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Cyclophosphamide promotes engraftment of gene-modified cells in a mouse model of Fanconi anemia without causing cytogenetic abnormalities

Jennifer E. Adair, Xin Zhao, Sylvia Chien, Min Fang, Martin E. Wohlfahrt, Grant D. Trobridge, Jason A. Taylor, Brian C. Beard, Hans-Peter Kiem, and Pamela S. Becker

Divisions of Hematology and Medical Oncology, Department of Medicine, University of Washington, Box #358056 and Box #358080, Seattle, WA 98195, USA. Division of Clinical Research, Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue North, Seattle, WA, 98109-1024, USA. Seattle Cancer Care Alliance, 825 Eastlake Avenue East, Seattle, WA 98109-1023, USA. Portland VA Medical Center, 3710 SW US Veterans Hospital Road, Portland, OR 97239

Abstract

A major hurdle for hematopoietic stem cell (HSC) gene therapy for inherited bone marrow disorders, including Fanconi anemia (FA), is adequate engraftment of gene-modified cells. A phenotypic defect in DNA repair renders FA patients sensitive to alkylating agents such as cyclophosphamide (Cy); however, at lower doses Cy is well tolerated in the FA transplant setting. We tested whether non-alkylating agents could replace Cy for pre-transplant conditioning to enhance engraftment of *FANCA* gene-modified hematopoietic cells. We compared Cy pre-conditioning with fludarabine (Flu) or cytarabine (AraC) or no conditioning as a control in *fanca*^{-/-} mutant mice receiving gene-modified bone marrow cells. Only mice conditioned with Cy exhibited appreciable engraftment of gene-modified cells by PCR and resistance to mitomycin C (MMC). Cy administration following transplantation increased gene marking levels in all animals treated, but highest gene marking and corresponding MMC resistance were observed in mice receiving Cy pre- and post-transplantation. Importantly, no cytogenetic abnormalities were observed in Cy-treated mice. We conclude that Cy is an effective and superior preparative regimen with respect to engraftment of lentivirus-transduced cells and functional correction in *fanca*^{-/-} mice. Thus, appropriately dosed Cy may provide a suitable conditioning regimen for FA patients undergoing HSC gene therapy.

Keywords

gene therapy; Fanconi anemia; lentivirus vector; cyclophosphamide; autologous transplantation

Correspondence should be addressed to: Hans-Peter Kiem, MD, Division of Clinical Research, Fred Hutchinson Cancer Research Center, 1100 Fairview Ave N Seattle WA 98109, hkiem@fhcrc.org and, Pamela S. Becker, MD, PhD, University of Washington Institute for Stem Cell and Regenerative Medicine, 815 Mercer St N415, Box 358056, Seattle WA 98109. pbecker@u.washington.edu.

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Introduction

Fanconi anemia (FA), a monogenic inherited bone marrow failure disorder that was considered a promising candidate for success in gene therapy early on, has witnessed a critical barrier in successful, persistent therapeutic engraftment of gene modified hematopoietic cells [1, 2]. This is, in part, due to a lack of identifiable and functional hematopoietic stem cells in FA patients [2, 3], as well as to the propensity of FA hematopoietic cells to undergo apoptosis when cultured *ex vivo* for extended periods of time [4, 5]. Recently, we have described efficient transduction and functional correction of FA patient bone marrow (BM)-derived hematopoietic cells with a safety-modified lentivirus that allows for short *ex vivo* culture (<24 hours) [6]. While this vector and transduction protocol are currently being translated into a Phase I clinical trial of FA gene therapy, further analysis of the long-term engraftment potential of these gene modified cells is warranted. Improvements to transduction efficiency and viability of transduced cells may not be sufficient to overcome the engraftment barrier if FA gene corrected cells do not have a selective growth advantage *in vivo* once transplanted.

