USE OF MURINE MODELS TO TEST NOVEL GENE TRANSFER STRATEGIES FOR THE TREATMENT OF FANCONI ANEMIA

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ABSTRACT

Anna C. Leath

USE OF MURINE MODELS TO TEST NOVEL GENE TRANSFER

STRATEGIES FOR THE TREATMENT OF FANCONI ANEMIA

The dawn of the genetic era has allowed for investigation of gene transfer therapy as a treatment for certain diseases. Fanconi anemia (FA) is a rare genetic disorder in which the majority of patients develops progressive bone marrow failure (BMF) and require bone marrow transplantation. A possible alternative treatment is autologous gene therapy; however, original clinical trials involving gene transfer for FA were unsuccessful. This has led to re-evaluation of the gene transfer protocols, the vectors and also a deeper investigation of the FA pathway itself. My work has focused on illuminating these areas to further advance gene transfer therapy for FA.

Many gene transfer protocols require the hematopoietic stem and progenitor cells (HSC/HPC) to be collected and then transduced ex vivo. The most common collection method is mobilization of the HSC/HPC to the peripheral blood (PB) using granulocyte colony-stimulating factor (G-CSF) and collection via

apheresis. In FA patients G-CSF fails to mobilize a sufficient number of HSC/HPC. This has led to research into agents such as AMD3100, a CXCR4 antagonist, which may replace or augment G-CSF mobilization. These data show in two FA murine models that AMD3100 synergizes with G-CSF resulting in a significant increase in mobilization as compared to G-CSF alone.

Previous work in our lab has shown that prototype foamy virus (FV) is an efficient gene transfer vector. Here a modified FV vector is used to transduce mobilized FA cells. The data indicate that long-term repopulating cells mobilized with both G-CSF and AMD3100 can be efficiently transduced by our FV vector. Clinically, FA is characterized mainly by BMF, but also by myelodysplasia (MDS) and acute myeloid leukemia (AML). However, current FA murine models do not display these disease phenotypes. These data show that double-mutant Fancc-/-;Fancg-/- mice spontaneously develop BMF, MDS and complex random chromosomal abnormalities that the single-mutant mice do not. Importantly, this model closely recapitulates the phenotypes found in FA patients and may be useful as a preclinical platform to evaluate the molecular pathogenesis of spontaneous BMF and MDS in FA and novel gene transfer protocols for FA.

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AML	Acute myeloid leukemia
ANOVA	Analysis of variance
APOBEC3	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3
BFU-E	Blast forming unit-erythroid
BMC	Bone marrow cell(s)
BMF	Bone marrow failure
BoyJ	WT B6.SJL-Ptrca Pep/BoyJ
C3	Complement component 3
C5	Complement component 5
CAS I	Cis-acting sequence I
CAS II	Cis-acting sequence II
CD26	Cluster of differentiation 26
CFU	Colony forming unit
CFU-GEMM	Colony forming unit-granulocyte, erythrocyte, monocyte, megakaryocyte

CFU-GM	Colony forming unit-granulocyte, macrophage
CG	Cathepsin G
CLAD	Canine leukocyte adhesion deficiency
CMV	Cytomegalovirus
CXCR4	Chemokine (C-X-C motif) receptor 24, also known as fusin
DEB	Diepoxybutane
DMEM	Dulbecco’s modified Eagle’s medium
DNA	Deoxyribonucleic acid
EGFP	Enhanced green fluorescence protein
EMCV	Encephalomyelocarditis virus
FA	Fanconi anemia
FBS	Fetal bovine serum
Flt-3	Fms-like tyrosine kinase 3
FV	Prototype foamy virus(es)
GCOS	GeneChip operating software
G-CSF	Granulocyte colony-stimulating factor
H&E	Hematoxylin and eosin
HPC	Hematopoietic progenitor cells

HSC	Hematopoietic stem cells
ID complex	FANCI/FANCD2 complex
IMDM	Iscove’s modified Dulbecco’s medium
IP	Infectious particles
IRES	Internal ribosome entry site
LDMC	Low-density mononuclear cell(s)
LMO2	LIM domain only 2
LTR	Long terminal repeat
MDS	Myelodysplastic syndrome
MMC	Mitomycin C
MMP9	Matrix metalloproteinase 9
MOI	Multiplicity of infection
NE	Neutrophil elastase
NOD/SCID	Non-obese diabetic/severe combined immunodeficiency
PB	Peripheral blood
PBS/0.1% BSA	Phospho-buffered saline/0.1% bovine serum albumin
PCR	Polymerase chain reaction
PGK	Phosphoglycerate kinase

PKR	Protein kinase regulated by RNA
P/S	Penicillin/streptomycin
Redox	Reduction-oxidation reaction
RNA	Ribonucleic acid
RU	Repopulating units
s.c.	Subcutaneous
SDF-1	Stromal cell-derived factor 1, also known as CXCL12
SEM	Standard error of the mean
SFFV	Spleen focus-forming virus
TNF-α	Tumor necrosis factor alpha
VCAM-1	Vascular cell adhesion molecule 1
VLA-4	Very late antigen 4
WT	Wildtype C57BL/6J mice

CHAPTER 1

Introduction

In the last 60 years the dawn of the genetic age has occurred. The knowledge and understanding of genes, their products and molecular techniques have allowed us to delve further into genetic understanding and manipulation than ever before. This new era allows for identification of individual genes and their products' roles in normal cellular functions and also in disease phenotypes. For those diseases that are caused by a single gene defect, it has been hypothesized that adding a functional copy of that single gene would result in correction of the disease, a process termed gene therapy.

Perhaps the most straightforward organ system in which to perform this type of correction is the hematopoietic system. The hematopoietic system is rather unique in that it is continually replenished throughout the life of an individual with new cells derived from hematopoietic stem cells (HSC). In order to correct a disease phenotype that mainly affects the hematopoietic system, focus can be put on correcting the defective gene in the HSC, which will then result in the repopulation of the entire hematopoietic system with cells containing the

corrected gene. The practice of removing part of the HSC population is common (bone marrow aspiration, mobilization and apheresis), which allows for exploration of the collection of the HSC for gene transfer therapy (Rosenbeck, Srivastava et al. 2010). Therefore, diseases that affect the hematopoietic system due to a mutation in a single gene are prime candidates for gene transfer therapy.

While various gene transfer strategies provide potential treatments for patients with these types of diseases, the protocols have not been as straightforward to implement as originally hoped. Collectively, the scientific community has found that there are overall challenges when attempting gene transfer as well as specific challenges for each different disease.

When considering gene transfer therapy for the hematopoietic system, one essential question is how to eliminate the diseased hematopoietic system. One approach is to start with a disease that does this on its own. In Fanconi anemia (FA) the majority of patients present with a progressive bone marrow failure (BMF), where the hematopoietic system stops making new blood cells (Auerbach, Buchwald et al. 2002). Also, each patient with this disease has been found to have a defect in only a single gene (Auerbach, Buchwald et al. 2002).

For FA patients, there are few curative options available (Bagby and Alter 2006). In FA patients (with a spontaneous reversion to correct the gene defect) (Waisfisz, Morgan et al. 1999; Gregory, Wagner et al. 2001; Gross, Hanenberg et al. 2002; Soulier, Leblanc et al. 2005; Mankad, Taniguchi et al. 2006) and also in FA mouse models it has been shown that wildtype (WT) cells have a competitive advantage over FA cells (Gush, Fu et al. 2000; Galimi, Noll et al. 2002; Haneline, Li et al. 2003). Together, this evidence indicates that FA is a good target disease for gene transfer therapy.

While the basic concept of gene transfer therapy for FA is straightforward, actual implementation is extremely complex. Clinical trials failed to detect long-term engraftment of transduced cells in patients (Liu, Young et al. 1997; Liu, Kim et al. 1999; Kelly, Radtke et al. 2007). Additionally, the use of murine models found that the ex vivo manipulation necessary for the gamma-retrovirus transduction protocol led to increased apoptosis of the FA cells and an increase in the development of clonal evolution (Li, Le Beau et al. 2005). This necessitated the re-evaluation of both the gene transfer vector and protocol in relation to FA and also a more complete understanding of the FA pathway.

Fanconi anemia

FA is a heterogeneous genetic disease. To date, 13 genes have been identified as FA genes (*Fanca*, *-b*, *-c*, *-d1*, *-d2*, *-e*, *-f*, *-g*, *-i*, *-j*, *-l*, *-m*, *-n*) (Strathdee, Duncan et al. 1992; Strathdee, Gavish et al. 1992; Wevrick, Clarke et al. 1993; Pronk, Gibson et al. 1995; Whitney, Thayer et al. 1995; Lo Ten Foe, Rooimans et al. 1996; Sinoula Apostolou 1996; Joenje, Oostra et al. 1997; de Winter, Waisfisz et al. 1998; de Winter, Leveille et al. 2000; de Winter, van der Weel et al. 2000; Hejna, Timmers et al. 2000; Joenje, Levitus et al. 2000; Joenje and Patel 2001; Timmers, Taniguchi et al. 2001; Taniguchi and Dandrea 2002; Bagby 2003; Meetei, de Winter et al. 2003; Meetei, Sechi et al. 2003; Levitus, Rooimans et al. 2004; Levitus, Waisfisz et al. 2005; Levran, Attwooll et al. 2005; Meetei, Medhurst et al. 2005; Niedernhofer, Lalai et al. 2005; Bagby and Alter 2006; Taniguchi and D'Andrea 2006; Dorsman, Levitus et al. 2007; Rahman, Seal et al. 2007; Reid, Schindler et al. 2007; Sims, Spiteri et al. 2007; Smogorzewska, Matsuoka et al. 2007; Xia, Dorsman et al. 2007). Another 2 genes have been identified as having gene products that associate with the pathway (FA associated protein 24 and FA associated protein 100), but have not yet been found as a defective gene in a FA patient, which is a requirement to be

classified as an FA gene (Ciccia, Ling et al. 2007; Ling, Ishiai et al. 2007). Recently, RAD51C was identified as defective in a patient whose cells are hypersensitive to MMC, one of the initial diagnostic tests for FA (Meindl, Hellebrand et al. 2010; Vaz, Hanenberg et al. 2010). This patient has not yet developed BMF, so this is not officially an FA gene yet, however, it is assumed that this will be *Fanco* (Meindl, Hellebrand et al. 2010; Vaz, Hanenberg et al. 2010).

FA is primarily an autosomal recessive disease, only one complementation group, *Fancb*, is X-linked recessive (Meetei, Levitus et al. 2004; Bagby and Alter 2006). All of the FA mutations result in chromosomal instability (Bagby and Alter 2006). The overall incidence of FA in the population is 1 in 350,000 births, so FA is a very rare disorder with approximately 3000 patients listed in the International Fanconi Anemia Registry (Kutler, Singh et al. 2003).

Diagnosis is based on hypersensitivity of the patient's cells to cross-linking agents and then further analysis to determine complementation group (Green and Kupfer 2009). *FANCA* is mutated in the majority of patients and together with

FANCC and *FANCG* these three complementation groups account for over 90% of FA patients (Kutler, Singh et al. 2003).

Currently the best option for an FA patient is HSC transplantation (Gluckman, Broxmeyer et al. 1989; Gluckman, Auerbach et al. 1995; Gluckman and Rocha 2005; Green and Kupfer 2009). However, the majority of patients are not able to find an HLA-matched donor, which leaves them with few options (Kutler, Singh et al. 2003). In the last decade a purine analog, fludarabine, has been used as part of a conditioning regimen before transplantation, and has been shown to reduce the incidence of graft-versus-host disease and transplantation-related mortality for both HLA-matched and unmatched transplantation (MacMillan and Wagner 2010). However, even though HSC transplantation is often referred to as a “curative” option, the incidence of solid tumors and squamous cell carcinomas among these patients is very high (Rosenberg, Socie et al. 2005). A reduction in myelopreparation may decrease the chance of these malignancies; however, addressing whether myelopreparation is necessary in patients with BMF is difficult. A mouse model of FA that develops BMF would be useful for these experiments. Other therapies such as androgen supplementation also provide some therapeutic benefit to

patients (Muller and Williams 2009); however, autologous transplantation of gene transfer corrected HSC would be a welcome treatment option.

In order to understand the approach taken for gene transfer it is necessary to understand the underlying causes of FA. FA was originally described by Dr. Guido Fanconi as a genetic disorder that resulted in anemia (Fanconi 1967). Further studies have found that patients can also present with myeloid malignancies and solid tumors (Bagby and Alter 2006; Green and Kupfer 2009).

The genes that are mutated in FA patients are spread throughout the genome (Schmid and Fanconi 1978; Strathdee, Duncan et al. 1992; Pronk, Gibson et al. 1995; Whitney, Thayer et al. 1995; Sinoula Apostolou 1996; Joenje, Oostra et al. 1997; de Winter, Waisfisz et al. 1998; Cheng, van De Vrugt et al. 2000; Hejna, Timmers et al. 2000; Joenje and Patel 2001; Timmers, Taniguchi et al. 2001; Yang, Kuang et al. 2001; Meetei, de Winter et al. 2003; Tonnies, Huber et al. 2003; Meetei, Levitus et al. 2004; Levitus, Waisfisz et al. 2005; Dorsman, Levitus et al. 2007). The FA gene products lack homology with other known proteins that may give clues to their function. The exceptions are *Fancl,* which is an E3 ubiquitin ligase, and *Fancj* and *Fancm,* which have DNA helicase domains (Meetei, de Winter et al. 2003; Levitus, Waisfisz et al. 2005; Levran, Attwooll et

al. 2005; Meetei, Medhurst et al. 2005). The Fanconi anemia proteins are known to function in a pathway together which is thought to primarily function in the repair of DNA cross-links caused by agents such as mitomycin C (MMC) or diepoxybutane (DEB) (de Winter and Joenje 2009). The pathway is an active area of investigation. Some of the FA proteins form a core nuclear complex (thought to consist of FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL and FANCM) that functions to monoubiquitinate the phosphorylated FANCD2/FANCI complex (ID complex) in response to DNA damage (Dorsman, Levitus et al. 2007; Grompe and van de Vrugt 2007; Sims, Spiteri et al. 2007; Smogorzewska, Matsuoka et al. 2007; Ishiai, Kitao et al. 2008). The monoubiquitinated form of the ID complex then translocates to chromatin where it functions with BRCA1, BRCA2 and RAD51 in nuclear foci thought to be sites of DNA repair (Figure 1) (Dorsman, Levitus et al. 2007; Grompe and van de Vrugt 2007; Sims, Spiteri et al. 2007; Smogorzewska, Matsuoka et al. 2007; Ishiai, Kitao et al. 2008). Inactivation of any of the FA core complex proteins results in complete failure to monoubiquitinate the ID complex, leading to cellular hypersensitivity to DNA cross-linking agents (de Winter and Joenje 2009).

