Gene therapy of Fanconi anemia: preclinical efficacy using lentiviral vectors

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Fanconi anemia (FA) is an inherited cancer susceptibility syndrome caused by mutations in a DNA repair pathway involving at least 6 genes (FANCA, FANCC, FANCD2, FANCE, FANCF, and FANCG). The clinical course of the disease is dominated by progressive, life-threatening bone marrow failure and high incidence of acute myelogenous leukemia and solid tumors. Allogeneic bone marrow transplantation (BMT) is a therapeutic option but requires HLA-matched donors. Gene therapy holds great promise for FA, but previous attempts to use retroviral vectors in humans have proven ineffective given the impaired proliferation potential of human FA hematopoietic progenitors (HPCs). In this work, we show that using lentiviral vectors efficient genetic correction can be achieved in quiescent hematopoietic progenitors from Fanca−/− and Fancc−/− mice. Long-term repopulating HPCs were transduced by a single exposure of unfractionated bone marrow mononuclear cells to lentivectors carrying the normal gene. Notably, no cell purification or cytokine prestimulation was necessary. Resistance to DNA-damaging agents was fully restored by lentiviral transduction, allowing for in vivo selection of the corrected cells with nonablative doses of cyclophosphamide. This study strongly supports the use of lentiviral vectors for FA gene therapy in humans. (Blood. 2002;100:2732-2736)

Introduction

Fanconi anemia (FA) is a rare autosomal recessive disorder caused by mutations in a DNA repair pathway that involves at least 7 genes (FANCA, FANCB/D1, FANCC, FANCD2, FANCE, FANCF, and FANCG).1-8,31 Fanconi anemia type A and type C combined account for approximately 80% of all FA cases.5 Clinical features of FA are considerably heterogeneous and include congenital abnormalities of the skeleton and other organs (kidney, heart), life-threatening bone marrow failure, and increased cancer susceptibility. Life expectancy is on average reduced to 20 years, with patients with serious illness dying before they reach adulthood and patients with mild forms surviving until the fifth decade of life. The clinical presentation is usually dominated by hematological deficiencies derived from bone marrow failure, which can progressively lead to profound aplastic anemia. If the supportive treatment for the anemia is successful, patients are at high risk for acute myelogenous leukemias and solid tumors.9-11 Allogeneic bone marrow transplantation (BMT) is a therapeutic option but requires HLA-matched donors.12

Gene therapy holds great promise for patients with FA. In spite of successful retroviral-mediated genetic correction of FA knockout (KO) mouse hematopoietic progenitors,13 attempts to use retroviral vectors in patients have proven to be ineffective. This difference most likely results from the impaired proliferation potential specific to human FA hematopoietic progenitors (HPCs).14 It must be noted, in fact, that the phenotype of FA KO mice differs from the clinical syndrome. Their hematopoiesis is virtually normal under stationary conditions and becomes impaired only under extreme proliferative stress.15,16 Given that ex vivo expansion is not an option for FA gene therapy in humans, transplantation protocols in mice are relevant for clinical application only if modeled while taking into account the differences between KO mice and FA patients.

High-efficiency gene delivery into nondividing hematopoietic progenitors is a major requirement for FA gene therapy in humans because bone marrow cells from FA patients, unlike those from mouse KO cells, are fragile, grow poorly in vitro, and are therefore refractory to transduction by retroviral vectors,14 which can transduce only dividing cells. On the other hand, lentiviral vectors can transduce early hematopoietic progenitors capable of in vivo engraftment, and efficient transduction occurs in the absence of cytokines and other growth factors.17

In this work, we show that using lentiviral vectors, efficient genetic correction can be achieved in hematopoietic progenitors from Fanca−/− and Fancc−/− mice15,18 using a transduction protocol that does not include ex vivo progenitor expansion. Long-term repopulating HPCs were transduced by a single exposure of unfractionated bone marrow mononuclear cells to lentivectors carrying the normal gene. Notably, no cell purification or cytokine prestimulation was necessary. Resistance to DNA-damaging agents was fully restored by lentiviral transduction, allowing for in vivo selection of the corrected cells with nonablative doses of cyclophosphamide. This study strongly supports the use of lentiviral vectors for FA gene therapy in humans.

