

Retroviral-mediated Transduction of the Fanconi Anemia C Complementing (FACC) Gene in Two Murine Transplantation Models

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ABSTRACT. Fanconi anemia (FA) is a well-known genetic syndrome manifested by bone marrow failure, variable physical anomalies, and cancer susceptibility. This disorder is marked by genotypic and phenotypic heterogeneity and consists of four distinct complementation groups A, B, C, and D. The defective gene responsible for the C group of FA, FACC, was identified by cDNA complementation cloning, and we have recently proposed a trial of gene therapy for group C FA. No animal model yet exists for FA. Consequently, we have studied the effects of constitutive expression of human FACC in two murine transplantation models. In the first model, we demonstrated transduction of FACC to reconstituting stem cells of mutant W/W^V mice. In the second model, we demonstrated transduction of FACC to hematopoietic cells transplanted to the bone marrows and spleens of non-myeloablated BALB/c mice. Our data suggest that retroviral-mediated transfer of the normal human FACC cDNA to hematopoietic progenitor and stem cells of mice is feasible and not associated with direct harmful effects to the hematopoietic organ.

Key words: Fanconi anemia, gene transduction, mouse bone marrow transplantation

INTRODUCTION

Fanconi anemia (FA) is a genetic syndrome diagnosed in patients by cellular sensitivity to DNA cross-linking agents and manifested by bone marrow failure, variable congenital abnormalities, and cancer susceptibility (1). FA is caused by defects in at least four different genes (A, B, C, D), and a cDNA for the C group (FACC) was recently identified (2,3). The most common mutation in FACC is an A-to-T mutation in the fourth intron that leads to an in-frame deletion of 38 amino acids (4). The DNA and protein sequence of FACC shows no structural motifs or homologies with proteins of known function (3). Consequently, the biochemical function of FACC is not entirely clear. The FACC polypeptide is primarily localized to the cytoplasm (5,6), suggesting an indirect role in regulating DNA damage. FACC messenger RNAs are expressed at low levels ubiquitously. In the developing mouse, FACC is expressed in bone, brain and soft tissues (7,8).

We recently documented the feasibility of *ex vivo* transfer of the FACC cDNA to CD34-enriched hematopoietic progenitor cells derived from FA(C) patients (9,10). Transfer and expression of the normal FACC cDNA improved the clonogenic growth of the mutant hematopoietic cells, suggesting that defective FA(C) hematopoiesis could be rescued by gene therapy. Although these and other of our experiments (11) suggested that our retroviral FACC vector was capable of infecting purified populations of hematopoietic progenitors, we were interested in determining whether it could transduce long-term reconstituting stem cells of animals and whether over expression of FACC had adverse effects on these normal animal hosts. We chose two distinct murine bone marrow transplantation models including the well-known W/W^V paradigm (12) as well as a model of serial cell transplantation into non-myeloablated BALB/c mice (13). The latter was chosen because our proposed human gene therapy trial (14) makes use of a repetitive autologous

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transplantation schedule without prior ablative chemotherapy. Testing gene transduction in animal models both present and absent selective pressure for engraftment was a primary objective.

MATERIALS AND METHODS

FACC vector and cell lines. The vector used in our studies, G1FASVNa, was previously described and is based on the G1xSVNa backbone (15). It contains a copy of the normal FACC cDNA obtained from the pFAC3 plasmid (3). The FACC gene is driven by the Moloney murine retrovirus long terminal repeat element.

We previously described the construction and isolation of an amphotropic producer clone (no. 52-19) of G1FASVNa. Briefly, an ecotropic producer line was established using GP+ E86 cells(16). Supernatant from one of the ecotropic producer clones was used to sequentially infect cells from an amphotropic producer line derived from PA317 cells(17). The no. 52-19 producer cell line was used for all experiments in this report.

Mice. Female WBB6F₁-W/W^V (recipients for long-term gene-transfer experiments), female C57BL/6J (donors for long-term experiments), and male or female BALB/c mice (non-ablated experiments) were purchased from The Jackson Laboratory (Bar Harbor, ME). All experiments with mice were performed according to protocols reviewed and approved by the Animal Care Use Committee of the National Institutes of Health. Mice were housed in individual filtered cages.