Figure 1

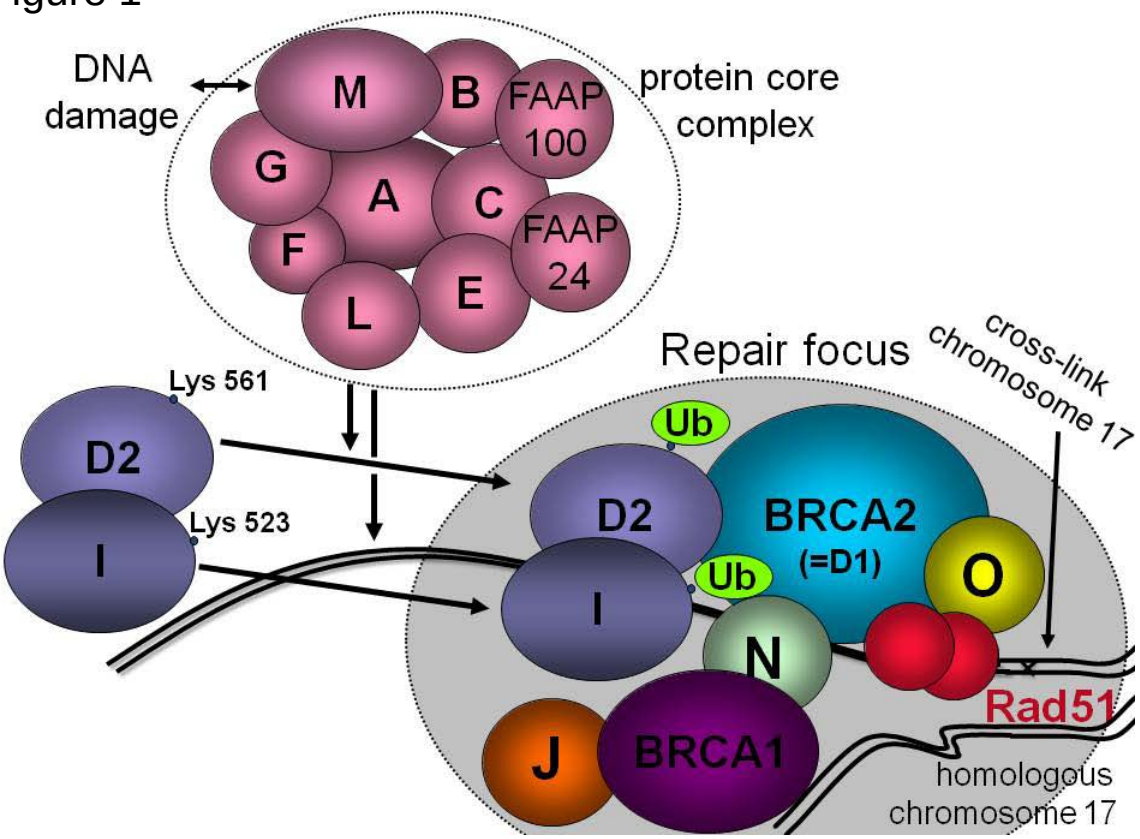
DNA
damage
M
B
FAAP
100
G
A
C
FAAP
24
F
E
L
protein core
complex
Repair focus
cross-link
chromosome 17
Lys 561
D2
Lys 523
I
Ub
D2
BRCA2
(=D1)
Ub
I
O
N
Rad51
J
BRCA1
homologous
chromosome 17

Figure 1. The hypothesized Fanconi anemia DNA repair pathway. Upon DNA damage the FA core complex forms in the nucleus (known interacting proteins: FANCA, -B, -C, -E, -F, -G, -L, -M, FAAP24 and FAAP100). The FA core complex monoubiquitinates both FANCD2 and FANCI (ID complex). The ID complex then translocates to the DNA repair focus and functions with other Fanconi proteins (FANCD1, -J, -N, -O) as well as non-Fanconi DNA repair proteins (BRCA1, RAD51) in the repair of DNA cross-links. Currently the FA pathway is not fully understood and an active area of investigation (modified from H. Hanenberg).

The FA core complex proteins were originally thought to have a singular function: their interactions in the core complex that resulted in the monoubiquitination of the ID complex. However, recent evidence has pointed to functions for these core complex proteins in maintenance of normal hematopoiesis independent of the core complex (Pang, Fagerlie et al. 2000; Pang, Christianson et al. 2001; Zhang, Li et al. 2004; Hussain, Wilson et al. 2006; Leveille, Ferrer et al. 2006; Mukhopadhyay, Leung et al. 2006; Wilson, Yamamoto et al. 2008). However, this hypothesis has not yet been evaluated using a genetic model. Murine knockouts of the homologues of *FANCA* (*Fanca*), *FANCC* (*Fancc*), *FANCG* (*Fancg*), *FANCD1* (*FancD1*), *FANCD2* (*Fancd2*) and *FANCM* (*Fancm*) have been established (Bakker, van de Vrugt et al. 2009; Parmar, D'Andrea et al. 2009). Although cells from all strains of FA knockout mice are hypersensitive to MMC, none of these single knockout mice display spontaneous aplastic anemia or myeloid malignancies characteristic of FA in patients (Bakker, van de Vrugt et al. 2009; Parmar, D'Andrea et al. 2009).

Mobilization

Currently, the most common method used to collect HSC/hematopoietic progenitor cells (HPC) from patients is mobilization of the HSC/HPC from the bone marrow to the peripheral blood (PB). Mobilization is preferable to bone marrow aspiration in most cases because the procedure is safer, less painful, results in a shorter anemic period after transplantation and also results in a decreased incidence of disease after transplantation (To, Haylock et al. 1997; Korbling and Anderlini 2001). Agents that can be used for mobilization include cytokines such as G-CSF (Duhrsen, Villeval et al. 1988; Socinski, Cannistra et al. 1988), chemotherapy (Richman, Weiner et al. 1976) and even exercise (Barrett, Longhurst et al. 1978). While evidence that HSC/HPC exist at low levels in the peripheral blood has been published (Goodman and Hodgson 1962; McCredie, Hersh et al. 1971), the realization that HSC/HPC could be mobilized from the bone marrow to the PB resulted directly from observations in patients. The first notice of mobilization came from a group who quantified an increase in the number of peripheral blood colony forming units after chemotherapy (Richman, Weiner et al. 1976).The discovery of the use of cytokines, as opposed to or in addition to chemotherapy, for the mobilization of stem cells allowed for more

extensive study of mobilization (Socinski, Cannistra et al. 1988; Haas, Ho et al. 1990; Bensinger, Clift et al. 1996). During this time, the level of $CD34^+$ cells as a measure for the enrichment of HSC/HPC was being established (Bensinger, Longin et al. 1994; Weaver, Hazelton et al. 1995). Currently, granulocyte colony-stimulating factor (G-CSF) is the standard mobilizing agent used in patients. Unfortunately, G-CSF is not effective as effective in mobilizing $CD34^+$ cells in FA patients as in normal patients (Croop, Cooper et al. 2001). This has also been found true for other genetic disorders such as chronic granulomatous disease and adenosine deaminase-deficient severe combined immunodeficiency patients (Sekhsaria, Fleisher et al. 1996), and also in up to 15% of normal individuals (Moncada, Bolan et al. 2003).

These observations led to research into the mechanism of G-CSF mobilization, which provided new directions for mobilization protocols. Many papers pointed to matrix metalloproteinases as the main mechanism for G-CSF induced mobilization, specifically neutrophil elastase (NE) and cathepsin G (CG) (Levesque, Takamatsu et al. 2001; Levesque, Hendy et al. 2002) or matrix metalloproteinase 9 (MMP9) (Pruijt, Fibbe et al. 1999; Heissig, Hattori et al. 2002; Levesque, Hendy et al. 2002). Their hypothesis was that the neutrophils

were degranulating in the bone marrow stroma and that the disruption of the HSC/stromal cell interactions by the proteinases released in the environment resulted in mobilization. This hypothesis was further supported by the observations that NE and CG both act on vascular cell adhesion molecule-1 (VCAM-1) and that disruption of the interaction of VCAM-1 with very late antigen-4 (VLA-4) results in mobilization of HSC/HPC (Levesque, Takamatsu et al. 2001; Levesque, Hendy et al. 2002; Levesque, Liu et al. 2004). However, mice deficient in NE and CG still had normal mobilization in response to G-CSF (Levesque, Liu et al. 2004), which led to investigation of other HSC/stromal cell interactions. Other ligands to receptors known to function in stem cell tethering have been shown to induce mobilization, such as soluble c-Kit receptor (Nakamura, Tajima et al. 2004) and Fms-like tyrosine kinase 3 (Flt-3) ligand (Reeves, Wei et al. 2010). Another involves chemokine (C-X-C motif) ligand 12 (CXCL12) also known as stromal cell-derived factor-1 (SDF-1) interacting with CXCR4, also known as fusin. The release of NE and CG disrupts the interaction between the HSC and the stromal cell by disrupting the SDF-1 chemotactic gradient in the bone marrow (Petit, Szyper-Kravitz et al. 2002; Levesque, Hendy et al. 2003) and also by decreasing the responsiveness of the HSC to the SDF-1 chemotactic

gradient (Levesque, Hendy et al. 2003; Levesque, Liu et al. 2004). Dipeptidyl peptidase-4, also known as cluster of differentiation 26 (CD26), has also been shown to disrupt the SDF-1 gradient. CD26, a peptidase expressed on the surface of myeloid cells and bone marrow HSC/HPC can cleave and inactivate SDF-1 (Bongers, Lambros et al. 1992). Additionally, HSC/HPC mobilization is reduced by 50 to 80% in CD26-/- mice (Christopherson, Cooper et al. 2003; Christopherson, Cooper et al. 2003). Cells treated with CD26 inhibitors have been shown to have increased engraftment (Christopherson, Hangoc et al. 2004; Broxmeyer, Hangoc et al. 2007; Campbell, Hangoc et al. 2007).

The above observations led to research into the administration of antagonists to molecules known to help in retention of HSC in the stroma. One such inhibitor is AMD3100 (more recently known as plerixafor), a small molecule that is an antagonist to CXCR4, the receptor for SDF-1. Previous studies have shown that administration of AMD3100 in addition to G-CSF results in a synergistic increase in the mobilization of HSC in WT mice and HPC in *Fancc-/-* mice (Broxmeyer, Orschell et al. 2005). Ideally this method can be used to obtain sufficient numbers of HSC/HPC from FA patients; however, whether this combination of agents will result in increased mobilization of HSC/HPC in our FA

mouse models needs to be determined first. Additionally, there is evidence that the mobilized cell population does not respond to transduction in the same manner as the BMC population (Hematti, Sellers et al. 2003; Thomasson, Peterson et al. 2003), perhaps due to mobilization's effects on cell surface receptors or other biochemical changes in the cells. Therefore studying the transduction of mobilized HSC/HPC in our murine model is necessary.

Interestingly, recent evidence points to a role for reduction-oxidation reaction (redox) regulation of stem cell mobilization. Many of the signal transduction pathways leading to cytokine expression are redox regulated (Lekli, Gurusamy et al. 2009). In the bone marrow niche, HSC are in a hypoxic environment and upon mobilization they enter an oxygenic environment (Lekli, Gurusamy et al. 2009). FANCG is known to interact in redox pathways in the mitochondria (Mukhopadhyay, Leung et al. 2006). Further investigation into the redox pathways' regulation of mobilization may provide a link that helps explain the FA mobilization defect.

Additional evidence has recently been published supporting the argument for the innate immune response also having a role in mobilization (Lee, Wu et al. 2009; Jalili, Marquez-Curtis et al. 2010; Jalili, Shirvaikar et al. 2010; Lee,

Wysoczynski et al. 2010). Both AMD3100 and G-CSF mobilization were found to depend on complement activation, but at different steps in the pathway. AMD3100 activates complement component 5 (C5) via granulocyte protease release (Lee, Wysoczynski et al. 2010), whereas G-CSF activates complement at some point upstream of complement component 3 (C3) (Lee, Wu et al. 2009; Jalili, Shirvaikar et al. 2010; Lee, Wysoczynski et al. 2010). Since FA mice mobilize well in response to AMD3100, but not to G-CSF, there may be some disruption in the complement pathway before the C5 activation step.

The mechanism of HSC mobilization is an active area of investigation. The studies here have focused on evaluating AMD3100 for the augmentation of G-CSF mobilization in FA murine models.

Prototype foamy virus

Foamy virus was first isolated from *Rhesus macaque* kidney cells in 1955 (Rustigian, Johnston et al. 1955). Foamy virus has since been found in many other mammals including: African green monkey (Neumann-Haefelin, Fleps et al. 1993), chimpanzee (Herchenroder, Renne et al. 1994), gorilla (Bieniasz, Rethwilm et al. 1995), baboons (Broussard, Comuzzie et al. 1997), and many others. In 1971 a human foamy virus was isolated from the lymphoblastoid cells in a nasopharyngeal carcinoma from a Kenyan patient (Achong, Mansell et al. 1971; Achong, Mansell et al. 1971). The sequence of “human foamy virus” was found to be greater than 95% identical to the simian foamy virus isolated from *Pan troglodytes schweinfurthii* and was renamed SFVcpz(hu) (Herchenroder, Renne et al. 1994) and is now referred to as prototype foamy virus (FV).

FV is in the retrovirus family, genus spumavirus. The virus reverse transcribes its RNA genome to DNA either before leaving the host cell or in the virion form (before entering the cell it is infecting) (Moebes, Enssle et al. 1997; Yu, Sullivan et al. 1999). This is also characteristic of hepadnaviruses (reviewed in (Beck and Nassal 2007)). Because the virions contain a DNA genome it is thought that they may be more stable than retrovirus or lentivirus vectors

(Moebes, Enssle et al. 1997; Yu, Sullivan et al. 1999). The FV genome is approximately 13kb and contains 5 genes: gag, pol, env, tas and bet (Figure 2) (Lochelt, Zentgraf et al. 1991). The gag gene encodes structural proteins and differs from other retroviruses in that it retains an immature morphology, meaning it is not cleaved (Meiering and Linial 2001). The pol gene encodes a protein with reverse transcriptase, integrase and protease domains (Meiering and Linial 2001). The env gene encodes the envelope protein, which contains an endoplasmic reticulum retention signal (Goepfert, Wang et al. 1995). The tas gene (also known as bel-1) is required for replication, it encodes the protein that transactivates the long terminal repeat (LTR) promoter (Lochelt, Zentgraf et al. 1991; Mergia, Shaw et al. 1991; Rethwilm, Erlwein et al. 1991). The last gene, bet, codes for the alternative splicing products: bet, bel-2 and bel-3, whose function in the FV life cycle is an active area of investigation. It is thought that bet may function in disrupting the antiviral activity of apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3 (APOBEC3) (Perkovic, Schmidt et al. 2009). There are 2 “packaging” signals in the genome, cis-acting sequences I and II (CAS I and CAS II), when these are not present the RNA genome will not be packaged into the virion (Meiering and Linial 2001).