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Materials and methods

Animals

_Fanca<sup>−/−</sup>_ and _Fancc<sup>−/−</sup>_ mice (129SvJ) were bred for 8 generations to obtain a syngeneic background. Genotyping of the mice was performed using 3-primer polymerase chain reaction (PCR), as described previously.16,18 All mice used in this study were 6 to 12 weeks old and were housed in the Department of Comparative Medicine at Oregon Health Sciences University. All experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee.

Cells, culture media, and reagents

The lymphoblastoid cell lines used in this study were obtained from Dr G. Bagby (HSC536), Dr M. Buchwald (HSC99), and the NIA Aging Cell Culture Repository (AG09391). Mitomycin c (MMC) was from Sigma (St Louis, MO). Media for liquid culture and colony-forming assays were from Stem Cell Technologies (Vancouver, BC, Canada).

Lentiviral vectors

Yamada et al19 described a first-generation HIV-derived lentiviral vector carrying an expression cassette for the FANCC gene. In our work, we chose a self-inactivating vector design and a third-generation, 4-plasmid packaging system.20 Plasmids were cotransfected in 293T cells, and the medium was collected after 48 hours and concentrated by ultracentrifugation at 19 400 rpm in a Beckman SW55 rotor. The virus pellet was resuspended in Hanks balanced salt solution (HBSS), and the content of p24 was determined by enzyme-linked immunosorbent assay (ELISA; NEN, Perkin Elmer Life Sciences, Boston, MA). Vector batches were tested for the absence of replication-competent virus by monitoring p24 antigen expression in the culture medium of transduced SupT1 lymphocytes for at least 3 weeks.

Lentiviral transduction of _Fancc<sup>−/−</sup>_ and _Fancc<sup>−/−</sup>_ bone marrow

Bone marrow cells were harvested from the femora of donor _Fanca<sup>−/−</sup>_ or _Fancc<sup>−/−</sup>_ mice in RPMI supplemented with penicillin/streptomycin. Red cells were depleted in ACK lysis buffer (Bio-Fluids, Rockville, MD). Bone marrow was reconstituted in FANCA or FANCC lentiviral supernatant at 37°C for 24 hours, at a multiplicity of infection (MOI) between 10 and 100.

Bone marrow transplantation

After lentiviral transduction, BM cells were washed and transplanted in _Fanca<sup>−/−</sup>_ or _Fancc<sup>−/−</sup>_ recipients (n = 6 per group). Each animal received 1 × 10<sup>6</sup> cells through the retro-orbital plexus, without any preconditioning. One week after transplantation, they were given a single dose of 40 mg/kg cyclophosphamide (CPA) intraperitoneally. Mice that underwent transplantation were bled every 4 weeks for analysis of the integrated vector. The same protocol was used for secondary transplantation of bone marrow harvested from reconstituted primary recipients.

Semiquantitative PCR for the detection of the FANCA/FANCC provirus

The proviral copy number in peripheral blood DNA of lenti-FANCC animals that underwent transplantation was measured as described21 using a 4-primer PCR, including one primer pair specific for the proviral DNA (human FANCC cDNA) and a second pair specific for an unrelated autosomal locus (murine Fanca gene). PCR products were analyzed by Southern blotting.22 Hybridization signals were visualized using a Molecular Dynamics PhosphorImager SI (Molecular Dynamics, Sunnyvale, CA) and quantitated using the software IPLab Gel (Signal Analytics, Vienna, VA).