For other bone marrow disorders wherein there is not a sufficient, naturally occurring selective pressure to enhance bone marrow repopulation with gene corrected cells, some effort is made to clear defective bone marrow cells using cytotoxic agents such as chemotherapy or chemoradiotherapy, thereby creating space for the gene-modified cells to engraft and expand. However, patients diagnosed with FA phenotypically display a DNA repair defect, making the use of cytotoxic agents, which elicit their effects via DNA damage (i.e. alkylating agents), concerning. This is primarily due to the risk of extrahematopoietic toxicity in these patients and, possibly, secondary solid tumors as a consequence of exposure to DNA damaging (e.g. alkylating) agents.

For this reason, we sought to investigate engraftment of lentivirus-transduced hematopoietic cells, both in the absence of a preparative regimen as well as in the setting of conditioning agents that do not cause double-stranded DNA crosslinks (the type of DNA damage that is unable to be repaired because of defects in the Fanconi pathway) and an alkylating agent, cyclophosphamide (Cy), which does cause these double-stranded DNA crosslinks. As FA cells are exquisitely sensitive to DNA double-stranded crosslinks, administration of Cy after transplant of gene-corrected cells also allows for selective killing of residual, non-gene-corrected cells and thus, a selective advantage for gene-modified cells *in vivo* [7–9]. For these studies, we have used the *fanca*^{-/-} mouse model developed by Grompe and colleagues [10]. This and similar models recapitulate certain aspects of FA, such as sensitivity to agents that cause DNA double-stranded cross-links (mitomycin C; MMC), and have been used to phenotype other functional abnormalities as well as elucidate the involvement of other Fanconi proteins in the Fanconi pathway [10, 11]. They have also been used previously to demonstrate phenotypic correction after gene transfer by retroviral or lentiviral vector [12].

Previously, Navarro *et al.* demonstrated that there was no engraftment of 20 million donor wild-type cells in *fanca*^{-/-}-recipient animals without prior myeloablation [13], whereas low dose Cy was quite effective as a preparative regimen, allowing engraftment in the *fancc*^{-/-} murine mutant [14].

Here we wished to optimize engraftment of transduced cells by examining the effectiveness of the improved transduction protocol with the previously described safety-modified lentiviral vector in the context of no preparative regimen, conditioning with Cy or conditioning with non-alkylating agents known to induce myelosuppression. Both fludarabine (Flu) and cytarabine (AraC) are non-alkylating, myelosuppressive agents, which historically have been used in the stem cell transplant setting, alone and in combination, in

FA patients who have developed acute myeloid leukemia [15]. To compare these agents, we performed two types of experiments: (1) transplantation of heterozygous *fanca*^{+/-} mouse marrow cells transduced with a lentiviral vector encoding green fluorescence protein (GFP) into homozygous *fanca*^{-/-} recipients (Fig. 1), and (2) transplantation of homozygous *fanca*^{-/-}-deficient cells transduced with the lentiviral vector encoding the human *FANCA* gene (lenti-FANCA), developed for use in a clinical trial of gene therapy for FA [6], into homozygous *fanca*^{-/-} recipients (Fig. 4).

Materials and methods

Lentiviral vectors

The HIV-1-based pRRL.sin.cPPT.PGK.GFP.wpre self-inactivating lentiviral vector [16], which contains a human phosphoglycerate kinase promoter (PGK) driving expression of enhanced green fluorescent protein (GFP) was used for transduction of *fanca*^{+/-}-cells in the first experiment. The vector also contains a central polypurine tract (cPPT) to enhance transduction of quiescent cells [17]. There is a 400 bp deletion of the U3 region of the 3' LTR that contains all of the major determinants responsible for regulating the HIV-1 LTR promoter including the TATA box.

Using this GFP-expressing vector, a third generation self-inactivating lentiviral vector containing the full-length human *FANCA* gene was assembled that would be suitable for a gene therapy clinical trial, pRRL.sin.cPPT.PGK.FANCA.wpre. The human *FANCA* cDNA was originally provided by Dr. Hans Joenje in the mammalian expression vector pREP4 (Invitrogen). Lentiviral vector stocks of VSV-G-pseudotype were prepared transiently by calcium phosphate-or phenylethylamine-mediated three plasmid transfection of 293T cells as previously described [18].