Figure 2

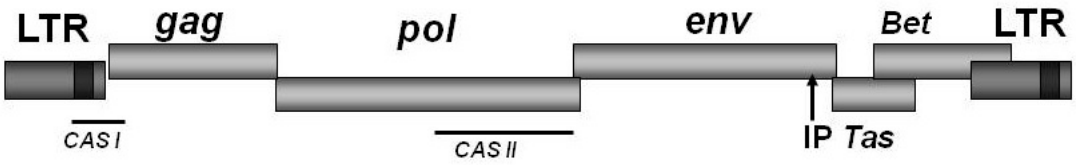

Figure 2. FV genome. Schematic representation of the FV genome with the viral genes *gag*, *pol*, *env*, *Tas* and *Bet*. IP is the internal promoter for the expression of Tas. *CAS I* and *II* depict the discontinuous packaging signal residing in the cis-acting sequences I and II.

Upon entering the cell, the virus will locate near the centrosome and wait, still capsulated, for up to 2 weeks (Lehmann-Che, Renault et al. 2007). Upon the cell dividing, the virion will shed its capsule and the POL protein will coordinate the viral genome integration into the cellular DNA. Therefore, unlike retrovirus vectors, FV does not require immediate cell cycling to integrate its genome so there is no need to stimulate cells to cycle in vitro (Lehmann-Che, Renault et al. 2007). Upon integration the tas transactivator will then drive transcription from the LTR promoter (Meiering and Linial 2001). Transcription of the viral genome begins and the proteins are synthesized. The virions exit the cell via exocytosis using vesicles from the endoplasmic reticulum (Meiering and Linial 2001).

While infection with FV results in cytopathic effects including rapid syncytium formation, cytoplasmic vacuolization, and cell death (Meiering and Linial 2001), current self-inactivating gene transfer vectors have not reported these cytopathic effects (Bauer, Allen et al. 2008; Si, Pulliam et al. 2008; Erlwein and McClure 2010). Foamy viral transmission is not fully understood, although it is thought to be through saliva (Johnson, de la Rosa et al. 1988; Broussard, Comuzzie et al. 1997; Meiering and Linial 2001) and blood (Brooks, Merks et al. 2007). Humans are not typically seropositive for FV unless directly in contact with

non-human primates (Achong and Epstein 1978; Brown, Nemo et al. 1978; Nemo, Brown et al. 1978; Loh, Matsuura et al. 1980; Muller, Ball et al. 1980; Achong and Epstein 1983; Mahnke, Kashaiya et al. 1992; Schweizer, Turek et al. 1995; Ali, Taylor et al. 1996). There is no evidence of horizontal transfer from human to human (Erlwein and McClure 2010). FV results in chronic, persistent infections in both simians and humans (Johnston 1961; Hooks and Gibbs 1975; Johnson, de la Rosa et al. 1988; Kertayadnya, Johnson et al. 1988; Schweizer, Falcone et al. 1997; Heneine, Switzer et al. 1998) and also in certain cell types in culture such as myeloid, lymphoid and erythroid (Cringle, Yu et al. 1996; Mergia, Leung et al. 1996; Mikovits, Hoffman et al. 1996). Chronic infections may mean that the FV gene transfer vector will result in the protein being expressed long-term in the patient's cells.

FV is not associated with any diseases or symptoms in natural hosts or humans and it has broad host and tissue tropism (Mergia, Shaw et al. 1991; Hill, Bieniasz et al. 1999). FV has a large genome, so it can carry and transfer large genes, up to 9.2kb (Lochelt, Zentgraf et al. 1991; Erlwein and McClure 2010). Also, FV has a high genetic stability which is important for gene transfer because the transgene sequence needs to remain intact (Schweizer, Schleer et al. 1999).

Past gene therapy trials for severe combined immunodeficiency using a retrovirus vector resulted in integration by the LIM domain only 2 (LM02) promoter, resulting in leukemia (Hacein-Bey-Abina, Von Kalle et al. 2003). Many recent studies have addressed the integration profile of FV, to determine the risk of insertional mutagenesis. Unlike the gamma-retroviral vectors which have been shown to integrate preferentially near proto-oncogenes, FV has a small preference toward transcription start sites and CpG islands (Trobridge, Miller et al. 2006; Beard, Keyser et al. 2007; Rethwilm 2007; Bauer, Allen et al. 2008), which may mean that FV will be less likely to induce insertional mutagenesis.

Further analysis of the FV integrase protein is ongoing and may lead to a greater understanding of integration and even allow for targeting integration to specific areas. Additionally, development of integration assays to detect activation of nearby genes have also indicated that FV, when combined with a promoter such as the phosphoglycerate kinase (PGK) promoter, is much less likely to result in the activation of nearby genes than retrovirus also with a PGK promoter (Hendrie, Huo et al. 2008).

CHAPTER 2

Materials and methods

Experimental animals

Fanca-/- mice (Cheng, van De Vrugt et al. 2000), *Fancc-/-* mice (Chen, Tomkins et al. 1996) and *Fancg-/-* mice (Yang, Kuang et al. 2001) (C57Bl/6xSV129) were backcrossed 10 generations into a C57BL/6J strain ($CD45.2^+$) as described previously (Haneline, Li et al. 2003; Si, Ciccone et al. 2006). Mice deficient in both *Fancc* and *Fancg* were generated by mating *Fancc+/-* with *Fancg+/-* to obtain double heterozygous mice (F1). These mice were then used to generate the homozygous *Fancc-/-;Fancg-/-* mutant mice (F2).

Congenic wildtype C57BL/6J (WT) mice ($CD45.2^+$) and wildtype B6.SJL-Ptrca Pep/BoyJ (BoyJ) mice ($CD45.1^+$) were originally purchased from Jackson ImmunoResearch Laboratories (Bar Harbor, ME) and are maintained in our animal facility. The Institutional Animal Care and Use Committee of Indiana University approved all studies.

Drug administration and isolation of low-density mononuclear cells

G-CSF (Amgen Corp., Thousand Oaks, CA) was administered at a dose of 3µg subcutaneously (*s.c.*) in 0.1ml phospho-buffered saline/0.1% bovine serum albumin (PBS/0.1% BSA) every 12 hours for four consecutive days as described previously (Broxmeyer, Orschell et al. 2005). Control animals received a similar volume of PBS/0.1% BSA for four consecutive days. AMD3100 (provided by Dr. Gary Bridger, AnorMed, Inc., Langley, British Columbia) was administered at a dose of 5mg/kg *s.c.* 14 hours following the last dose of G-CSF and one hour prior to the collection of PB. Low-density mononuclear cells (LDMC) were isolated from the PB using Ficoll Hystopaque-1119 (Sigma-Aldrich, St. Louis, MO), washed in PBS/0.1% BSA and resuspended in Gibco Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO).

Hematopoietic progenitor assays (colony assays)

Clonogenic methylcellulose assays were performed in triplicate in 35mm plates (Becton Dickinson, Franklin Lakes, NJ). LDMC from 0.1ml PB for mobilization experiments or 2.0 X 10^4 for bone marrow cells (BMC) were cultured

per plate. Cells were cultured in 1.04% methylcellulose (Stem Cell Technologies, Vancouver, BC) in Gibco IMDM (Invitrogen, Carlsbad, CA) supplemented with 30% FBS (Sigma-Aldrich, St. Louis, MO), BioWhittaker penicillin (100U/ml)/streptomycin (100µg/ml) (P/S) (Invitrogen, Carlsbad, CA), 2mM BioWhittaker L-glutamine (Invitrogen, Carlsbad, CA) and 71.5µM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). Assays were supplemented with the following cytokines from Peprotech (Rocky Hill, NJ): 200ng/ml murine stem cell factor, 10ng/ml murine granulocyte macrophage colony-stimulating factor, 5ng/ml murine interleukin 3 and with 4U/ml human recombinant erythropoietin from Amgen (Thousand Oaks, CA). Cells were cultured for 7 days at 37ºC, 5% CO_2 and then scored for the presence of hematopoietic progenitor colonies. When indicated, assays were also supplemented with MMC (Sigma Aldrich, St Louis, MO) or tumor necrosis factor alpha (TNF-α) (Sigma Aldrich, St Louis, MO) at the indicated concentrations as described previously (Haneline, Broxmeyer et al. 1998; Koh, Hughes et al. 1999). In order to count the cells according to hematopoietic cell lineage, the colonies were stained with 1.5mg/ml benzidine in water with 5% glacial acetic acid and 25µl 30% H_20_2.

Competitive repopulation assays

Competitive repopulation experiments were conducted as previously described (Haneline, Li et al. 2003; Broxmeyer, Orschell et al. 2005; Li, Le Beau et al. 2005). LDMC collected from 1 ml (cohort 1) or 2 ml (cohort 2) of the PB of donor WT, *Fanca-/-* or *Fancc-/-* mice treated with G-CSF, AMD3100 or both were transplanted along with 7.5 x 10^5 BoyJ bone marrow LDMC into lethally irradiated recipients. Two independent experiments with 3-5 recipients/genotype/treatment were analyzed.

For the transduced mobilized cells, after transduction cells were washed 2 times with PBS/0.1% BSA. LDMC from the equivalent of 1 ml of peripheral blood were injected into lethally irradiated recipients along with 5 X 10^5 BoyJ bone marrow LDMC (n=7 per group).

For the *Fancc-/-;Fancg-/-* experiments, bone marrow LDMC were isolated using Ficoll Hystopaque-1119 (Sigma-Aldrich, St. Louis, MO), washed in PBS/0.1% BSA and resuspended in Gibco Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO) as described previously (Haneline, Gobbett et al. 1999; Si, Pulliam et al. 2008). The cells were injected into the tail vein of

lethally irradiated recipient mice along with competitor cells from congenic BoyJ mice at the indicated concentrations.

Lethally irradiated recipients

Eight to twelve week old WT were used as recipients. These mice were irradiated (1100 rads) using the gammacell-4 exactor (Nordion, Ottawa, ON, Canada) containing a ^{137}Cs source.

Chimerism

Chimerism was measured at the indicated time points as described previously (Haneline, Li et al. 2003). Briefly, PB was collected from the tail of recipient mice. The samples were treated with red blood cell lysis buffer for 15 minutes and then washed 2 times in PBS/0.1% BSA. They were then stained with FITC-conjugated CD45.2 antibodies (BD Biosciences, San Jose, CA) for 30 minutes at 4ºC in the dark. They were then washed with PBS/0.1% BSA once and the percentage of positive cells was determined by flow cytometry. Mean donor chimerism was analyzed to evaluate for significant differences between groups.

Secondary transplantation

Four months after transplantation, LDMC were collected from the bone marrow of primary recipients and 2 x 10^6 LDMC were transplanted into lethally irradiated recipients to test self-renewal capacity as described previously (Broxmeyer, Orschell et al. 2005).

Multi-lineage flow analysis

Multi-lineage analysis was performed on the indicated cell population using FITC-conjugated CD45.2 and PE-conjugated CD3 or Mac-1 and APC-conjugated Gr-1 or B220 antibodies (BD Biosciences, San Jose, CA). The percentage of cells double positive for CD45.2 and the specific lineage marker was determined using flow cytometry.

Repopulating units

Repopulating units (RU) were calculated using the following equation (Harrison 1980; Haneline, Li et al. 2003; Li, Le Beau et al. 2005): RU = (competitor number x % donor chimerism)/(100 - % donor chimerism).

Statistical analysis

The indicated statistical test was performed using GraphPad Prism 4. (La Jolla, CA).

Foamyviral vectors

The foamyviral constructs used in our studies were derivatives of the MD9 construct (Heinkelein, Dressler et al. 2002), 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 8) 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 (Heinkelein, Dressler et al. 2002). A linker was cloned into the Not I 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 BamH I and Spe I, thereby creating the MD9-*EGFP* construct. The human *FANCC* cDNA was cloned into the BamH I site resulting in the MD9-*FANCC/EGFP* vector.

Foamy viral production

Self-inactivating FV vectors were produced by transfection of 293T cells. Briefly, 8 X 10^6 293T were plated on 10cm tissue culture treated plates. The following morning, media was aspirated off and replaced with 4ml fresh Gibco high glucose Dulbecco's modified eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 15% FBS (heat inactivated) (Sigma-Aldrich, St. Louis, MO), 2mM BioWhittaker L-glutamine (Invitrogen, Carlsbad, CA) and 0.15% BioWhittaker sodium bicarbonate. Per plate, 5µg pcoGAG, 1µg pcoPOL, 1µg pcoPE01 and 10µg of the vector plasmid were mixed. The volume was brought up to 1ml per plate with plain high glucose DMEM. Polyethylenimine (1mg/ml) was diluted to 51µg/ml with plain high glucose DMEM. 1ml of 51µg/ml polyethylenimine was added to the plasmid mix for each plate, flash vortexed and incubated at room temperature for 20 minutes. After incubation, 2ml was added to each plate. The plates were incubated overnight at 37ºC, 5% CO_2. The next day the media was changed on the plates (high glucose DMEM supplemented with 10% FBS (heat inactivated), 2mM BioWhittaker L-glutamine (Invitrogen, Carlsbad, CA), 100U P/S) and they were again incubated overnight at 37ºC, 5% CO_2. The next morning the supernatant was collected and filtered through a

45µm low protein-binding filter. High-speed centrifugation (>15000g, 2 hours) was performed to concentrate the virus, which was then resuspended in media (high glucose DMEM supplemented with 10% FBS (heat inactivated), 2mM BioWhittaker L-glutamine (Invitrogen, Carlsbad, CA), 100U P/S). Aliquots were taken for the titer and remaining samples were frozen at -80ºC.

Viral titer

The following titer procedure is used to determine the number of infectious viral particles (IP) present in the supernatant. The day prior to collection a set number of HT1080 cells are plated. The next morning, the virus is collected, filtered, and concentrated. An aliquot is taken for serial dilution. Each of the serial dilution aliquots is flash frozen and then thawed and added to the HT1080 cells. The flash freeze allows for close approximation of how many IP will be present when the frozen samples are thawed for use. The media is changed after 24 hours. At 48 hours post transduction the cells are collected and the percentage of cells that are GFP^+ is determined using flow cytometry. This percentage is used to calculate how many IP were originally present: % GFP^+ X starting cell # X 1/(dilution factor) = IP/ 1 ml. Now that the number of IP per ml in our frozen

samples of virus is known, a specific multiplicity of infection (MOI) to transduce our target cells can be calculated. This is a measure of how many IP are present per cell. The titers of the viral supernatant were 1-5 x 10^7 viral particles/ml for MD9-*EGFP* and 4-10 x 10^6 viral particles/ml for MD9-*FANCC/EGFP* construct after concentration.