Semiquantification of the FANCA provirus was performed similarly to that of the FANCC provirus. Briefly, murine ST3 cells transduced or nontransduced with the FANCA lentivirus were used as the standards for the determination of the copy number of the provirus from the peripheral blood of mice that underwent transplantation with lentiviral-transduced BM. Southern blot analysis verified that the transduced cell line used as the standard contained only a single copy of the FANCA provirus, and a standard curve was generated as described previously.23 Primers specific for human FANCA were 5′-GCAGTTTGGCCAGC-GATTCC-3′ and 5′-CTTCTTGATGGTTTCTTCTTGTA-3′, resulting in a 120-bp product. Primers 5′-TTTCCTCATTCAACTGCTG-3′ and 5′-CAGGTACATCCCTCTCGATACCC-3′ were designed to amplify a 942-bp region of the _fanca_ gene as an internal control; 5′-TTTGCATACCAAAAGTT- CAGGG-3′ was used as probe for the lenti-FANCA provirus, and 5′-AGACACACTACCTTGATGATTCT-3′ was used as a probe for _Fanca_. The percentage contribution of each allele was determined as explained elsewhere.21

Twelve-day spleen colony-forming unit assay

Forty thousand unfractionated BM cells from the animals reconstituted with lenti-FANCA–transduced HSCs were transplanted into lethally irradiated wild-type recipients killed on day 12 after transplantation. Genomic DNA was prepared from spleen colonies,22 and 4-primer PCR was performed as described above.

Southern blot analysis was performed on each colony to determine the proviral copy number. For FANCC, 5 μg DNA from each spleen colony-forming unit (CFU-S) DNA was Spel-digested and probed with a NotI-Xhol fragment (596 bp) from the human FANCC cDNA. Southern blot analysis for _Fanca_ provirus was performed in the same way, but a Xhol-Xhol fragment (2100 bp) from the human FANCA cDNA was used as a probe.

FANCA and FANCC protein expression

FANCA and FANCC protein expression were analyzed in the BM of the recipients of _Fanca/Fancc_ lentiviral-transduced bone marrow by Western immunoblotting, using anti-FANCA and anti-FANCC polyclonal antisera, kindly provided by Drs Alan D’Andrea and Grover Bagby.

Hematological protection against MMC toxicity

Untreated _Fanca<sup>−/−</sup>_ and _Fancc<sup>−/−</sup>_ mice (n = 6 each), control wild-type mice (n = 6), and secondary recipients of _Fancc_ or _Fanca_ lentiviral-transduced bone marrow (n = 6 each) were exposed to MMC (0.3 mg/kg body weight, single intraperitoneal injection per week for 6 weeks). Peripheral blood was collected before MMC treatment and every 2 weeks thereafter. Samples were analyzed at Antech Diagnostics (Portland, OR) on an ABC Vet automated blood counter.

Results

Lentivector design and characterization

To explore the potential of lentiviral vectors in the management of FA, we designed third-generation,22 self-inactivating,22 HIV-derived lentiviral vectors carrying expression cassettes for the _FANCA_ and _FANCC_ genes, respectively, under the control of the cytomegalovirus promoter/enhancer (Figure 1A). High-titer recombinant lentiviruses were generated by transient transfection and concentrated by ultracentrifugation (more than 10<sup>9</sup> transduction units per milliliter). Biological activity of the proteins encoded by the lenti-FANCA and lenti-FANCC vectors was first assessed by their ability to phenotypically correct human Epstein-Barr virus–immortalized lymphoblastoid cell lines derived from FA patients. These cells undergo growth arrest, chromosomal abnormalities, and cell death when cultured in the presence of DNA–cross-linking agents such as MMC or diepoxybutane.24,25 HSC99 (FANCA<sup>−/−</sup>), kindly provided by Dr Buchwald) and HSC536 (FANCC<sup>−/−</sup>)<sup>26</sup> cell lines were transduced with the appropriate lenti-FA or a control lenti-GFP (green fluorescent protein) vector at an MOI between 10 and 50 and were subsequently grown in the presence of increasing concentrations of MMC. The number of viable cells was assessed after 5 days by flow cytometry analysis with propidium iodide exclusion. Corrected cells showed a fully restored resistance to DNA damage, with an increase of the LD<sub>50</sub> for MMC of approximately 20-fold compared with controls (Figure 1B-C). Lentiviral vectors also reverted cell cycle abnormalities...
in the presence of MMC, as shown by the sharp decrease in late S/G2-arrested cells after growth in 100 nM MMC (Figure 1D).