Infection and transplantation into W/W^V mice. Bone marrow cells from C57/BL6 mice were infected with recombinant virus containing the FACC gene (9). First, donor mice were treated with 150 mg/kg of 5-FU injected intravenously through the tail vein. Second, bone marrow cells were harvested; pre-stimulated for 48 hours with recombinant mouse interleukin-3 (IL-3, 50 units/ml, Biosource International, Camarillo, CA), mouse stem cell factor (SCF, 100 ng/ml, Biosource International), human interleukin-6 (IL-

6, 100 ng/ml, GIBCO BRL, Grand Island, NY); and co-cultured at a concentration of 5×10^5 /ml with producer cells. Polybrene was added at a final concentration of 6µg/ml to the transduction medium. After 48 hours of co-culturation, 2×10^6 infected bone marrow cells were injected into recipient W/W^V mice.

Hemoglobin electrophoresis. To confirm engraftment in W/W^V mice, hemoglobin electrophoresis was performed on peripheral blood of transplanted mice. Cystamine-treated samples were electrophoresed on Titan gel strips (Helena Laboratories, Beaumont, TX) (19).

Blood count determination. Automated erythrocyte and leukocyte blood counts were determined using a Coulter counter.

Infection and serial transplantation into BALB/c mice. Bone marrow cells from female BALB/c mice were infected by co-culturation as described above. Again, pre-stimulation for 48 hours was performed with IL-3, SCF, and IL-6. After 48 hours of co-culturation, $2-5 \times 10^6$ infected bone marrow cells were injected into recipient male BALB/c mice.

DNA analysis. Mouse peripheral blood was collected and expelled into cold 1x RBC lysing buffer, followed by incubation with cell lysis buffer (320 mM sucrose, 5 mM MgCl₂, 10 mM Tris, 1% Triton X) and nuclear dropping buffer (24 mM EDTA, 75 mM NaCl) After mixing, proteinase K (10 mg/ml) and 10% SDS were added to the samples. After incubation at 55°C for 4 hours, DNA was extracted by adding phenol/chloroform followed by chloroform. 5M NaCl and 100% EtOH were used to precipitate DNA. The DNA pellet was washed with 70% EtOH and resuspended in 1x TE and 10 mM NaCl. DNA was analyzed by polymerase chain amplification using primer pairs specific for the neomycin resistance gene: 5'-TCC ATC ATG GCT GAT GCA ATG CGG C-3' and 5'-GAT AGA AGG CGA TGC GCT GCG AAT CG-3'. The

percentage of blood mononuclear cells infected with the virus was assayed by making dilutions of DNA samples and comparing them to a known standard. The PCR conditions were as follows: denaturation at 95°C for 2min, step cycling at 95°C for 1min, 55°C for 1min, 72°C for 2min for 30 cycles, extension at 72°C for 8min, and soak at 4°C.

Mouse spleen, liver, bone marrow, and kidney were isolated and ground up using a mortar and pestle. The fine tissue samples were resuspended in 1.5ml LST(MgCl₂, NaCl, Tris)/VRC (vanadyl ribonucleoside complex)(10:1) on ice. The samples were further treated with 4x NPLB (NP40, sucrose, LST) to separate DNA from RNA. Tail buffer (5mM EDTA, 200mM NaCl, 100 mM Tris, 0.2% SDS) and proteinase K (2mcg/μl) were added, and the samples were incubated overnight at 55°C. The samples were treated with phenol/chloroform followed by 70% ETOH to extract and precipitate the DNA. The DNA pellets were resuspended in 1x TE. The DNA from the mouse tissues were analyzed by PCR methods described above.

DNA PCR for amphotropic envelope sequences. Primers used to identify amphotropic envelope sequences (20) include: 5'-GGA GTA GGG ATG GCA GAG AGC-3' and 5'-CTG TCT CCC TGC GGG GTA CTT-3'.

RNA analysis. Mouse peripheral blood was collected and expelled into RNA STAT-60 (Tel-Test B, Friendswood, TX). Chloroform extraction followed by isopropanol precipitation were used to isolate RNA. After washing RNA with 75% EtOH, the pellet was resuspended in DEPC-treated water. RT-PCR was used to analyze FACC gene expression using primer pairs which distinguish between the transduced human FACC transcript and the endogenous mouse FACC transcript (5'-AGA GCA CAG ACT ATG GTC CA-3' and 5'-TGC AGG AGC TCT GAG GTC TGT-3') as described in the RT PCR kit by Perkin-Elmer. The first round of PCR was as

follows: 42°C for 30min, 99°C for 5min, 4°C for 5min. The second round of PCR was the same as above in the DNA analysis.