Animals

All animal procedures conformed to protocols approved by the Fred Hutchinson Cancer Research Center Institutional Animal Care and Use Committee (IACUC). 129/SvJ-derived Fanconi Complementation Group A knockout (*fanca*^{-/-}) mice¹² were kindly provided from Dr. Marcus Grompe, Oregon Health Sciences University, Portland, OR, and a colony was maintained. Genotyping was performed to identify the homozygous and heterozygous affected offspring mice.

Overnight lentiviral transduction

Donor female bone marrow was harvested by flushing of the tibiae, femurs, and iliac bones. Bone marrow cells were hemolyzed and transduced as a single batch at an MOI of 10 in Iscove's Modified Dulbecco's Medium (IMDM) containing 10% fetal bovine serum and 1mM N-acetyl cysteine in the presence of growth factors including GCSF, SCF, Flt3 ligand, and TPO at 100 ng/ml each, on non-treated tissue culture vessels coated with recombinant fibronectin peptide (Retronectin: Takara Bio, Inc). Transduction cultures were incubated overnight at 37°C under hypoxic conditions (5% O₂).

Conditioning and transplantation

All donor bone marrow cells were from female mice of either *fanca*^{+/-} (Experiment 1) or *fanca*^{-/-} (Experiment 2) lineage. For each experiment, recipient *fanca*^{-/-}-mice were divided to 3 or 4 preconditioning groups (n = 5 mice per group). Conditioning groups included no preconditioning (control); combination Flu + AraC [200 mg/kg daily × 3 days and 300 mg/kg daily × 3 days, respectively, by intraperitoneal injection (IP)]; AraC alone [1000 mg/kg IP]; and Cy alone [120 mg/kg IP] (Figs.1b and 4b).

Engraftment and post transplant monitoring

Batch-transduced cells were administered through tail vein injection or a combination of tail vein injection and retro-orbital sinus delivery to recipient mice such that each mouse received a total dose of $0.5\text{--}1 \times 10^6$ cells from the same transduction procedure. Mice that received $<0.5 \times 10^6$ total cells (Experiment 1; 2 mice in the Cy group and 1 mouse in the AraC group) were used as controls for MMC sensitivity studies (discussed below). Mice were bled via the retro-orbital sinus at regular intervals following transplantation to monitor kinetics of hematopoietic recovery and to collect samples for gene marking and RIS studies. At day +35, survival bone marrow aspirates were performed to assess gene marking in WBCs and CFCs.

At day +42, single-dose Cy [120mg/kg] was administered IP to achieve impose selective pressure and allow expansion of the transduced cell population. Mice were monitored for hematopoietic recovery and to collect samples for gene marking and RIS studies following Cy administration. At days +85 through +92, mice were euthanized by CO₂, necropsies were performed, and blood, bone marrow and spleens were collected for additional gene marking analyses. Transduction efficiency and percent transduced cells was determined by flow cytometry for GFP (Experiment 1) or quantitative real-time polymerase chain reaction of DNA isolated from transduced cells (Experiment 2) or methylcellulose colonies (both experiments).

Methylcellulose and MMC resistance assays

Hemolyzed BM cells were plated at a concentration of thirty thousand cells per 35 mm dish containing 1.2 ml methylcellulose (Stem Cell Technologies), and mitomycin C (MMC; Ben Venue Laboratories, Inc., Bedford OH) at 0, 10 nM, or 20 nM in triplicate. Plates were incubated at 37°C inside humidified (85%) and hypoxic (5% O₂) chambers. Colony numbers were counted after 14 days in culture, by light microscopy (both experiments) and scored for transduction efficiency (GFP expression) by fluorescence microscopy (Experiment 1).

An average of 24 to 36 colonies from each treatment condition was picked up and the colony DNA was subjected to PCR using lentivirus-specific primers to determine the presence or absence of the transduced lentiviral vector (both experiments) and also genotyping primers to determine the total number of engrafted heterozygous cells (Experiment 1). Alternatively, cells were also assayed for MMC resistance by suspension cell culture. Hemolyzed BM and spleen cells were cultured in IMDM medium containing 10% fetal bovine serum in the presence of growth factors including GCSF, SCF, Flt3 ligand, and TPO at 100 ng/ml each, on treated tissue culture vessels in the absence or presence of MMC. The surviving cell fraction was determined at 48 hours and 96 hours by the CellTiter Glo™ (Promega) luminescent cell viability assay.