Foamyvirus-mediated transduction

The mobilized LDMC were washed and resuspended in the viral supernatant (MOI=1) supplemented with murine interleukin 6 (200U/mL) and murine stem cell factor (100ng/mL) (both from Peprotech, Rocky Hill, NJ) and incubated on the recombinant human fibronectin fragment CH296, RetroNectin™ (TAKARA BIO INC., Otsu, Japan) as described previously (Hanenberg, Batish et al. 2002). Cells were incubated at 37ºC with 5% CO_2. Cells were harvested 14 hours following transduction and washed with PBS/0.1% BSA.

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'-ATGGTGAGCAAGGGCGAGGAG-3', Reverse 5'-AAGTCGTGCTGCTTCATG TG-3' with the following program: 95ºC, 5 minutes; 95ºC, 40 seconds; 55ºC, 30 seconds; 72ºC, 1 minute; cycled to step 2 for 31 cycles; 72ºC, 10 minutes and then stored at 4ºC and analyzed on a 1% agarose gel. The amplified product has a size of approximately 250bp. In addition, PCR for the genotype of the progenitor cells was performed. Three primers are used: 5'-GAGCAACACAAAT GGTAAGG-3', 5'-CCTGCCATCTTCAGAATTGT-'3 and 5'-TTGAATGGAAGGAT TGGAGC-3' with the following program: 95ºC, 5 minutes; 95ºC, 30 seconds; 55ºC, 2 minutes; 72ºC, 1.5 minutes; cycled to step 2 for 31 cycles; 72ºC, 10 minutes and then stored at 4ºC and analyzed on a 1% agarose gel. The amplified product of the WT copy of the *Fancc* gene is approximately 800bp, whereas the knockout gene PCR product is approximately 600bp.

Southern blot for junctional fragment analysis

Genomic DNA from bone marrow and spleen specimens was isolated using phenol-chloroform extraction and digested with Xho I (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 Pst I (New England BioLabs, Ipswich, MA) and the 1655bp fragment was isolated using QIAquick gel extraction kit (Qiagen, Valencia, CA), labeled using the Prime-It II Random Primer labeling kit (Stratagene, La Jolla, CA) and purified using a micro-spin 30 column (Bio-Rad, Hercules, CA). The membrane was pre-hybridized for 2 hrs at 42ºC with the hybridization solution (6X SSC, 50% Formamide, 5X Denhardt's, 0.5% SDS in water) supplemented with 100µg/ml denatured salmon sperm DNA (Stratagene, La Jolla, CA). After pre-hybridization, the membrane was hybridized for 16 hrs at 42ºC with the hybridization solution supplemented with 100µg/ml denatured salmon sperm DNA (Stratagene, La Jolla, CA) and denatured labeled probe. The next day, the membrane was washed four times for 15 minutes at 42ºC with the wash solution (2X SSC, 0.1% SDS in water) and exposed to film (BioMax MS film Kodak, Rochester, NY) at -80ºC with a Cronex Lightning Plus intensifying screen (DuPont, Wilmington, DE).

Complete blood counts and chimerism

PB was collected from the tail vein of mice and stored in microtainer brand tubes with EDTA (Becton Dickinson, Franklin Lanes, NJ). Complete blood counts were performed using a Hemavet950 (Drew Scientific Group, Dallas, TX). The remaining blood was then treated with red cell lysis buffer (Gentra Systems, Minneapolis, MN) for 15 minutes at room temperature. The cells were washed in PBS/0.1% BSA and split into 2 aliquots. Each aliquot was stained with either FITC-conjugated CD45.2 or CD45.1 antibodies (BD PharMingen) at 4ºC for 20 minutes. The cells were then washed in PBS/0.1% BSA and analyzed by flow cytometry.

Histology

Spleens and humeri or tibiae from selected animals were fixed in 10% buffered formalin and embedded in paraffin. Sections were obtained and stained with hematoxylin and eosin (H&E). All histological sections were analyzed in a blinded fashion.

Microscopy

Pictures were taken on a Zeiss Axioscope (Zeiss, Ontario, NY) with the Plan Neofluar 20x/0.50 or the Ph3 Neofluar 40X/1.30 oil (Zeiss, Ontario, NY). 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, McHenry, IL).

Cytogenetic Analysis

Cytogenetic analysis was performed on BMC from non-competitive transplantation recipients as described (Lee, Warburton et al. 1990). Briefly, short-term, synchronized cultures were initiated and metaphase cells were prepared using standard cytogenetic techniques following mitotic arrest with 1% colcemid (10µg/ml, Invitrogen Life Technologies, Grand Island, NY). Between five and 15 G-banded metaphase cells were completely analyzed for each culture. Mouse chromosomes were classified utilizing standardized mouse chromosome ideograms and following nomenclature standards of the International Committee on Standard Genetic Nomenclature for Mice (http://www.informatics.jax.org/mgihome/nomen/anomalies.shtml).

SKY analysis

Spectral karyotyping (SKY) was performed utilizing mouse reagents (SkyPaint for mouse, Applied Spectral Imaging (ASI), Carlsbad, CA) and following the manufacturer's protocol for mouse chromosome analysis with the exception of a longer hybridization time of 42 hours. Fluorochromes utilized included: rhodamine, Texas red, Cy5, FITC and Cy5.5. An interferogram for each metaphase was generated using a SD200 Spectracube (ASI) mounted to a Leica DM 5000 fluorescence microscope. Three to ten metaphase cells were captured and karyotyped for each culture.

RNA isolation and GeneChip Array

RNA was isolated using the RNeasy Mini Kit (Qiagen, Germantown, MD) from whole BMC obtained from 3-month-old syngeneic mice (WT, *Fancc-/-*, *Fancg-/-* and *Fancc-/-;Fancg-/-*). Microarray assays were performed in the Affymetrix Microarray Core, a unit of the Gene Microarray Shared Resource located in the Knight Cancer Institute at Oregon Health and Science University, Portland, OR. Hybridization targets were prepared from 2µg total RNA using the one cycle cDNA, IVT amplification/labeling protocol procedure as described in

the Affymetrix GeneChip Expression Analysis Technical Manual, rev.5 (Affymetrix, Santa Clara, CA). After fragmentation, each sample target was hybridized to an MOE 430 2.0 GeneChip array, a chip containing 45,000 probe sets that measure the expression level of >39,000 transcripts and variants from >34,000 well-characterized mouse genes (Affymetrix, Santa Clara, CA). Scanning was performed with the Affymetrix 3000 GeneArray Scanner and image processing and expression analysis were performed using Affymetrix GeneChip Operating Software (GCOS) v1.2 software. The quality of project hybridizations was assessed using: 1) performance metrics extracted from the GCOS report file for each array and 2) pair-wise scatter plots of the 'absolute analysis' data from each array assay.

Microarray Data Management and Analysis

At the 1.5 fold threshold in double mutant cells, 3,197 genes were differentially expressed in the pairwise comparison with WT cells. Only 520 and 304 genes were differentially expressed (as compared to WT) in *Fancc* and *Fancg* single mutant cells, respectively. Of the 520 (*Fancc* mutant), 263 were found in the set of 3,197 genes. Of the 304 (*Fancg* mutant), 216 were found in

the set of 3,197. The processed image files (.CEL) were imported into GeneSifter (Seattle, WA) and Partek Genomics Suite (Partek, Inc., St. Louis, MO) software programs. Hierarchical clustering was performed using GeneSifter for multiple data sets filtered by thresholds of 1.5, 2, 3, 4 and 5-fold using ANOVA ($p<0.01$) and the Benjamini-Hochberg correction for multiple comparisons. The dendrograms for each threshold were identical (Figure 13E shows the dendrogram for the 1.5-fold threshold). Principal component analysis was performed using both GeneSifter and Partek Genomics Suite (Figure 13F shows the results, for the results filtered at the 1.5-fold threshold, displayed using the Partek ellipsoid graphic tool).

CHAPTER 3

AMD3100 synergizes with G-CSF to mobilize repopulating stem cells in Fanconi anemia knockout mice

Introduction

FA patients usually present with BMF during childhood, making bone marrow aspiration a difficult process. Mobilization of stem cells from the bone marrow into the PB for collection would be ideal. G-CSF is an FDA approved therapy utilized for mobilization of HSC/HPC for transplantation in a variety of clinical settings (Mohle and Kanz 2007). However, patients with FA show a markedly decreased response to G-CSF (Croop, Cooper et al. 2001). The inability of G-CSF to adequately mobilize HSC/HPC in FA patients has prompted the search for alternative methods.

The interaction between bone marrow stroma and HSC via SDF-1 and CXCR4, respectively, plays an important role in HSC chemotaxis and retention within the marrow (Lapidot and Kollet 2002). AMD3100, a bicyclam derivative, is a competitive antagonist of this interaction and thus serves as a potential agent for HSC/HPC mobilization (Broxmeyer, Orschell et al. 2005). AMD3100 works

synergistically with G-CSF to induce mobilization of the following: wild type murine HSC, non-obese diabetic/severe combined immunodeficiency (NOD/SCID) repopulating cells from healthy human donors and HPC from *Fancc-/-* mice (Broxmeyer, Orschell et al. 2005). Further, during phase III clinical trials, AMD3100 has been administered to patients with multiple myeloma and non-Hodgkin's lymphoma and has been shown to be an efficacious and safe regimen for stem cell mobilization and gained FDA approval in December 2008 (DiPersio, Stadtmauer et al. 2009; Stiff, Micallef et al. 2009).

There is also evidence that AMD3100 may be useful as a chemosensitizing agent in acute myeloid leukemia (Nervi, Ramirez et al. 2009) and it is in phase I/II trials for chemosensitization for chronic myelogenous leukemia (Pusic and DiPersio 2010). Use of AMD3100 alone in healthy patients is also being studied (Devine, Vij et al. 2008). Many more studies of AMD3100 are currently ongoing (Pusic and DiPersio 2010).

Here, the effect of AMD3100 and G-CSF on HSC mobilization is examined in FA types A and C murine models. The data show that the combination of AMD3100 and G-CSF works synergistically to mobilize HSC with long-term repopulating ability and self-renewal capacity in two murine models of FA.

Results

In previous studies, increased mobilization of hematopoietic progenitors in *Fancc-/-* mice was assessed; however, the broad applicability of this approach to other FA genotypes was not determined. Given potential subtle differences in the phenotypes of FA genotypes in patients and various FA knockout mice, these studies were validated and extended in *Fanca-/-* mice, the murine homologue of the most prevalent FA complementation group in patients. Analogous to previous studies in WT and *Fancc-/-* mice, administration of G-CSF and AMD3100 resulted in at least an additive mobilization of lineage restricted and multipotent hematopoietic progenitors as compared to use of either compound alone (Figure 3). Furthermore, an increase in the mobilization of lineage restricted and multipotent progenitors was observed in *Fanca-/-* and *Fancc-/-* mice.

Previous studies in *Fancc-/-* mice examined the mobilization of progenitors but not repopulating HSC (Broxmeyer, Orschell et al. 2005). Given that mobilization of HSC is required for effective gene therapy in FA, competitive repopulation was used to evaluate the ability of G-CSF and AMD3100 alone or in combination to mobilize repopulating stem cells in WT, *Fanca-/-* and *Fancc-/-* mice. A schematic of the experimental design is shown in Figure 4.

Figure 3

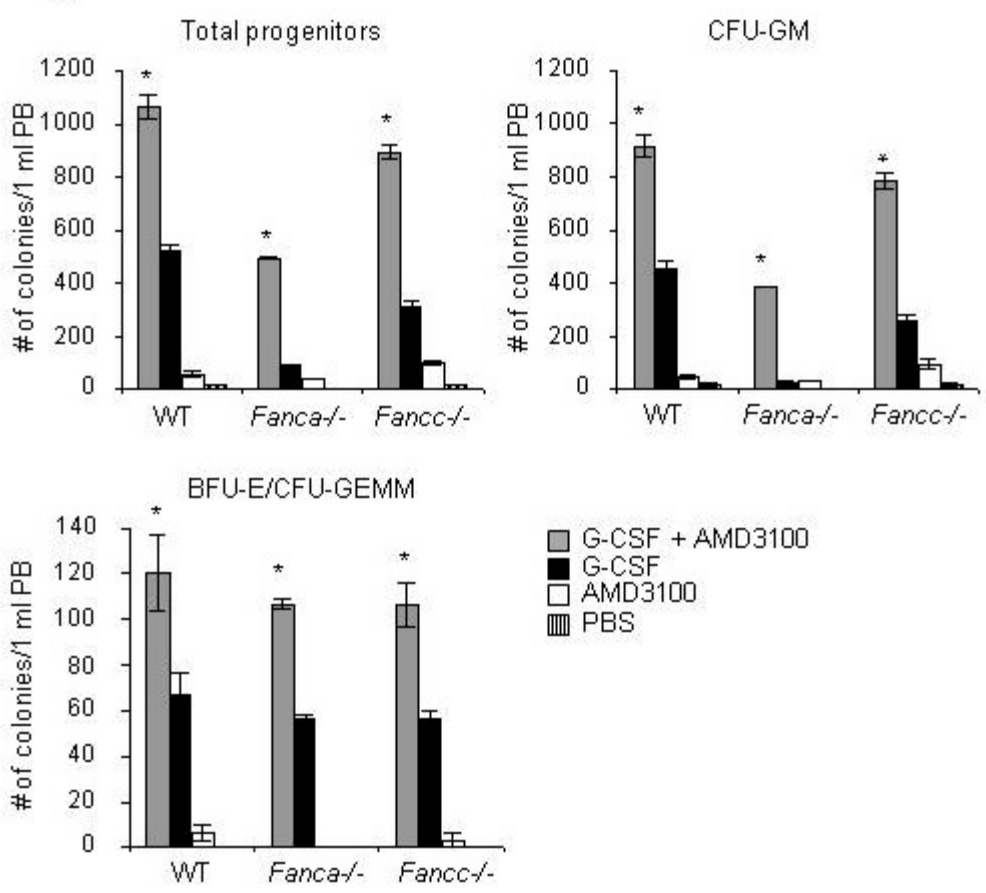
Total progenitors
CFU-GM
BFU-E/CFU-GEMM
# of colonies/1 ml PB
1200
1000
800
600
400
200
0
140
120
100
80
60
40
20
WT
Fanca-/-
Fancc-/-
G-CSF + AMD3100
G-CSF
AMD3100
PBS

Figure 3. Mobilization of HPC is enhanced when both G-CSF and AMD3100 are used. The mean total number of HPC/ml PB ± SEM of the indicated lineages and genotypes are shown. n=3/genotype, *p<0.05 by ANOVA with Bonferroni correction.