After the mouse tissues were minced and treated with LST/VRC and 4x NPLB, the RNA in solution was separated from the DNA for each of the samples. ACE (NaOAc, EDTA)/SDS (10:1), phenol, and chloroform/isoamyl alcohol were added to the samples and further treated with NaOAc and 100% ETOH to precipitate the RNA. The RNA pellet was washed with 80% ETOH and resuspended in DEPC-treated water. The mouse tissue RNA was analyzed by the RT-PCR method as described above.

Histologic analysis of mouse tissues. Mouse tissues were dissected and removed for histologic analysis. Samples were fixed in a 10% formaldehyde/PBS solution. Tissue blocks were prepared and cut for hematoxylin and eosin staining (American Histolab, Gaithersburg, MD).

RESULTS

Experiments with W/W^v mice

Bone marrow cells from C57/BL6 mice were infected with the FACC vector as described in the Methods section. 1-2 x 10⁶ infected mononuclear cells were injected intravenously into eight female W/W^v recipient mice. Eight weeks after transplantation, we analyzed hemoglobin electrophoresis patterns from these transplanted mice. All mice analyzed showed the donor pattern of hemoglobin electrophoresis, indicating successful engraftment. These animals remained healthy, with no evidence of deleterious effect, until sacrifice at 6 months. Blood counts were evaluated and found to be normal. Representative values are shown in Table I. WBC counts did not differ between control and transplanted mice.

TABLE I

		Hematocrit	Coulter WBC	Manual WBC
Control	#1	34%	27,300*	80,000*
Control	#2	32%	12,300	2,500
Mouse	#1	46%	19,900	ND
	#2	45%	17,400	ND
	#3	48%	8,200	10,000
	#4	33%	9,700	8,500
	#5	52%	ND	ND
	#6	36%	8,200	6,500
	#7	43%	11,600	ND
	#8	48%	6,300	10,000

*Control #1 was found on histologic examination to be suffering from septicemia with bacterial emboli.

Representative hematocrits and WBC counts were obtained from control and test mice. WBC counts were determined by Coulter automated and manual quantitations. ND-not determined.

We performed DNA analyses on peripheral blood of all animals at approximately 3.5 months following transplantation. Using primer pairs specific for the neomycin resistance gene, we detected proviral DNA by PCR in the peripheral blood cells of all seven animals tested (Fig. 1). By using dilutions of peripheral blood DNA from marked animals with mouse DNA and comparing PCR signals to that generated from a transduced cell line, we estimated that most animals had between 10-30% of peripheral blood cells marked with the FACC retrovirus.

We then designed specific primer pairs to distinguish the transduced human FACC transcripts from the endogenous mouse FACC transcripts in RNA analyses. Blood samples obtained between 4.5 to 6 months following transplantation were analyzed for RNA expression by RT-PCR. As shown in Fig. 2, five of the seven evaluable mice (mice #4, 6, 1, 3, 7) showed detectable RNA at 4.5 to 6 months. RNA from the cell line PD4 served as a positive control for human FACC. These results confirmed transduction and gene expression in long-term reconstituted animals. As expected bone marrow mononuclear cells from the mice also showed detectable FACC expression at 6 months (Fig. 2).

One mouse (#5) was sacrificed at approximately 2 months following transplantation. Tissues which were marked with the FACC retrovirus included bone marrow and spleen. The

kidney and liver were negative by PCR (Fig. 1). The bone marrow, spleen, kidney, and liver were negative for amphotropic envelope gene sequences as determined by PCR (data not shown). The other mice were sacrificed at 6 to 6.5 months following transplantation. The following organs were harvested from all seven mice: spleen, thymus (if present), lymph node (if any), brain, bowel, kidney, lung, muscle, genitourinary tissue, heart. All tissues were analyzed for RNA expression by the RT-PCR method (with +/- reverse transcriptase controls for each tissue). All tissue RNAs were also analyzed for β -actin expression (with +/- reverse transcriptase controls for each tissue) to confirm integrity of RNA. Table II indicates tissues scored positive for FACC RNA from each mouse.