Cytogenetic analysis

Approximately 2 million mouse bone marrow cells were used to inoculate 5 ml of RPMI media supplemented with 16% fetal bovine serum and 10% PEN/Strep or MarrowMax media. The cultures were incubated at 37°C with 5% CO₂ for 15 to 30 hours before addition of colcemid (final concentration 0.04 µg/mL) to arrest the cells in metaphase. Cells were harvested with the hypotonic solution (0.075 M KCl) pre-warmed to 37°C and fixed in methanol and glacial acetic (2.5:1 ratio). Fixed cells were dropped on glass slides, treated with 0.025% trypsin in 0.9% NaCl, and stained with 1:4 diluted Wright's stain (pH 6.8) for GTW banding. Metaphases were analyzed under a microscope at a magnification of about 1250X for chromosome count and structural integrity. A minimum of twenty metaphases were analyzed for each sample. Karyotypes were written according to the ISCN2009

guideline using the mouse chromosome band designation specified in ideogram found from the following web link: <http://www.informatics.jax.org/silver/images/figure5-2.gif>

Statistical analysis

P values were determined by the Student's *t* test.

Results

Engraftment and Cy-mediated selection of lenti-GFP transduced *fanca*^{+/-} bone marrow cells transplanted into *fanca*^{-/-} mouse recipients

First, we wanted to assess whether engraftment of gene-modified cells was affected by the choice of conditioning regimen and compare the results to mice who received no conditioning prior to transplantation. To simplify tracking of transplanted cells, a lentivirus vector encoding GFP was used to transduce heterozygous (*fanca*^{+/-}) bone marrow cells which simulate genetic correction by the presence of one wild-type murine *fanca* allele (Fig. 1a). Bulk transduced cells (52% GFP⁺ by flow cytometry) were then infused into recipient pre-conditioned mice. Human dose equivalents were calculated for each conditioning agent used in these studies (Fig. 1b).

In this experiment, mice conditioned with Cy displayed the most efficient engraftment with 3 out of 5 mice exhibiting circulating GFP⁺ white blood cells (WBCs) by flow cytometry at day +35, compared to 2 out of 5 mice in the other groups (AraC+Flu, AraC alone and Control) (Fig. 2a). Two of the Cy group mice and one of the AraC+Flu-conditioned mice which did not display any gene marking at this time point had received only partial cell doses during transplantation due to complications associated with tail vein injections. The range of GFP⁺ cells observed in the engrafted Cy-conditioned mice at this time point (2.6–52.7% of circulating WBCs) also exceeded that observed for the other test groups (Fig. 2b) and was most dramatically different for the control and AraC+Flu group, (marking range <1% of WBCs). Flow cytometry assays performed using bone marrow WBCs from these mice showed demonstrable marking in only the Cy-conditioned group as assessed by GFP expression (Fig. 2c, white bars). Given the sensitivity of *fanca*^{+/-} cells to DNA cross-linking agents, post-transplant Cy (120 mg/kg) was administered to all mice on day +45 as a method of inducing selection of *fanca*^{+/-} cells. Following post-transplant Cy administration, circulating WBC marking increased across all groups, with the highest observed in the Cy group mice at 77.4%, 24.1% and 77% GFP⁺ WBCs (Fig. 2b). This observation was confirmed in BM-derived WBCs at day +85, with Cy-conditioned mice displaying 67.9%, 15.8% and 56.3% GFP⁺ cells, respectively.

Since non-transduced *fanca*^{+/-} BM cells were also transplanted in these mice initially, we performed genotyping PCR as well as GFP scoring in CFCs isolated from each mouse (Fig. 3). We did not observe any homozygous colonies positive for GFP in these assays that would suggest unintentional gene transfer via replication-competent lentivirus. We observed the greatest mean number of GFP⁺ heterozygous CFCs in the Cy-conditioned mice (Fig. 3a). When CFU sensitivity to MMC was examined, mice in the Cy-conditioned group that exhibited high level marking by flow cytometry for GFP (*n* = 3) also exhibited more CFCs in 10 or 20 nM MMC than those mice which received suboptimal cell doses during transplant and displayed no gene marking (*n* = 2) (Fig. 3b).