Figure 4

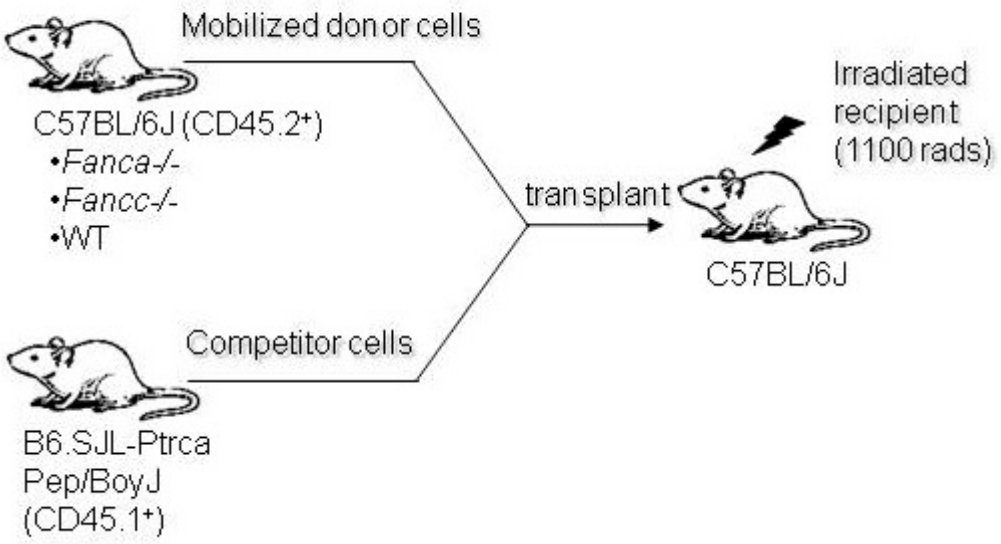
Mobilized donor cells
C57BL/6J (CD45.2+)
•Fanca-/-
•Fancc-/-
•WT
Competitor cells
B6.SJL-Ptrca
Pep/BoyJ
(CD45.1+)
transplant
Irradiated
recipient
(1100 rads)
C57BL/6J

Figure 4. Competitive repopulation. Mobilized donor cells ($CD45.2^+$) are collected from the PB following mobilization and transplanted along with BoyJ competitor BMC ($CD45.1^+$) into lethally irradiated recipients. The percentage of donor-derived cells in the PB was measured monthly.

Lethally irradiated recipients received mobilized peripheral blood cells from donor mice ($CD45.2^+$) along with Boy J ($CD45.1^+$) competitor BMC. The amount of competitor cells transplanted was sufficient to rescue lethally irradiated recipients approximately 30% of the time, any survival higher than this can be attributed to rescue from the donor cell population. For the *Fanca-/-* and *Fancc-/-* genotypes, there was a significant increase in survival when both G-CSF and AMD3100 were used to mobilize the transplanted cells as compared to either agent alone (Figure 5).

CD45.1 and CD45.2 chimerism was analyzed monthly in order to determine the competitive repopulating ability of mobilized test cells. The repopulating ability of fresh BMC from the three respective genotypes was also calculated (Figure 6 and Table I). As expected, the repopulating ability of the BMC from *Fanca-/-* and *Fancc-/-* mice was only 35-40% of syngeneic WT controls (Table I). Strikingly, there was a much greater defect in the repopulating activity of *Fanca-/-* and *Fancc-/-* PB cells following mobilization with G-CSF alone (15-30% of WT controls) analogous to the paucity of $CD34^+$ phenotypic cells mobilized using G-CSF only in FA patients (Croop, Cooper et al. 2001) and the reduced numbers of lineage restricted and multipotent progenitors.

Figure 5

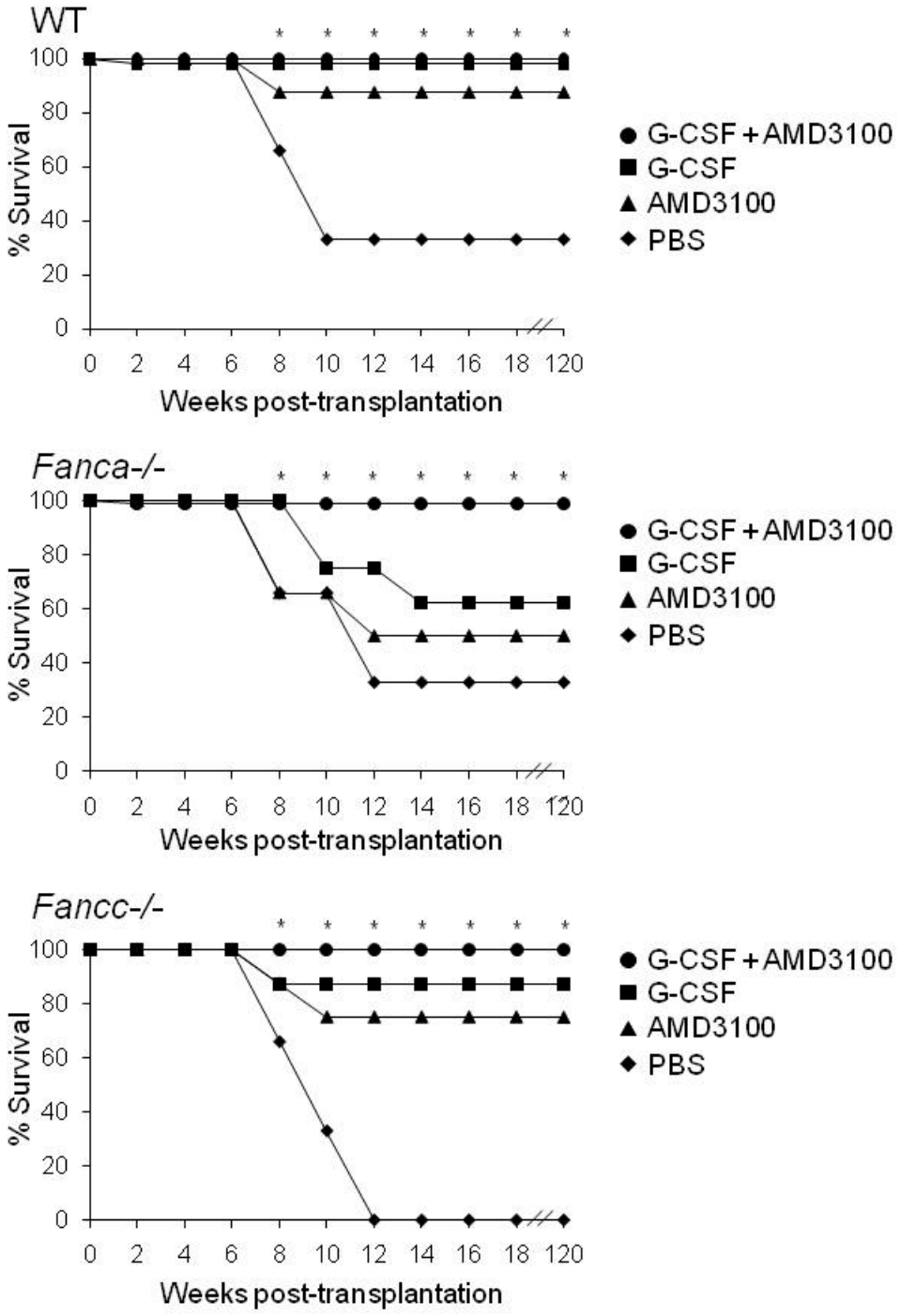
WT
100
80
60
40
20
0
% Survival
0 2 4 6 8 10 12 14 16 18 120
Weeks post-transplantation
G-CSF + AMD3100
G-CSF
AMD3100
PBS
Fanca-/-
100
80
60
40
20
0
% Survival
0 2 4 6 8 10 12 14 16 18 120
Weeks post-transplantation
G-CSF + AMD3100
G-CSF
AMD3100
PBS
Fancc-/-
100
80
60
40
20
0
% Survival
0 2 4 6 8 10 12 14 16 18 120
Weeks post-transplantation
G-CSF + AMD3100
G-CSF
AMD3100
PBS

Figure 5. Transplantation with HSC/HPC mobilized by both AMD3100 and G-CSF resulted in an increase in survival. Transplantation with HSC/HPC mobilized by both AMD3100 and G-CSF resulted in an increased survival over time as compared to either AMD3100 alone or PBS for each respective genotype by log-rank test for trend. n=8, *$p<0.05$.

Figure 6

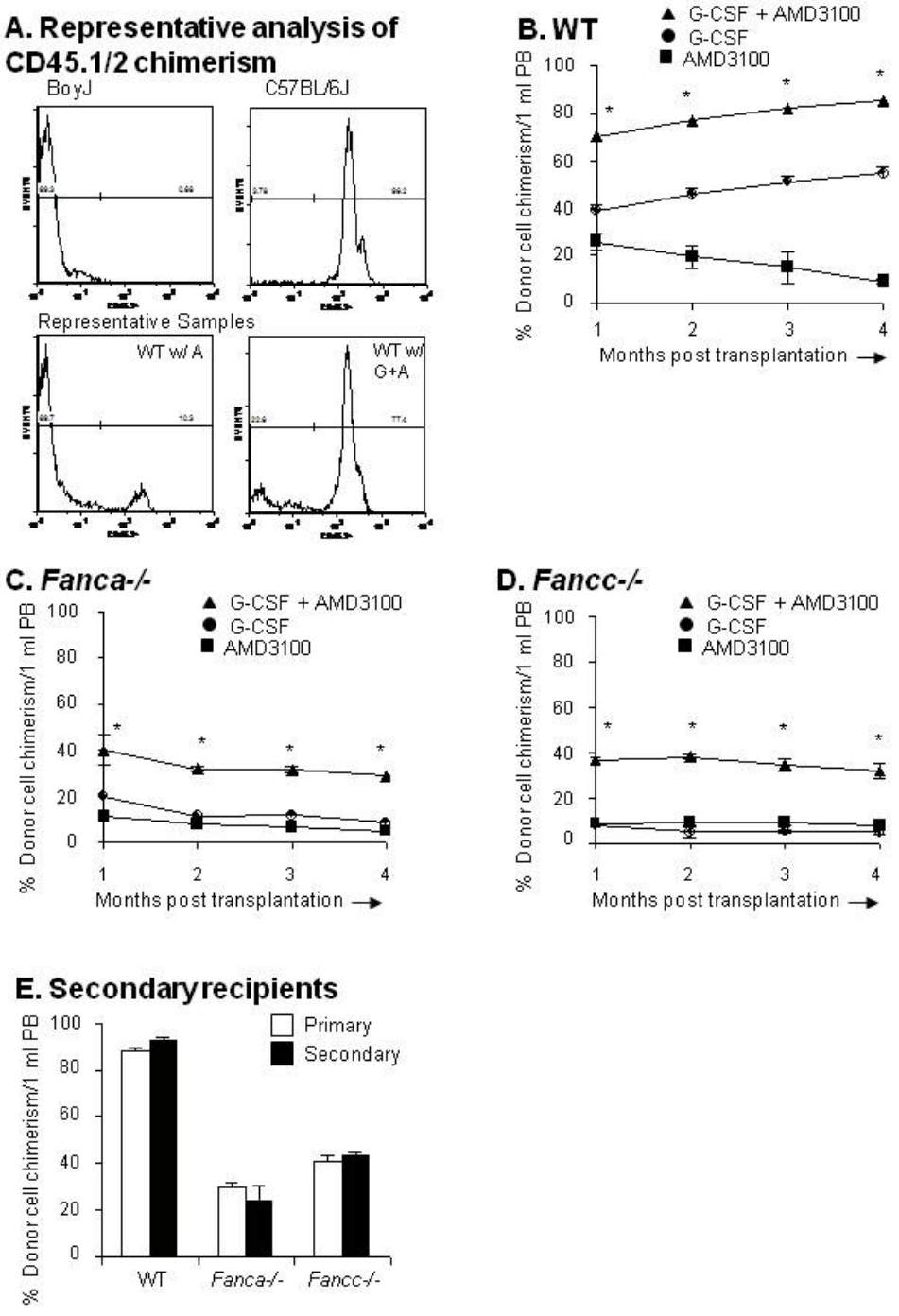
A. Representative analysis of CD45.1/2 chimerism
BoyJ
C57BL/6J
Representative Samples
WT w/ A
WT w/ G+A
B. WT
G-CSF + AMD3100
G-CSF
AMD3100
% Donor cell chimerism/1 ml PB
Months post transplantation
C. Fanca-/-
D. Fancc-/-
E. Secondary recipients
Primary
Secondary
WT
Fanca-/-
Fancc-/-

Figure 6. Mobilization of HSC is enhanced when G-CSF and AMD3100 are used in combination. Flow cytometry analysis was used to determine percentage of donor-derived chimerism in the PB of recipient mice (A). Mean percent donor cell chimerism of each genotype and mobilization group are indicated (B-D). Use of both AMD3100 and G-CSF resulted in a significant increase in chimerism as compared to either agent alone for all genotypes at all time points. Results are shown as mean ± SEM. n=5; *$p<0.05$ by ANOVA. (E) Secondary transplantations showed no significant change in chimerism, implying the mobilized cells had self-renewal capacity. Results depict mean chimerism 4 months post transplantation for primary recipients (white bars) receiving G-CSF plus AMD3100 mobilized cells and mean chimerism 4 months post transplantation for secondary recipients (black bars). n=3 for primary recipients, n=6 for secondary recipients, mean ± SEM, $p>0.05$.

Table I. Repopulating units of bone marrow and mobilized cells from WT, *Fanca-/-* and *Fancc-/-* mice.

	BM	RU/1 ml PB		
	RU/Femur	G-CSF	AMD3100	G-CSF & AMD3100
WT	72.30±0.17	4.12±0.89	1.90±0.62	10.66±0.58
Fanca-/-	25.29±0.06	0.67±0.03	0.51±0.13	2.57±0.12
Fancc-/-	26.02±0.10	1.23±0.57	0.47±0.12	3.21±0.32

Though stem cells mobilized with AMD3100 alone resulted in low chimerism in all experimental groups (Figure 6B-D), addition of G-CSF to AMD3100 resulted in a greater than additive increase in the chimerism and repopulating ability of *Fanca-/-* and *Fancc-/-* HSC (Figure 6C-D, Table I).

To verify that repopulating HSC with self-renewal capacity were mobilized to PB, primary recipients were sacrificed four months following transplantation and bone marrow LDMC from these recipients were transplanted into lethally irradiated secondary recipients. No significant change in chimerism occurred following transplantation into secondary recipients (Figure 6E). To determine whether the donor cells contributed to multiple blood lineages, multi-color flow cytometry was performed. Analysis of $CD45.2^{+}$ PB cells with B220, CD3, Gr-1 and Mac-1 antibodies demonstrated the presence of multi-lineage lymphoid and myeloid derived $CD45.2^{+}$ donor cells. An analysis from a representative recipient is shown in Figure 7 and the data is summarized in Table II. Collectively, the results support the hypothesis that AMD3100 plus G-CSF resulted in mobilization of long-term, multi-lineage, self-renewing HSC.