Correction of committed hematopoietic progenitors from Fanca-/- and Fancc-/- knockout mice

We next tested the lenti-FANCA and the lenti-FANCC vectors for their ability to correct the genetic defect of committed colony-forming precursor cells (CFCs) in Fanca-/-18 and Fancc-/-15 mice. Bone marrow mononuclear cells obtained from mice were transduced with lenti-FANCA or lenti-FANCC, respectively, at an MOI between 10 and 100. Cells were then assayed for their ability to form colonies in semisolid media in the presence of MMC. Under these conditions, the colony growth from bone marrow of Fanca-/- and Fancc-/- mice is strongly impaired, mimicking the deficient hematopoiesis of patients with FA. Genetic correction by lentiviral vectors resulted in a significant increase in CFC growth in MMC (Figure 2A).

Ex vivo correction of stem cells from Fanca-/- and Fancc-/- mice: primary and secondary transplantation

We previously showed that the genetic defect in FA is at the stem cell level.16 To demonstrate lentiviral-mediated genetic correction of hematopoietic progenitors capable of in vivo engraftment, we carried out transplantation experiments. Bone marrow mononuclear cells from Fanca-/- or Fancc-/- mice were infected ex vivo with lentivectors, in the absence of any growth factor or cytokine stimulation. Recipient mice (n = 6 for each mutant strain) underwent transplantation with 1 × 10^6 lentiviral-transduced cells, without any preconditioning, and received a single dose of 40 mg/kg cyclophosphamide (CPA) 1 week after transplantation for in vivo selection of the corrected cells.21 We then used semiquantitative PCR23 to measure the proviral copy number in peripheral blood

![Figure 1. FA lentiviral vectors.](image1.png)

![Figure 2. Lentiviral correction of KO mouse hematopoietic tissue.](image2.png)
DNA over time (Table 1). Before CPA selection, the proviral signal could not be detected in peripheral blood DNA of either the Fanca<sup>−/−</sup> or the Fancc<sup>−/−</sup> recipients, indicating less than 0.05 proviral copies/genome. Four months after CPA administration, the proviral copy number in Fancc<sup>−/−</sup> recipients had increased to 1.10 ± 0.24 copies/genome and persisted for the duration of the experiment (1 year after transplantation). The proviral copy number in Fanca<sup>−/−</sup> recipients ranged between 0.4 and 0.8 copies/genome (Table 1) and was sustained throughout the experiment. The relatively worse performance of the lenti-FANCA virus was possibly attributed to the size of the expression cassette, which may lower packaging efficiency during vector production. These data show long-term engraftment by CPA-selected, lentiviral-transduced, hematopoietic progenitors. Expression of the FANCA and FANCC proteins was detectable by Western blot on the bone marrow primary and secondary recipient animals (Figure 2B).

We also determined the proviral copy number in secondary recipients, 2 months after transplantation, using semiquantitative PCR. Consistent with selection at the HSC level, the proviral copy number remained stable after secondary transplantation (Table 2).

### Twelve-day spleen colony-forming unit analysis of corrected bone marrow

Bone marrow was harvested from primary recipient mice and was serially transplanted into lethally irradiated wild-type mice. We isolated genomic DNA from spleen colonies at day 12 and determined the presence of the provirus in each colony using PCR. Of the colonies examined, 49 of 50 (98%) were provirus positive in the Fanca experiment, and 37 of 50 (74%) were provirus positive in the Fancc experiment. Colonies that yielded a sufficient amount of genomic DNA were also analyzed by Southern blotting, showing that most had a single proviral integration site (Figure 3A).