Due to the potential for DNA cross-linking with Cy treatment, we performed cytogenetic analysis on BM-derived WBCs from male mice in the Cy conditioning group at day+85 (data not shown). No clonal cytogenetic abnormalities were observed in these mice and male:female chimerism was confirmed.

These data demonstrate that Cy conditioning promotes engraftment of gene-modified *fanca*^{+/-} hematopoietic progenitors more efficiently than non-alkylating agent regimens including AraC and/or Flu in *fanca*^{-/-} mice without inducing cytogenetic abnormalities. As heterozygous cells may not have accumulated cellular and genomic instability associated with homozygous *fanca* knockout, we next tested whether engraftment of transduced *fanca*^{-/-} cells was affected by conditioning with Cy.

Engraftment and Cy-mediated selection of lenti-FANCA transduced *fanca*^{-/-} bone marrow cells transplanted into *fanca*^{-/-} mouse recipients

Given the promising results observed with transplanted heterozygous cells, we next wanted to compare engraftment of corrected *fanca*^{-/-} cells into *fanca*^{-/-} recipient mice, emulating a clinical gene therapy approach. Owing to the cross-species functionality of the human *FANCA* gene in mice, a clinical grade lentivirus vector expressing the *FANCA* gene was employed for these studies using a transduction protocol optimized for human gene transfer into FA patient hematopoietic cells [6]. As the combination of AraC and Flu did not increase overall engraftment and gene marking in the previous studies, this combination was not tested further as a preparative regimen in this experiment (Fig. 4b). Instead, we focused on the comparison of Cy versus AraC versus no preparative regimen.

In these studies, gene marking was assessed by real-time quantitative PCR (taqman) using primer sets specific to integrated provirus sequences. Bulk transduced cells administered to conditioned mice in this experiment demonstrated a provirus copy number of 0.62, which corresponds to a transduction efficiency of 62% assuming a single provirus vector copy per cell by taqman PCR and a transduction efficiency of 22.7% in CFCs by colony PCR (5 provirus⁺ colonies out of 22 colonies assessed). By day +35 following transplant, 3 of 5 mice in the control group, 3 of 5 mice in the AraC group, and 4 of 5 mice in the Cy group were evaluable for marking analysis by PCR of circulating WBC DNA. All four mice in the Cy group displayed efficient engraftment of gene-corrected cells (WBC marking range of 0.14 to 0.53 provirus copies per cell), while no mice in the control group and only one mouse in the AraC group displayed detectable marking (0.05 provirus copies per cell on day +22). These results were confirmed by PCR of DNA from PB-derived WBCs on day +38 (Fig. 5a, white bars). As in the prior experiment, following post-transplant Cy-mediated selection (120mg/kg), WBC marking increased across all groups, with the Cy group again displaying the most prominent marking (5 of 5 mice, range of 0.42 to 1.3 provirus copies per cell). This difference was again confirmed in PB-derived WBCs (Fig. 5a, black bars). While colony plating efficiency from BM at day +72 was equivalent across all groups in methylcellulose colony forming assays containing no MMC, in 20 nM MMC there was a significant difference between the Cy group (44.6 ± 28.3 colonies/plate) as compared to the Control (4.8 ± 1.7, p=0.014) or AraC (5.4 ± 6, p=0.031) groups (Fig. 5a). Similarly, for day +72 BM cells assessed in liquid culture at 96 hr, cells from the Cy-conditioned mice had the highest live cell numbers in the presence of 0, 10 and 20 nM MMC, as compared to the control (p=0.0002 for 20 nM MMC) or AraC groups (p=0.0015 for 20 nM MMC) (Fig. 5c). This was also true for spleen-derived cells from the same mice (Fig. 5d). Colony PCR confirmed BM marking in transplanted mice and corresponded with MMC sensitivity and male:female chimerism (data not shown).