Figure 7

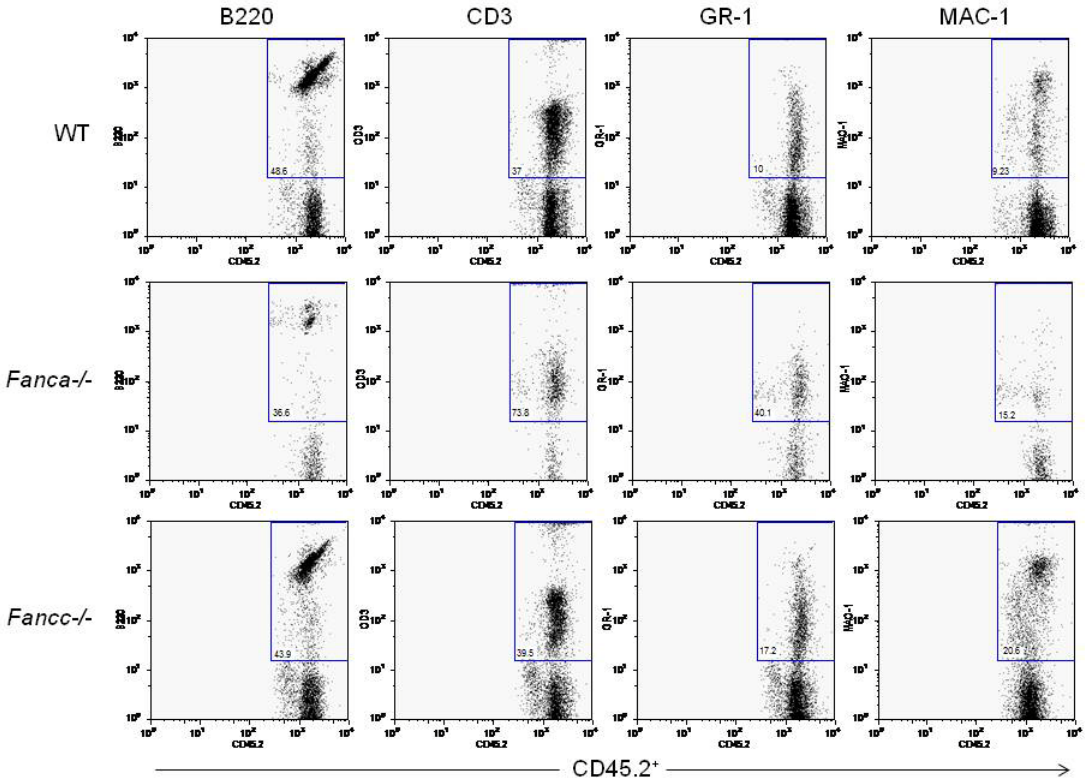
B220
CD3
GR-1
MAC-1
WT
Fanca-/-
Fancc-/-
48.6
37
10
9.23
36.6
73.8
40.1
15.2
43.9
39.5
17.2
20.6
CD45.2
CD45.2+

Figure 7. G-CSF plus AMD3100 mobilized HSC are capable of sustaining multi-lineage reconstitution. CD45.2^{+} PB cells from primary recipients receiving WT, *Fanca-/-* or *Fancc-/-* HSC mobilized with G-CSF and AMD3100 were analyzed for CD3, B220, Gr-1 and Mac-1 expression. Results depicted above are representative samples gated on CD45.2^{+} in order to show the percentage of CD45.2^{+} PB cells expressing the respective antigen.

Table II. Multi-lineage differentiation of AMD3100 and G-CSF mobilized PB cells in recipient mice. CD45.2^{+} PB cells from primary recipients receiving either WT, *Fanca-/-* or *Fancc-/-* mobilized HSC were analyzed for CD3, B220, Gr-1 and Mac-1 expression 4 months post transplantation. Results depicted are mean percentages of chimeric CD45.2^{+} PB cells expressing the respective antigen ± SEM. n=5.

	CD3	B220	GR-1	MAC-1
WT	21 ± 6	24 ± 7	31 ± 7	24 ± 7
Fanca-/-	26 ± 4	22 ± 6	21 ± 6	30 ± 5
Fancc-/-	29 ± 4	34 ± 3	16 ± 5	30 ± 6

Discussion

FA remains a complex genetic disorder with significant morbidity and mortality (Kutler, Singh et al. 2003) and few treatment options (Gluckman, Auerbach et al. 1995). There is a great need to develop other treatments. Genetic correction and transplantation of autologous HSC is a potential therapy; however, mobilization of adequate HSC in FA patients is a key prerequisite for non-invasive collection. Previous trials in FA patients using extended G-CSF administration mobilized a limited number of $CD34^+$ cells (Croop, Cooper et al. 2001). Whether the relative inability of G-CSF to mobilize $CD34^+$ cells in FA patients is a consequence of bone marrow hypoplasia or a defect in stem cell/stromal cell interactions is unclear. This question is difficult to address in human patients; however, the availability of syngeneic FA knockout mice provided the opportunity to address this issue in a preclinical setting.

Here, the repopulating ability was quantitatively examined for the bone marrow and the mobilized PB HSC in syngeneic FA and WT mice. As expected from previous studies (Carreau, Gan et al. 1999; Haneline, Gobbett et al. 1999; Si, Ciccone et al. 2006), both *Fanca-/-* and *Fancc-/-* mice had a significant decrease in bone marrow repopulating ability as compared to the repopulating

ability of WT mice. However, in response to G-CSF alone, this decrease in repopulating ability was accompanied by an additional decrease in the proportion of repopulating units mobilized from the bone marrow to the peripheral blood. Our results demonstrate that mobilization with both AMD3100 and G-CSF results in a synergistic increase in the mobilization of long-term, multi-potential, self-renewing HSC. These studies did not directly compare the repopulating ability when equal numbers of mobilized cells were transplanted from the different treatment groups. This experiment would be an interesting future direction to determine if the mobilized FA cells have an engraftment and repopulation advantage as compared to FA BMC as has been shown for normal mobilized cells (Nademanee, Sniecinski et al. 1994; Schmitz, Linch et al. 1996; Champlin, Schmitz et al. 2000). Additionally, in the *Fancc-/-* cells the multi-lineage analysis showed a lymphoid bias that is sometimes associated with aging (Gruver, Hudson et al. 2007; Miller and Cancro 2007). It is now necessary to address the potential of these mobilized cells as targets for gene transfer in FA murine models and in human NOD-SCID repopulating cells.

Data from this study demonstrate that mobilization of PB repopulating stem cells with G-CSF is clearly defective in FA knockout mice. One possibility is

that FA deficient HSC have an inability to respond to a number of chemokines that are important for stem cell mobilization. However, in preliminary studies, the responsiveness of myeloid progenitors to a number of cytokines and chemokines (including stem cell factor, G-CSF and stromal cell-derived factor-1) did not display such a phenotype (unpublished data, 2007). Alternatively, recent data show that HSC/HPC mobilization is dependent on a complex model involving both protease dependent and independent mechanisms (Robinson, Pisarev et al. 2003; Levesque, Liu et al. 2004; Pelus, Bian et al. 2004; Robinson, Seina et al. 2005; Lee, Wu et al. 2009; Jalili, Shirvaikar et al. 2010). Given our observations in these studies and the fidelity with which the murine models recapitulate the human mobilization defects, the FA murine models used here may be useful in future studies to discern the basic mechanisms underlying this clinical phenotype.

CHAPTER 4

Overnight transduction with FV vector containing the *FANCC* gene restores the long-term repopulating activity of mobilized *Fancc-/-* cells

Introduction

Spontaneous genetic correction of a germline mutation leading to repopulation of the entire hematopoietic system with normal progeny has been identified in a few FA patients (Waisfisz, Morgan et al. 1999; Gregory, Wagner et al. 2001; Gross, Hanenberg et al. 2002; Soulier, Leblanc et al. 2005; Mankad, Taniguchi et al. 2006). Additionally, in mice with targeted disruptions of FA genes, the hematopoietic system can be functionally corrected by retroviral vectors expressing human analogues of the targeted mouse genes in stem cells (Gush, Fu et al. 2000; Galimi, Noll et al. 2002; Haneline, Li et al. 2003). These observations have led to three clinical stem cell gene therapy phase I studies in *FANCA*-/- and *FANCC*-/- patients. So far, neither long-term marking/correction of cells nor clinical benefits for the patients were observed (Liu, Young et al. 1997; Kelly, Radtke et al. 2007). Due to the biological characteristics of the gammaretroviral vectors used for transduction of stem cells (Roe, Reynolds et al.

1993; Trobridge and Russell 2004), optimal gene transfer protocols for delivery of genes to mammalian stem cells require a pre-stimulation period of 1-2 days with cytokines that promote the proliferation and survival of stem/progenitor cells. This is followed by a 2-3 day exposure of the target cells to vector containing supernatant on the recombinant fibronectin fragment CH-296 (Hanenberg, Xiao et al. 1996; Hanenberg, Hashino et al. 1997). This gene transfer protocol was successful in transducing hematopoietic stem cells in humans, primates/monkeys and mice (Cavazzana-Calvo, Lagresle et al. 2005; Macchiarini, Manz et al. 2005; Trobridge, Beard et al. 2005). However, in murine FA models, prolonged in vitro culture of *Fancc-/-* bone marrow results in a length-of-culture dependent reduction in myeloid progenitors and repopulating ability (Haneline, Gobbett et al. 1999; Li, Le Beau et al. 2005), and the surviving untransduced *Fancc-/-* repopulating cells have an increased risk of developing cytogenetic abnormalities and myeloid malignancies (Haneline, Li et al. 2003). Therefore, limiting the in vitro culture would be predicted to both enhance the efficacy and the safety for genetic therapies of FA stem cells.

Our lab has previously demonstrated the ability of FV vectors encoding the human *FANCC* transgene to completely correct murine *Fancc-/-* myeloid

progenitors and repopulating hematopoietic stem cells in a 14-hour transduction protocol without prestimulation. This short gene transfer protocol resulted in 1-2 proviral integrations in the reconstituting stem cells and was not associated with the development of myelodysplastic syndromes (MDS) or acute myeloid leukemia (AML) in *Fancc-/-* stem cells transduced with the reporter construct (Si, Pulliam et al. 2008). These characteristics support the hypothesis that FV vectors are a viable strategy for stem cell gene transfer strategies in FA.

However, very little research has been done on the ability of FV to transduce mobilized HSC (Josephson, Vassilopoulos et al. 2002; Kiem, Allen et al. 2007). There is evidence that the mobilized cell population does not respond to transduction in the same manner as the BMC population (Hematti, Sellers et al. 2003; Thomasson, Peterson et al. 2003), perhaps due to mobilization's effects on cell surface receptors or other biochemical changes in the cells. Therefore, the transduction of mobilized HSC/HPC in our murine model has been studied.

Results

Our lab has previously developed a FV construct (Si, Pulliam et al. 2008) derived from the pMD9 construct, a gift from Axel Rethwilm (Würzburg, Germany). In our construct, the coding capabilities of all of the FV genes and the enhancer elements have been eliminated by complete or partial deletions. There is a remaining non-coding 5' region of GAG and 3' region of POL, which retain the packaging signals (CAS I and II) that are essential for the production of the FV particles. The encephylomyelocarditis (EMCV) internal ribosome entry site (IRES) and the *enhanced green fluorescence protein* (*EGFP*) cDNAs were cloned into the construct (MD9-*EGFP)* (Figure 8). The *hFANCC* cDNA was cloned into the MD9-*EGFP* construct under the spleen focus forming virus (SFFV) promoter (MD9-*FANCC/EGFP)* (Figure 8).

These plasmid constructs are transfected using polyethylenimine into 293T cells along with the helper plasmids: pcoPOL, pcoGAG and pcoPE01; which provide the coding sequences for the polymerase, structural and envelope proteins respectively.

Figure 8

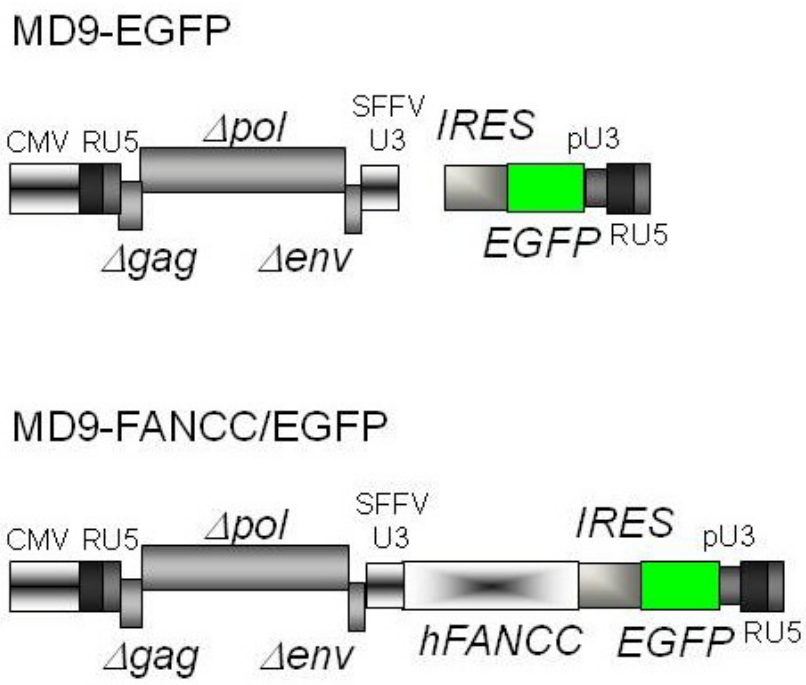
MD9-EGFP
CMV
RU5
Δpol
SFFV
U3
IRES
pU3
Δgag
Δenv
EGFP
RU5
MD9-FANCC/EGFP
CMV
RU5
Δpol
SFFV
U3
IRES
pU3
Δgag
Δenv
hFANCC
EGFP
RU5

Figure 8. Structure of the recombinant FV self-inactivating vectors MD9-*FANCC/EGFP* and MD9-*EGFP*. These vectors contain deletions in *gag*, *pol*, *env* and the U3 region of the 3' LTR. SFFV U3 is the promoter of the spleen focus-forming virus U3 region driving the human *FANCC* and the *EGFP* cDNAs (Si, Pulliam et al. 2008).