TABLE II

Mouse	#1	Lung, Liver, Spleen, Thymus
	#2	Lung, Spleen, Thymus
	#3	Spleen
	#4	Spleen
	#6	Lung, Liver, Spleen, Thymus
	#7	Lung, Liver, Spleen, Thymus, Kidney
	#8	Lung

Human FACC transcription was determined by the RT-PCR assay using primers specific for human sequences. Tissues positive for FACC mRNA are depicted. In all cases, β -actin-specific PCR primers amplifying endogenous β -actin sequences were used to check for integrity of samples.

Tissues from the mice were also reviewed by a veterinary pathologist. The bone marrows typically showed evidence of myeloid hyperplasia, and the spleens showed extramedullary hematopoiesis. However, there was no difference in the histology of bone marrow and spleen between transplanted and control (not transplanted) mice.

Experiments with non-ablated mice

Recent experiments have indicated high rates of engraftment of BALB/c marrow infused repetitively into female BALB/c hosts (13). Based in part on these studies, we tested transduction of FACC into seven unprepared host BALB/c

animals. A total of $2-5 \times 10^6$ transduced donor bone marrow cells were infused via tail vein injection once every two weeks, for four total injections, into recipient male BALB/c mice. All animals remained healthy until sacrifice at approximately three months following infusion. On two occasions, 2.5 and 3 months following infusion, peripheral blood was withdrawn by orbital puncture and tested for proviral DNA by PCR using the primer pairs described above. All peripheral blood DNAs were negative for proviral DNA at 30 and 35 cycles of PCR. Following sacrifice of test and control animals by cervical

dislocation, the bone marrow and spleen of the test and two control animals were dissected and analyzed by DNA PCR. As shown in Fig. 3, the majority of the marrow and spleen samples were marked by our FACC vector. Spleens from all test animals were compared with those from control animals by a veterinary pathologist.

Again, all spleens had evidence of extramedullary hematopoiesis, but there was no difference in histologies between transplanted and control mice. Because the peripheral blood was negative by PCR, non-hematopoietic tissues were not analyzed.

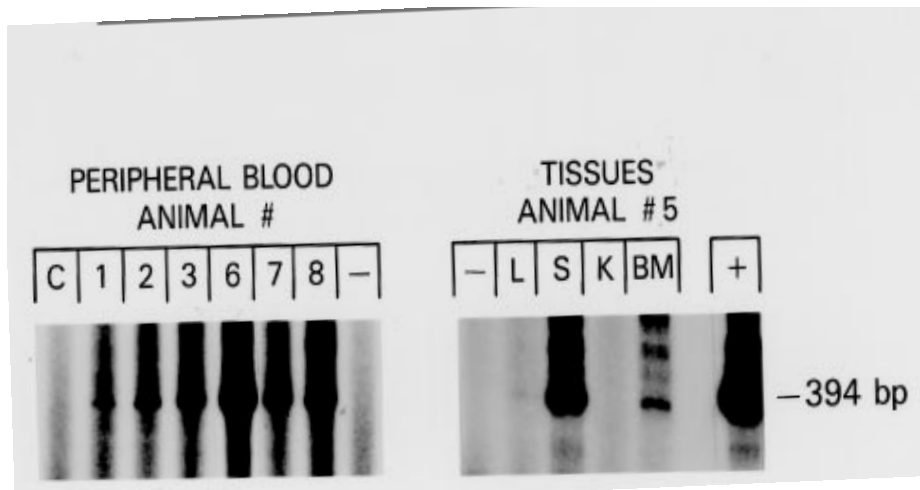


Figure 1. DNA polymerase chain reaction analysis of transplanted W/W mouse tissues. DNAs isolated from peripheral blood of animals which received donor bone marrow infected with the FACC retroviral vector were analyzed for Neo^R-specific sequences (left panel). DNA from a mock-infected animal (C) was used as a control. DNA from the hematopoietic organs of animal #5 were tested by PCR. Lung (L), spleen (S), kidney (K), and bone marrow (BM) DNAs were analyzed (right panel). The expected 394 bp signal generated from genomic DNA from a transduced cell line is shown (+). Water controls (-) are shown