As *fanca*^{-/-} cells are more sensitive to DNA damage and all mice received Cy either before and after transplantation or after transplantation only, we performed cytogenetic analysis of male mice from each conditioning group (Fig. 6). Four of the four recipients from the Cy group were found to be chimeras with predominantly donor female cells (>50% of metaphase spreads bearing duplicate X chromosomes), with one mouse exhibiting a non-clonal structural abnormality (single X and translocation t(8;9) believed to represent an artifact of culture. All other metaphases examined were normal.

Two out of the two AraC group recipients examined exhibited all host cells with normal male metaphases, with the exception of one metaphase that showed additional material attached to the centromere of chromosome 9 which was considered non-clonal as it appeared in less than 3 metaphases.

Three normal control mice that did not undergo transplant with transduced cells exhibited normal male karyotypes, with the exception of two non-clonal, structural abnormalities in single cells (data not shown). In one animal, two cells were missing a copy of chromosome 12 and one of these was also missing a copy of chromosome 17. Another animal displayed a single cell with a deletion of chromosome 1. The third animal displayed a single cell lacking chromosome 4, with a marker chromosome and extra material replacing part of chromosome 6. These changes were observed in animals that did not receive chemotherapy or gene-modified cells, and were considered non-clonal as they occurred in less than 3 metaphases.

As an additional measure of safety, we also performed retrovirus integration site (RIS) analysis of BM WBCs from Cy-conditioned mice both before and after Cy-mediated selection to determine whether Cy treatment after transplant skewed the clonal distribution of gene-modified cells. We were able to identify a total of 95 unique RISs between the two time points examined, with no decrease in the clonal repertoire identified after Cy-mediated selection based on the ratio of unique RISs identified to the total number of sequence reads obtained from the same sample (pre-selection ratio of 0.03; post selection ratio of 0.4).

These data confirm that Cy-mediated conditioning promotes engraftment and selection of gene-corrected hematopoietic cells in a clinically relevant transplant model, the *fanca*^{-/-} mouse. Furthermore, these data demonstrate that Cy-mediated conditioning and selection did not induce cytogenetic abnormalities or skew the gene-modified cell pool towards clones with a potentially harmful selective advantage in this model.

Discussion

Here we found that a Cy-based preparative regimen was superior to myelosuppressive, but non-alkylating, drugs Flu and AraC with regard to facilitating engraftment of lentivirus-modified hematopoietic cells in *fanca*^{-/-} mice. In addition, we also demonstrated that post-transplant administration of Cy could induce selection and expansion of gene corrected cells *in vivo*. Importantly, we did not observe cytogenetic abnormalities or abnormal gene-modified clonal contributions in Cy-treated mice during the course of these studies. Of note, the number of metaphases examined in each group limits the statistical significance of observing no cytogenetic abnormalities in conjunction with any of the agents used in this experiment. Furthermore, we cannot rule out the presence of an initial transforming event which is undetectable by cytogenetic analysis due to a lack of clonal outgrowth in the time frame following transplant during which these analyses were conducted.

While the data shown here are consistent between experiments, the numbers of mice used are too low to establish mathematical statistical significance within each experiment, owing to the poor breeding capability of *fanca*^{-/-} mice. Additionally, the length of follow-up included in these studies does not allow for thorough assessment of long-term repopulation capacity of the transplanted cells. In this regard, a follow-up study including long-term monitoring after transplant in the same mouse model is currently underway at the request of the Food and Drug Administration to provide additional long-term safety data in support of our current Phase 1 clinical trial using this FANCA-expressing lentivirus vector to gene-modify FA patient cells for autologous transplant. In this follow-up study, *fanca*^{-/-} recipient mice either did not receive any preparative regimen prior to transplant of gene-modified cells, modeled after our current clinical trial design, or were conditioned with 120 mg/kg Cy

on day -2. While long-term analyses are still being performed in these mice, initial marking studies corresponded with our findings summarized herein. Namely, mice receiving no preparative regimen (n = 10) displayed marking levels by taqman PCR ranging from 0%–1.2% in peripheral blood WBCs at 4 months after transplant, while mice receiving CY conditioning prior to transplant (n = 8) display gene marking levels ranging from 0.1%–30% in peripheral blood WBCs at the same time point. Gene marking analysis in bone marrow-derived CFCs at the same time point indicates an average marking of 9.3% (range: 1.6–26.8%) in non-conditioned mice and 19.4% (range: 3.6–32%) in Cy-conditioned mice. Given previous gene therapy trials for FA have failed to demonstrate persistent, therapeutic engraftment of gene corrected cells, these data suggest that Cy should be considered for facilitating engraftment and/or selection of gene corrected cells in FA patients undergoing gene therapy.