### Hematological correction of lentiviral-corrected mice

To demonstrate that both the Fanca<sup>−/−</sup> and the Fancc<sup>−/−</sup> mice treated with ex vivo lentiviral gene therapy and in vivo CPA selection were protected from hematopoietic toxicity, we exposed untreated wild-type mice, untreated mutant controls, and wild-type mice to weekly doses of MMC (0.3 mg/kg). Platelet counts (PCs) were analyzed at various time-points (Figure 3B). Normal mice were resistant to MMC and did not develop thrombocytopenia, whereas Fanca<sup>−/−</sup> and Fancc<sup>−/−</sup> mice rapidly developed PC abnormalities, leading most (4 of 6) of them to death within 6 weeks. All mutant mice treated by lentiviral gene therapy and subsequent CPA selection were protected from the thrombocytopenia and death induced by MMC.

### Discussion

In many respects, Fanconi anemia is a suitable candidate disease for ex vivo gene therapy. First, the syndrome has a serious clinical outcome, and the only available therapeutic option is allogeneic bone marrow transplantation, associated with considerable morbidity and mortality. Second, corrected cells can have a selective advantage in vivo over the defective ones, as suggested by the high incidence of mosaicism in patients. Third, the deficiency in the Fanconi DNA repair pathway specifically affects the hematopoietic stem cell compartment. Although the exact mechanisms accounting for this tissue-specific damage are unclear, the condition provides a valuable model disease for gene transfer into hematopoietic stem cells.

Genetic correction of human FA progenitors has proven to be a formidable challenge. Clinical attempts performed so far have

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**Table 2. Proviral copy number in the peripheral blood of Fanca<sup>−/−</sup> and Fancc<sup>−/−</sup> mice undergoing secondary transplantation**

<table>
<thead>
<tr>
<th>Animal</th>
<th>Fanca&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th></th>
<th>Fancc&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<td>After BMT (2 mo)</td>
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Transplantation was performed using mice in Table 1 as the donors.

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**Figure 3. Lentiviral correction of early progenitors and phenotypical correction in FA KO mice.** (A) Southern blot analysis on CFU-S-derived colonies. Genomic DNA was obtained from spleen colonies digested with appropriate restriction enzymes and hybridized with probes recognizing the lenti-FANCA (left) or the lenti-FANCC (right) proviruses (see “Materials and methods”). All the colonies show a single band, indicating a single integration site. Colonies 1 to 11 were isolated from the same mouse and show a band of the same apparent molecular weight, thus suggesting a monoclonal origin. (B) Phenotypical correction in lentiviral-corrected Fanca<sup>−/−</sup> and Fancc<sup>−/−</sup> mice. Platelet counts were determined at 2-week intervals after the intraperitoneal (ip) administration of 0.3 mg/kg MMC. Lentiviral-corrected mice were protected against MMC toxicity for the duration of the experiment, but all knockout mice experienced severe hematological toxicity, leading most (4 of 6) of them to death within 6 weeks.
relied on oncoretroviral vectors, with overall disappointing results. The main reason for this can be found in the fragility of human FA hematopoietic progenitors, in their inability to efficiently grow in vitro, and in their consequent resistance to retroviral transduction. The protocols for ex vivo cytokine expansion of hematopoietic progenitors, refined over the years to improve the efficiency of retroviral vectors with sometimes excellent results, have failed on human FA tissue.

FA knockout mice provide a valuable model for investigating the pathophysiolo

Algorithms

The protocol of transduction we used in this work, including no cytokine prestimulation, low viral dose, and overall minimal ex vivo manipulation followed by in vivo cyclophosphamide selection of corrected stem cells, is particularly relevant for future clinical applications to humans. The next step toward the design of clinical trials for lentiviral FA gene therapy will be to evaluate the efficiency of lentiviral vectors on human primary hematopoietic progenitors obtained from FA patients. These studies are under way.

To date, lentiviral vectors have not been approved for clinical use; however, we believe clinical trials for the correction of the FA defect using lentiviral vectors will offer an excellent opportunity because transduction is performed ex vivo, therefore posing fewer safety concerns than with systemic delivery, and correction of FA will be an impetus to attempt a genetic cure of other hematological diseases such as sickle cell anemia, thalassemia, and many immunodeficiencies.

Note added in proof. The genes for both FANCB and FANCD1 have recently been shown to be BRCA2.

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