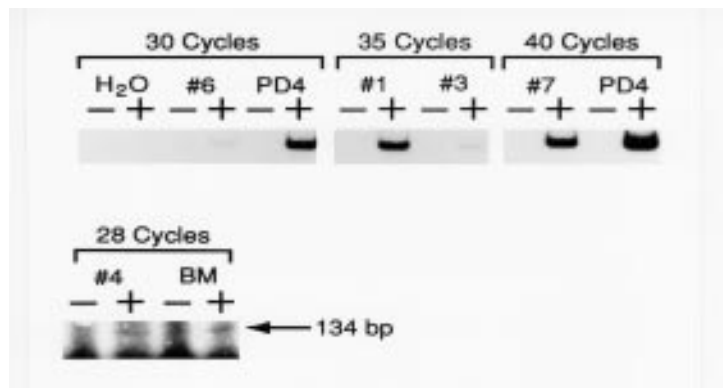


Figure 2. Reverse transcriptase polymerase chain reaction analysis of peripheral blood and bone marrow from FACC vector-transduced W/W mice. RNAs were isolated from peripheral blood and bone marrow and analyzed for expression of FACC message. RNAs isolated from the peripheral blood of mice #4, 6, 1, 3, and 7 were positive. RNA samples were performed in the presence (+) and absence (-) of reverse transcriptase. Positive control RNA was obtained from the FA(C) cell line PD4.

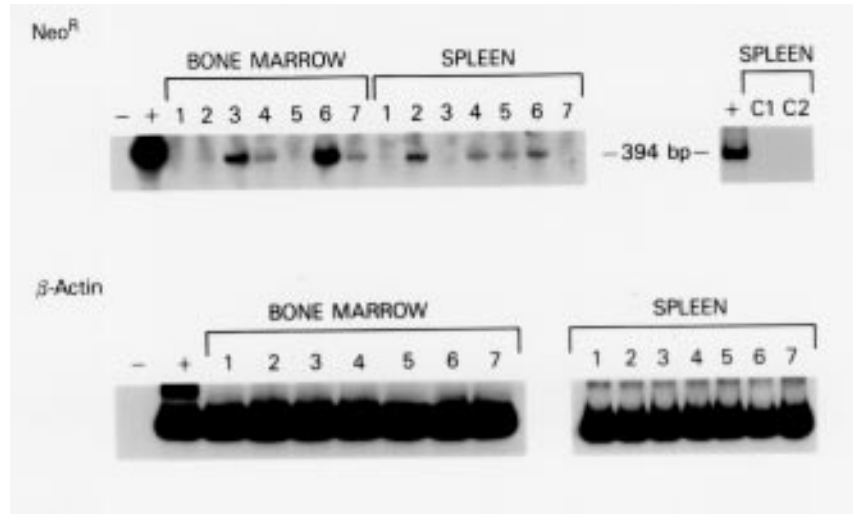


Figure 3. FACC vector transduction in non-ablated BALB/c mice. DNA PCR results of bone marrow and spleen DNAs of seven animals which received FACC vector-transduced donor marrow cells without prior bone host bone marrow ablation. Neo^R-specific primers were used for detection of 394 bp fragment. Results from mock-infected animals are shown at right. β-actin-specific PCR primers amplifying endogenous actin gene sequences were used to check for integrity of DNA samples.

DISCUSSION

Based on our experiments(9-11), we have recently proposed a clinical trial of gene therapy for FA(C) patients(14). To determine if the FACC vector was capable of transducing hematopoietic stem cells, we performed gene transfer studies in W/W^V and non-ablated BALB/c mice.