This poses a potential dilemma in the treatment of patients with FA, in that alkylating agents such as Cy are well known to cause the type of DNA cross-links to which the FA patients are susceptible due to defective DNA repair mechanisms. Nevertheless, the drug has been used quite effectively at decreased doses for FA patients undergoing allogeneic stem cell transplant and there has been no report of an increased risk of secondary myelodysplastic syndrome (MDS) or leukemia after allogeneic transplantation including Cy. In fact, most current transplant regimens include either busulfan or Cy or both as well as total body irradiation, albeit all at reduced doses [19–22]. Furthermore, the Cy dose used in these studies (120mg/kg in mouse) is equivalent to a dose of 14.4 mg/kg in a human child or 9.7mg/kg in a human adult patient based on a standard body surface area conversion [23], lower than that used for successful allogeneic transplantation in human FA patients (60–200mg/kg as a single agent; 20–40mg/kg in combination with total body irradiation and/or antithymocyte globulin) [24]. In contrast, the doses of AraC and Flu used in these studies are equivalent to or higher than doses typically used in human transplant patients (for AraC: 2–4 gm/m² alone or in combination with Cy and/or total body irradiation [25, 26]; for Flu: 120–180 mg/m² in combination with Cy and/or ATG and/or total body irradiation [27–29]).

In addition, Cy has been used extensively in mouse gene therapy studies by Grompe et al. with no report of Cy-associated MDS or leukemia in the *fanca*^{-/-} or *fancc*^{-/-} backgrounds [14]. We have confirmed this in the present studies, in which we have not observed any cytogenetic abnormalities associated with Cy treatment, nor have we observed secondary malignancies or losses in hematopoietic gene modified clonal diversity.

Other alternatives to Cy conditioning could include Busulfan, a myeloablative alkylating agent which functions similar to total body irradiation (TBI) in that it depletes non-cycling primitive stem cells [30, 31]. Likewise, low-dose irradiation has demonstrated success in other mouse models of gene-modified cell transplantation [32–35]. However, it remains to be determined whether either of these treatment modalities will prove as successful as cyclophosphamide in preclinical autologous gene therapy for Fanconi anemia.

While nonalkylating, myelosuppressive drugs may be more attractive from a theoretical point of view, the lack of effectiveness observed in these studies and the superiority of Cy-mediated engraftment would suggest that the use of Cy should be further explored and developed in this patient population. Of course, it would be ideal to find a dose of Cy that would eliminate all of the FA cells prior to infusion of gene-corrected cells, and this could likely be achieved with dose escalation protocols to determine the appropriate dose. The increased sensitivity of FA cells to Cy may, in fact, present a unique situation in which uncorrected cells can efficiently be eliminated with acceptable toxicity while corrected cells can be selected for expansion. In conclusion, these studies support the use of Cy to mediate

successful gene therapy for FA and warrant further studies to elucidate the most conservative Cy dose at which effective engraftment and/or selection can be accomplished.

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Fig. 1. Experimental strategy for comparing engraftment after various conditioning regimens in *fanca*^{-/-} mice transplanted with lentivirus-transduced *fanca*^{+/-} bone marrow cells (EXP 1). **a)** Schematic diagram of experimental design for assessing engraftment. **b)** Timeline of transplantation, corresponding conditioning regimens and post-transplant selection. Total drug doses administered and human equivalent dose (HED) calculated by body surface area conversion are also included.