None of the helper plasmids contain the packaging sequences, therefore only the MD9-*EGFP* or MD9-*FANCC/EGFP* construct RNA should be packaged into the FV virions. This, along with the deletion of the Tas transactivator, means that the virions should be replication incompetent.

The supernatant is collected, filtered, concentrated and either used fresh or frozen. For these experiments an MOI of 10 was used. The separation of the POL/GAG onto two separate plasmids and the isolation and cloning of a new envelope (pcoPE01) by our collaborator, Dr. Helmut Hanenberg are changes from our previous work (Si, Pulliam et al. 2008).

Our results show that the mobilized *Fancc-/-* cells transduced with the reporter construct had a significantly decreased number of progenitors as compared to the *Fancc-/-* cells corrected with the transgene (Figure 9). Additionally, the colony forming ability was corrected to levels comparable to WT (Figure 9). While this seems very promising, from this experiment the transduction efficiency was not determined. However, a portion of these cells was used for the following competitive repopulation experiments and post-transplantation assays were used to determine the transduction efficiency.

Figure 9

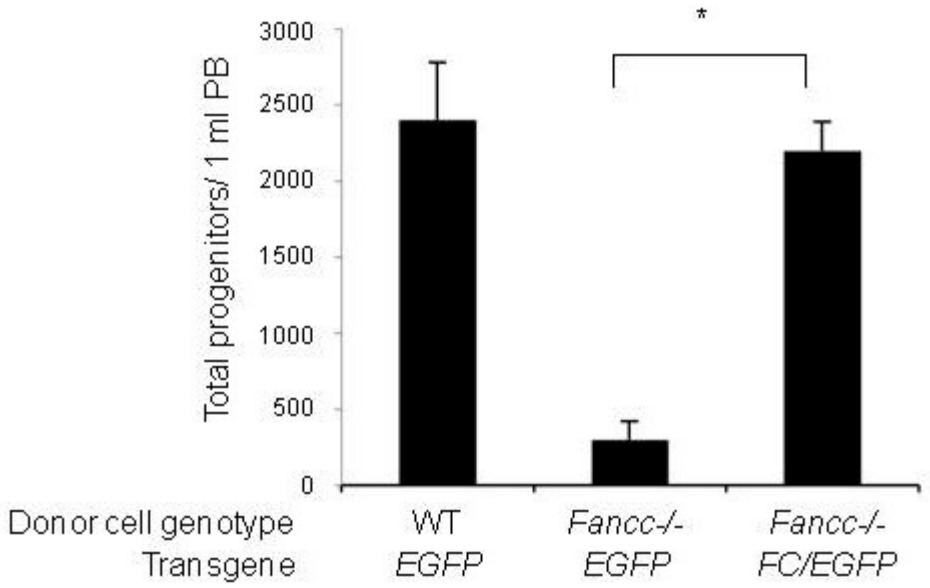
*
3000
2500
2000
1500
1000
500
0
Total progenitors/ 1 ml PB
Donor cell genotype
Transgene
WT
EGFP
Fancc-/-
EGFP
Fancc-/-
FC/EGFP

Figure 9. Correction of G-CSF and AMD3100 mobilized *Fancc-/-* LDMC results in a significant increase in progenitors/1ml PB. n=3, plated in triplicate. *p<0.01 by student's t-test.

Competitive repopulation is a very sensitive measure of the capabilities of a cell to repopulate a lethally irradiated recipient. Because the donor cells are competing against competitor cells from the same pool, chimerism levels can be compared as a measure of competitive repopulating ability. Here, competitive repopulation experiments were performed to determine the repopulating ability of the mobilized transduced cells.

A significant increase was observed in the *Fancc-/-* cells corrected with the *FANCC* transgene as compared to those transduced with only MD9-*EGFP* (Figure 10). The increase in competitive repopulating ability for the *Fancc-/-* cells corrected with the *FANCC* transgene resulted in a chimerism level comparable to WT (Figure 10). From the peripheral blood a very low expression of EGFP was observed. This is as anticipated and has been observed many times with this construct (Si, Pulliam et al. 2008). Because the competitive repopulation assay shows a correction in the function of the HSC/HPC, it is believed that the construct is present and the expression of the EGFP has decreased because it is the second gene expressed from the construct. To further confirm this, the viral construct DNA was detected as an indicator of transduction.

Figure 10

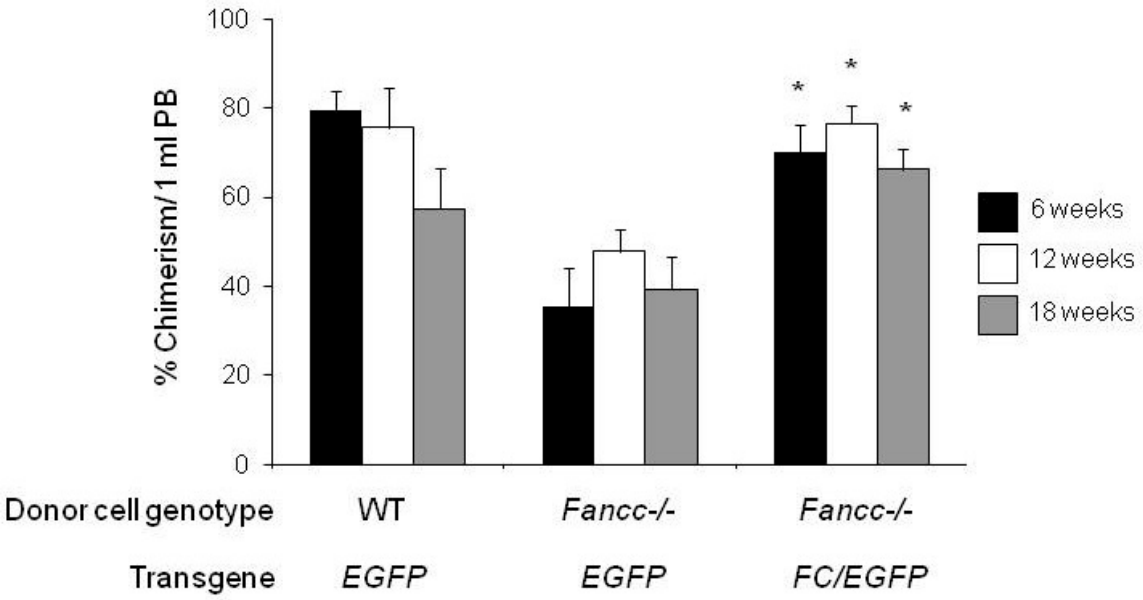
100
80
60
40
20
0
% Chimerism/1 ml PB
*
*
*
6 weeks
12 weeks
18 weeks
Donor cell genotype
WT
Fancc-/-
Fancc-/-
Transgene
EGFP
EGFP
FC/EGFP

Figure 10. Correction of *Fancc-/-* mobilized cells results in a significant increase in repopulating ability to levels comparable to WT. n=7 per group, *p<0.05 by student's t-test as compared to *Fancc-/-* with *EGFP* at the respective time point. There was not a statistically significant decrease in WT chimerism from 12 weeks to 18 weeks post transplantation (n=7, p>0.05 by student's t-test).

Four months post transplantation the LDMC from the bone marrow of a portion of these primary recipients was collected and sorted for the donor cells. These cells were plated in standard clonogenic assays. In order to be certain that the CD45.2^{+} chimerism observed was due to test cell repopulation and not endogenous recipient mouse cell repopulation, colonies were picked from these assays and genotyping was done to confirm that the CD45.2^{+} cells were indeed from the donor *Fancc-/- transduced* cells (Figure 11A). Representative samples are shown. In the results from 50 colonies from 5 different mice 82% were the correct genotype.

A PCR for the EGFP portion of the proviral integrant was performed to determine the percentage of colonies that were positive for the transgene. Representative samples are shown in Figure 11B and summary data is shown in Table II. 91% of colonies from mice transplanted with *Fancc-/-* cells transduced with MD9-*FANCC/EGFP* were positive for EGFP. Those *Fancc-/-* or WT cells transduced with the reporter construct only had lower percentages of colonies positive for the transgene. This is expected because the reporter construct does not confer a survival advantage.

Figure 11

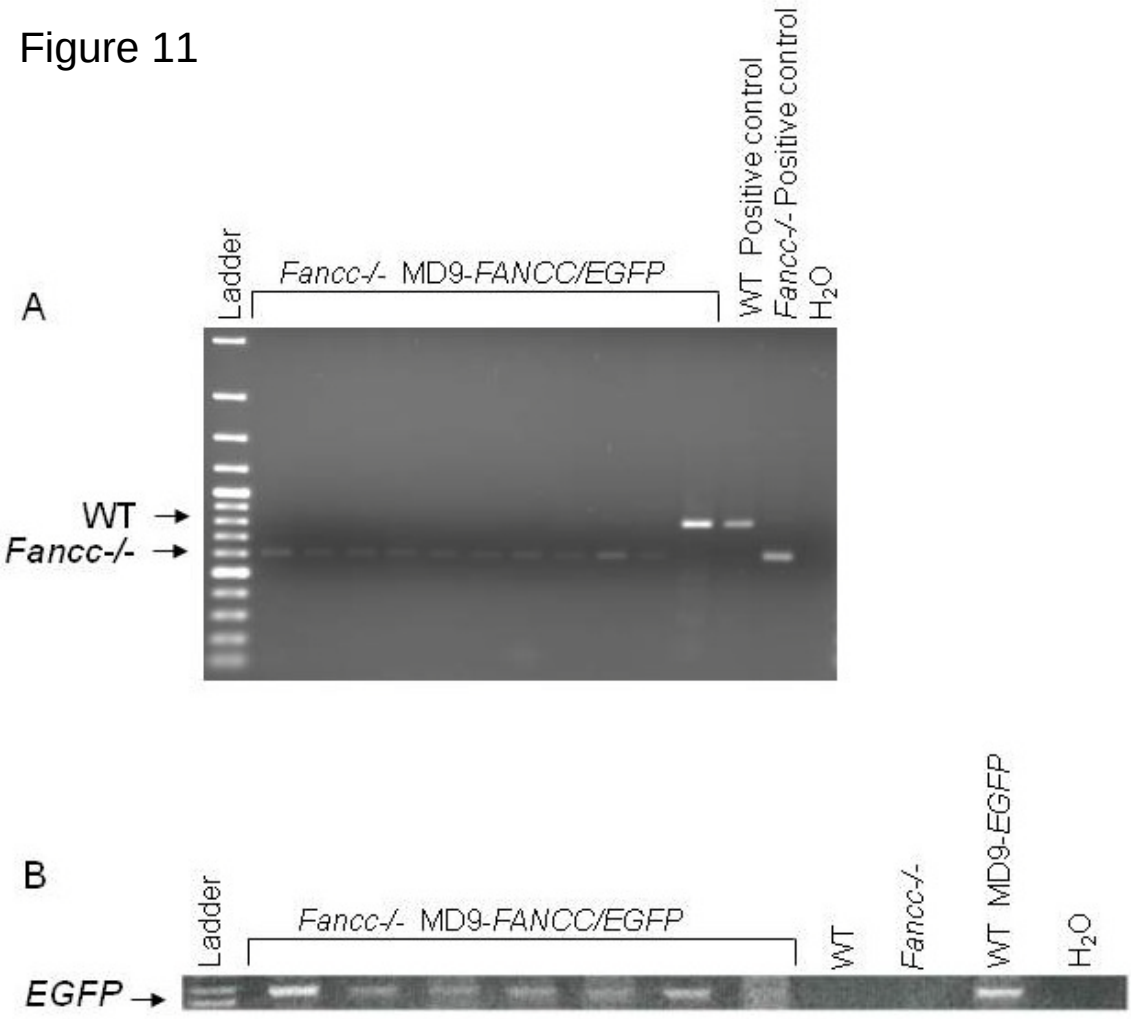
A
Ladder
Fancc-/- MD9-FANCC/EGFP
WT Positive control
Fancc-/- Positive control
H2O
WT
Fancc-/-
B
Ladder
Fancc-/- MD9-FANCC/EGFP
WT
Fancc-/-
WT MD9-EGFP
H2O
EGFP

Figure 11. Analysis of genotype and transduction efficiency of myeloid progenitors. CD45.2^{+} BMC were sorted and cultured in semisolid medium to grow myeloid progenitors. DNA from individual colonies was isolated and amplified to detect the genotype (A) and presence of the provirus (B). Representative gels of the amplified PCR products are shown. The test cell genotype and the provirus utilized are indicated.

Sensitivity to MMC and TNF-α is one characteristic of *Fancc-/-* cells. The colony assays assessing the CD45.2^{+} cells from the bone marrow of primary recipients after 4 months also included conditions with varying concentrations of MMC and TNF-α to determine the sensitivity of the cells. The *Fancc-/-* cells corrected with the transgene demonstrated WT levels of resistance, this implies that after 4 months in vivo the transgene is still being expressed and the protein is functioning in the FA pathway.

An equally important point is that the *Fancc-/-* cells transduced with the reporter construct only were still sensitive to these agents (Figure 12). The retroviral constructs used previously have resulted in myeloid malignancies that can be detected by a decrease in sensitivity to these agents (Haneline, Li et al. 2003), although the recipients will need to be assessed after a longer in vivo period to compare directly with the retroviral data, it is still promising to see that at this time point there is no decrease in sensitivity. Because it is very likely that not all of the transplanted cells will be transduced, it is especially important to perform this control to assess the non-corrected cells likelihood of developing myeloid malignancies.

Figure 12

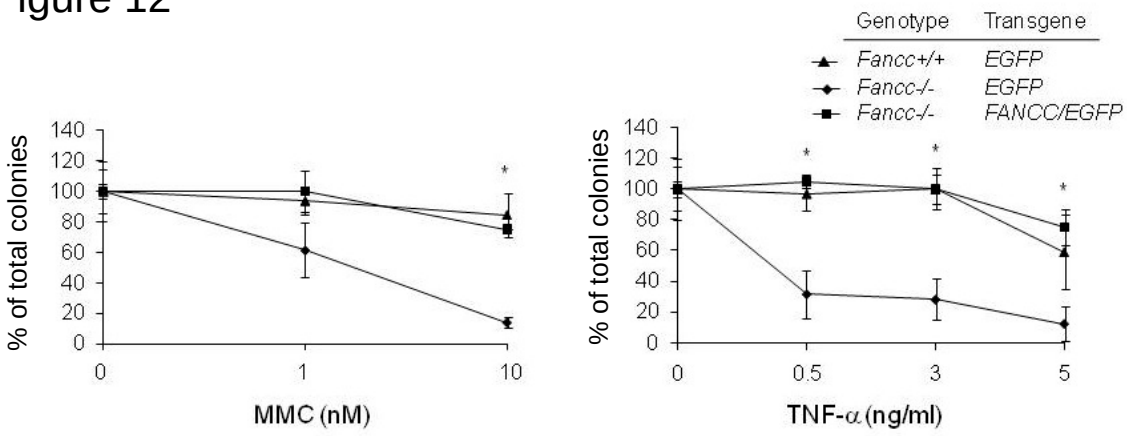
Genotype
Transgene
Fancc+/+
EGFP
Fancc-/-
EGFP
Fancc-/-
FANCC/EGFP
% of total colonies
140
120
100
80
60
40
20
0
0
1
10
MMC (nM)
*
% of total colonies
0
0.5
3
5
TNF-α (ng/ml)
*
*
*

Figure 12. MMC and TNF-α hypersensitivity is maintained in primary recipients of MD9-*EGFP* transduced *Fancc-/-* cells 4.5 months after transplantation. Bone marrow LDMC from *Fancc-/-* with MD9-*EGFP* (n=6), *Fancc-/-* with MD9-*FC/EGFP* (n=6) and WT MD9-*EGFP* (n=5) recipients were cultured in clonogenic assays in triplicate to determine their respective sensitivity to MMC or TNF-α at 18 weeks following transplantation. The genotypes and viral transgenes are indicated. Data represent the mean ± SEM growth of all recipients in each respective group. *p<0.05 indicates statistical significance between the non-corrected *Fancc-/-* cells and the other two experimental groups by ANOVA.