In the W/W^V transplantation model, a high transduction frequency was achieved in peripheral blood cells. W/W^V mice have a stem cell defect due to mutation in the c-kit gene and can be competitively reconstituted by wild type marrow (C57BL/6) in the absence of radiation treatment (12). In previous studies of retroviral gene transfer of human adenosine deaminase to W/W^V mouse hematopoietic stem cells, the majority of animals had detectable levels of adenosine deaminase after 5.5 months (21). That study made use of a retroviral construct similar in design to the FACC vector in our experiments: the cDNA was driven by the Moloney retroviral promoter, and the vector contained a neomycin-phosphotransferase gene as a selectable marker. Recent studies have suggested that inclusion of selectable marker sequences may diminish

expression of the nonselectable cDNA sequences (22). Our data indicated that the majority of mice expressed FACC mRNA in peripheral blood cells at 5-6 months after transplantation. Possibly, the lack of detectable expression in 2 of the 7 evaluable mice may be attributed to transcriptional down-regulation either from inclusion of Neo^R sequences or alternatively from methylation of the Moloney LTR (23).

In our experiments, we did not detect harmful effects on engraftment of stem cells or maturation and survival of hematopoietic cells attributable to human FACC over expression. Endogenous FACC has a ubiquitous pattern of transcription and a "housekeeping gene" promoter structure. Inclusion of endogenous regulatory sequences in vectors may not be required, since FACC expression seems not to be tightly controlled and over expression does not appear to be directly toxic.

Retroviral vectors in clinical use are replication-defective and routinely monitored for replication-competent retrovirus (RCR)(24,25). The potential for RCR to cause disease was recently established in a nonhuman primate transplantation model: three of eight monkeys developed a rapidly progressive lymphoma

induced by RCR viremia (20). We failed to detect amphotropic envelope sequences in tissues from mice infected with GIFASVNa and have not detected RCR in supernatants from this producer line (J Liu, unpublished data).

Previous analyses have suggested that repetitive infusion of large numbers of donor marrow cells can lead to engraftment into unprepared BALB/c hosts (13). Two obstacles to the application of these findings to gene therapy of nonmalignant blood disorders are the large number of hematopoietic cells required for engraftment in the non-ablated host and the difficulty in retroviral transduction of quiescent non-dividing cells (24). The original reports of transplantation into non-myeloablated mice called for infusion of more than 10^8 unselected cells or nearly two-thirds of the complement of stem cells in one mouse (13). For human stem cell transplantation, approximately 2×10^6 CD34+ cells/kg of body weight are sufficient for engraftment following myeloablation (26). Without prior myeloablation, the number of stem cells required to compete for engraftment in humans is unknown but may be proportionately as high as that required in non-ablated mice (two-thirds of the total complement). In our experiments here and elsewhere (27), only $2-5 \times 10^6$ unselected murine cells were transplanted with each cycle of transduction. This number of cells apparently was sufficient to compete for residence in bone marrow and spleen tissues as detected by PCR methods but insufficient to be detected in peripheral blood. In our experiments we were unable to analyze the extent of chimerism by Y-chromosome-specific probes but could assess engraftment by retroviral marking.

In our experiments with serially transplanted non-ablated mice, we were unable to document gene transduction of peripheral blood cells despite PCR evidence of engraftment in the bone marrow and spleen. We can think of at least two possibilities to explain these results. First, as

suggested above, engraftment into non-ablated hosts may be schedule- and dose-dependent (28). Possibly, the number of cells used in our experiments was insufficient to provide stem cells capable of contributing to peripheral blood reconstitution. Assuming, for example, that marrow and spleen engrafted cells represented fewer than 1% of the total number of cells, the transduced compartment may include committed progenitor cells with limited proliferative potential. Progeny from the few transduced stem cells may be insufficient in number to be detected in peripheral blood. An alternative possibility is that the PCR signal in the tissues arose from a population of cells that did not circulate to the peripheral blood.

Prestimulation of donor cells with either 5-FU or cytokines (IL-3, IL-6, IL-11, and SCF) may diminish the engraftment potential of treated hematopoietic stem cells, possibly by inducing cell cycle activation (29). Most purified mouse stem cells are in the G0 or G1 phases of the cell cycle (30), and the quiescent fraction of stem cells seems to contribute to engraftment in non-ablated hosts (31). Our experiments suggest that hypertransfusion of smaller numbers of prestimulated donor cells can lead to low-level engraftment in bone marrow and spleen. Prestimulation of donor cells was achieved by either 5-FU or cytokine treatment, manipulations which may lead to suboptimal stem cell engraftment. For clinical gene therapy in FA, however, we suggest that even low-level transduction of mutant FA(C) stem cells might lead to amplification of gene-corrected stem cells through expression of the normal FACC gene(9).

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