report using foamy viral vectors to treat
canine leukocyte adhesion deficiency (CLAD) found multiple integrants
in this large-animal model. A third possibility that we considered was that of clonal selection leading to MDS. To test for this possibility, we
examined the hematopoietic organs and evaluated clonogenic growth
from the myeloid progenitors of primary and secondary recipients over a
total of 18 months. From multiple recipients, no pathological abnormali-
ties were observed. The lack of pathological sequela is consistent with
the study in CLAD dogs where foamy viral vector integrants were found
within genes or near oncogenes at a much lower frequency as reported
for retroviral vectors.

A potential limitation to the use of FV vectors in the clinic to date is
that the fusogenic capacities of FV envelope proteins and packaging
proteins in the target cells.

In vivo, nonintegrated FV virions retain the potential for stable integra-
tion in quiescent cells for up to 30 days following transduction. Thus,
cells need to be exposed in vitro to virions for only a short time, allowing
cycling of the target cells and proviral integration to occur in vivo. Since
FA murine and human FA stem cells have increased in vitro time-
dependent induction of apoptosis and at least FANCE cells develop in
vitro dependent clonal transformation, this abbreviated transduction
protocol appears optimal for genetic correction of FA stem cells. Here,
we demonstrate that in vitro manipulation of FANCE/CD117 cells for 14
hours in the absence of prestimulation is sufficient for FV-mediated
delivery of FANCC to stem cells and correction of stem cell repopulating
activity in primary and secondary transplantations. In preliminary
studies, we have now found that efficient transduction can be observed
following in vitro culture in as few as 8 hours (data not shown).

We observed 1 to 2 proviral integrants in the repopulating cells of
reconstituted mice. There are at least 3 possibilities for this observation.
It is possible that analogous to rare cases of apparent spontaneous
correction of FA stem cells, correction of a subpopulation of
FANCE cells with a functional transgene leads to an in vivo selection
of the corrected cells. Future studies mixing ratios of transduced and
untransduced cells into irradiated recipients may allow formal testing of
this hypothesis. Alternatively, previous studies have shown that hematopo-
iesis in reconstituted lethally irradiated mice tends to be oligoclonal in
nature. This correlates with our data, which show a low number of
integrants, regardless of the transgene or stem cell genotype. In contrast
to our studies in mice, a recent report using foamy viral vectors to treat
canine leukocyte adhesion deficiency (CLAD) found multiple integrants
in this large-animal model. A third possibility that we considered was that of clonal selection leading to MDS. To test for this possibility, we
examined the hematopoietic organs and evaluated clonogenic growth
from the myeloid progenitors of primary and secondary recipients over a
total of 18 months. From multiple recipients, no pathological abnormali-
ties were observed. The lack of pathological sequela is consistent with
the study in CLAD dogs where foamy viral vector integrants were found
within genes or near oncogenes at a much lower frequency as reported
for retroviral vectors.

A potential limitation to the use of FV vectors in the clinic to date is
that the fusogenic capacities of FV envelope proteins and packaging
proteins in the target cells.


Overnight transduction with foamyviral vectors restores the long-term repopulating activity of $\text{Fancc}^{-/-}$ stem cells

Yue Si, Anna C. Pulliam, Yvonne Linka, Samantha Ciccone, Cordula Leurs, Jin Yuan, Olaf Eckermann, Stefan Fruehauf, Sean Mooney, Helmut Hanenberg and D. Wade Clapp