Fig. 2. Cy Conditioned animals display significantly higher gene marking both before and after post-transplant Cy-mediated selection

a) Representative flow cytometry dot plots of peripheral blood WBCs obtained from one mouse in each conditioning group demonstrating cell population gating by forward and side light scatter (top row) and percent of cells gated GFP+ as a function of side light scatter (bottom row). Middle row shows representative histograms depicting live cell gating by propidium iodide staining. Only live cells within the gated cell population are represented in GFP-gated plots. All data were obtained on Day 35 following infusion of gene-modified cells. **b)** Time course of the mean percentage of GFP-expressing peripheral blood WBCs from transplanted mice in each conditioning group by flow cytometry demonstrates

consistently increased gene marking in peripheral blood of Cy-conditioned mice over time compared to other conditioning groups. Arrow represents time point at which post-transplant Cy was administered for selection of gene-modified cells. Error bars represent one standard deviation from the mean. N=3 (Cy), N=5 (Control, AraC and AraC+Flu). **c)** Flow cytometry of bone marrow-derived WBCs demonstrates higher gene marking in Cy-conditioned mice compared to other treatment groups both before and after Cy-mediated selection. Bars represent mean percentage of GFP-expressing bone marrow WBCs from transplanted mice in each conditioning group. Error bars represent one standard deviation from the mean. N=3 (Cy), N=5 (Control, AraC and AraC+Flu).

Fig. 3. Colony forming cells derived from Cy-conditioned mice display higher gene-marking and increased MMC resistance

a) Graph represents individual marking in BM CFCs (open circles) from each transplanted mouse as a function of conditioning group. Bars represent the overall mean transduction efficiency for each group. Marking was determined by GFP scoring of colonies under fluorescence microscopy. **b)** BM-derived cells from the 3 Cy-conditioned animals display greater MMC resistance in colony forming assays compared to BM-derived cells from 2 homozygous knockout animals. Bars represent mean colony number for each treatment group. Error bars represent one standard deviation from the mean.

Fig. 4. Experimental strategy for comparing engraftment after various conditioning regimens in *fanca*^{-/-} mice transplanted with lentivirus-transduced *fanca*^{-/-} bone marrow cells (EXP 2)
a) Schematic diagram of experimental design for assessing engraftment. **b)** Timeline of transplantation, corresponding conditioning regimens and post-transplant selection.

Fig. 5. Cy-conditioned animals display significantly higher gene marking both before and after post-transplant Cy-mediated selection

a) Lentivirus-specific PCR of bone marrow-derived WBCs demonstrates higher gene marking in Cy-conditioned mice compared to other treatment groups both before and after Cy-mediated selection. Bars represent mean percentage of lentivirus+ bone marrow WBCs from transplanted mice in each conditioning group. Error bars represent one standard deviation from the mean. N=5 mice per group. **b)** Bone marrow-derived colonies from Cy-conditioned mice demonstrate greater MMC resistance compared to colonies from other treatment groups. Bars represent the mean number of colonies per dish for all animals in each treatment group. Error bars represent one standard deviation from the mean. Asterisk (*) indicates a significant difference between the MMC-treated colonies with p value <0.01. **(c and d)** Liquid culture of bone marrow-derived WBCs **(c)** and spleen-derived WBCs **(d)** demonstrates greater MMC resistance in Cy-conditioned mice compared to other treatment groups after Cy-mediated selection. Cells obtained from each mouse were cultured independently and bars represent mean viability as determined by luminescence intensity for all cultures. Error bars represent one standard deviation from the mean. N=5 mice (Control and Cy groups), N=4 mice per group (AraC).

Fig. 6. Normal karyotype observed in all animals treated with Cy-mediated conditioning and selection or Cy-mediated selection only

Representative metaphase spreads isolated from bone marrow at day 84 post-transplant of one male mouse from each conditioning group (a-Control, b-AraC and c-Cy). Chromosomal alignment and G-banding verified no chromosomal abnormalities were present in mice analyzed from each group. A total of 20 metaphase spreads were examined per mouse, N= 2 mice per group (both male). Panel c (Cy) shows female karyotype observed in one male mouse with >90% gene marking by PCR at the same time point in peripheral blood. All 20 metaphase spreads examined from this mouse demonstrated female karyotype of donor cells.