Junctional fragment analysis using southern blot was performed from the whole bone marrow and spleen and only 1 integration site was detected in each of 3 mice examined (data not shown). One explanation for this is the low MOI used. Also, multiple integration sites may result in apoptosis of the cells. Further integration site analysis will be beneficial in future experiments.

Discussion

Currently, the best method to treat BMF in FA patients is the transplantation of normal HSC, (Gluckman, Broxmeyer et al. 1989; Davies, Khan et al. 1996; Guardiola, Pasquini et al. 2000; Kutler, Singh et al. 2003; Wagner, Eapen et al. 2007). However, allogeneic bone marrow or cord blood transplantation is not without subsequent risk as the conditioning regimens cause genotoxic stress that predisposes patients to an increased incidence of squamous cell carcinomas especially when compounded by the presence of chronic graft-versus-host disease (Kutler, Auerbach et al. 2003; Rosenberg, Socie et al. 2005). 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 potential sequelae (Si, Ciccone et al. 2006).

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 (Waisfisz, Morgan et al. 1999; Gregory, Wagner et al. 2001; Gross, Hanenberg et al. 2002; Soulier, Leblanc et al. 2005; Mankad, Taniguchi et al.

2006). 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 (Liu, Young et al. 1997; Kelly, Radtke et al. 2007). One possible reason for this failure of genetic therapy is the low number of stem cell targets available for gene transfer (Williams, Croop et al. 2005). In addition, even in the presence of saturating concentrations of growth factors, FA cells have an increased propensity to undergo apoptosis (Li, Le Beau et al. 2005). Therefore, the prolonged culture required to induce stem cells into cycle and allow gammaretroviruses to integrate into the target cell genome predisposes untransduced FA stem cells to undergo apoptosis (Li, Le Beau et al. 2005).

In studies here, FV vectors expressing the human *FANCC* cDNA functionally corrected multiple defects in mobilized *Fancc-/-* stem cells capable of repopulating primary 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. As mentioned previously, FV is not associated with any disease in humans (Switzer, Bhullar et al. 2004). Also, in contrast to gamma-retroviruses that have a half-life of only 4-6 hrs in vivo (Andreadis, Brott et al.

1997), non-integrated FV virions retain the potential for stable integration in quiescent cells for up to 2 weeks following transduction (Lehmann-Che, Renault et al. 2007). Thus, cells only need to be exposed in vitro to virions for a short time allowing cycling of the target cells and proviral integration to occur in vivo. As mentioned previously, since FA murine and human FA stem cells have increased in vitro time dependent induction of apoptosis and at least *Fancc-/-* cells develop in vitro dependent clonal transformation (Haneline, Li et al. 2003; Li, Le Beau et al. 2005), this abbreviated transduction protocol efficiently corrects FA stem cells (Si, Pulliam et al. 2008). Also, in vitro manipulation of mobilized *Fancc-/-* LDMC for 14 hrs in the absence of prestimulation, as presented here, is sufficient for FV-mediated delivery of *FANCC* to cells and correction of long-term repopulating activity in primary transplantations.

One or two proviral integrations were observed in the bone marrow and spleen cells of reconstituted mice in these experiments as well as previous experiments (Si, Pulliam et al. 2008). There are at least three possibilities for this observation. One possibility is that analogous to rare cases of apparently spontaneous correction of FA stem cells (Waisfisz, Morgan et al. 1999; Gregory, Wagner et al. 2001; Gross, Hanenberg et al. 2002; Soulier, Leblanc et al. 2005;

Mankad, Taniguchi et al. 2006), correction of a subpopulation of *Fancc-/-* 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 hematopoiesis in reconstituted lethally irradiated mice tends to be oligoclonal in nature (Jordan and Lemischka 1990; Jordan, McKearn et al. 1990). This correlates with our previously published data (performed with an MOI of 20 as compared to the MOI of 10 used here), which show a low number of integrants, regardless of the transgene or stem cell genotype (Si, Pulliam et al. 2008). 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 (Bauer, Allen et al. 2008). A third possibility necessary to consider is that of clonal selection leading to MDS. In previous studies clonal selection was tested for extensively. The hematopoietic organs and clonogenic growth from the myeloid progenitors of primary and secondary recipients were examined over a total of 18 months. From multiple recipients, no pathological abnormalities were observed. Additionally, in these studies no pathological abnormalities were observed in the recipient mice (Si,

Pulliam et al. 2008). The lack of pathological sequelae is consistent with the study in CLAD dogs where FV integrations were found within genes or near oncogenes at a much lower frequency than as reported for retroviral vectors (Bauer, Allen et al. 2008).

A potential limitation to the use of FV vectors in the clinic to date is that the fusogenic capacities of FV envelope proteins (Stanke, Stange et al. 2005; Duda, Luftenegger et al. 2006), especially when cells are exposed to higher virus titers and for prolonged periods of time. The envelope used here, pcoPEO1, has not caused fusion of the hematopoietic target cells in these studies.

Collectively, these studies demonstrate that the functional expression of a transgene using a FV construct can cure a disease phenotype in a murine model of a human disease with cells collected in a manor similar to that used in patients. With some additional modifications this gene delivery system may be applicable for therapeutic use in humans.

CHAPTER 5

Genetic disruption of both *Fancc* and *Fancg* in mice recapitulates the hematopoietic manifestations of Fanconi anemia

Introduction

As mentioned previously, FA patients develop progressive BMF, ultimately requiring stem cell transplantation in the second or third decade of life (Gluckman, Auerbach et al. 1995). The progressive BMF can also lead to hematologic neoplasms, particularly MDS and AML (Gluckman, Auerbach et al. 1995; Alter, Greene et al. 2003; Kutler, Singh et al. 2003), both of which are almost universally associated with aneuploidy and cytogenetic abnormalities (Auerbach and Allen 1991; Tonnies, Huber et al. 2003). The interactions among the FA proteins are complex and incompletely understood. While certain FA proteins function in the core complex as described above, additional functions have been described for individual FA proteins suggesting a critical role in maintenance of normal hematopoiesis (Pang, Christianson et al. 2001; Pang, Christianson et al. 2002; Zhang, Li et al. 2004; Gordon, Alon et al. 2005; Hussain, Wilson et al. 2006; Leveille, Ferrer et al. 2006; Mukhopadhyay, Leung

et al. 2006; Wilson, Yamamoto et al. 2008), yet these mechanisms have not been evaluated using a genetic model. Murine knockouts of the homologues of *FANCA* (*Fanca*), *FANCC* (*Fancc*), *FANCG* (*Fancg*), *FANCD1* (*FancD1*), *FANCD2* (*Fancd2*) and *FANCM* (*Fancm*) have been established (Bakker, van de Vrugt et al. 2009; Parmar, D'Andrea et al. 2009). Although all strains of FA knockout mice are hypersensitive to MMC, none of these single knockout mice display spontaneous aplastic anemia or myeloid malignancies characteristic of FA in patients (Bakker, van de Vrugt et al. 2009; Parmar, D'Andrea et al. 2009). If FA proteins have divergent functions independent of ID complex monoubiquitination in hematopoietic cells, double knockout mice might display a more aggressive hematopoietic phenotype. Therefore, *Fancc-/-;Fancg-/-* mice were generated and these results show that these mice more faithfully recapitulate the hematological manifestations observed in FA patients.

Results

In initial experiments in the lab (performed by Dr. Samantha Ciccone), multipotent, erythroid and myeloid progenitors from syngeneic 3-month-old WT, *Fancc-/-* and *Fancg-/-* mice were found to have differences in clonogenic potential (Figure 13A-D). These functional results provided the first suggestion that FANCC and FANCG have non-overlapping roles in hematopoiesis.

To further evaluate this possibility, microarrays were conducted to test the hypothesis that loss of both *Fancc* and *Fancg* will result in a significant change in the expression profile (Figure 13E-G). To accomplish this, unsupervised hierarchical clustering and principal component analyses were performed on expression microarray data from BMC RNA from 3-month-old syngeneic mice: WT, *Fancc-/-*, *Fancg-/-* and double knockout *Fancc-/-;Fancg-/-* mice. The results demonstrated that double mutant cells diverged more widely from the WT cells than did either of the single mutants (Figure 13E-F). Hence, there were more commonalities found in the expression space between the two single mutants and also between them and the WT samples; but significantly greater dissimilarities between these three groups and the double mutant samples (Figure 13E-F).

Figure 13

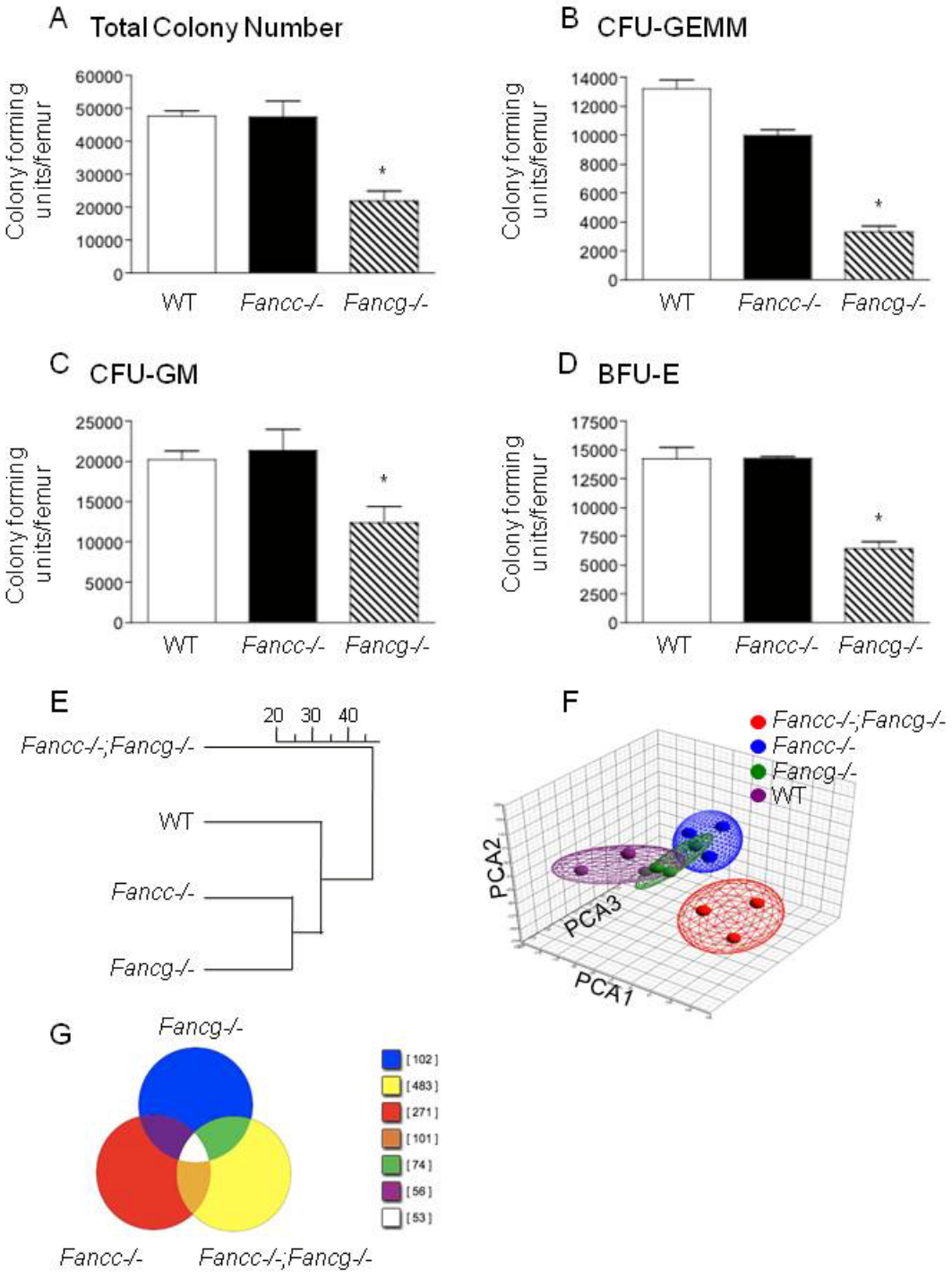
A
Total Colony Number
Colony forming units/femur
60000
50000
40000
30000
20000
10000
0
*
WT
Fancc-/-
Fancg-/-
B
CFU-GEMM
Colony forming units/femur
14000
12000
10000
8000
6000
4000
2000
0
*
WT
Fancc-/-
Fancg-/-
C
CFU-GM
Colony forming units/femur
25000
20000
15000
10000
5000
0
*
WT
Fancc-/-
Fancg-/-
D
BFU-E
Colony forming units/femur
17500
15000
12500
10000
7500
5000
2500
0
*
WT
Fancc-/-
Fancg-/-
E
20
30
40
Fancc-/-;Fancg-/-
WT
Fancc-/-
Fancg-/-
F
Fancc-/-;Fancg-/-
Fancc-/-
Fancg-/-
WT
PCA2
PCA3
PCA1
G
Fancg-/-
[102]
[483]
[271]
[101]
[74]
[56]
[53]
Fancc-/-
Fancc-/-;Fancg-/-

Figure 13. Loss of *Fancg* results in a more severe defect in multiple hematopoietic compartments than loss of *Fancc*. (A) Total number of progenitors per femur isolated from syngeneic 3-month-old mice with statistically similar weights (25 ± 0.6g**)** and bone marrow cellularity ($10 \times 10^6 \pm 0.7 \times 10^6$ cells per femur). *p=0.0009 by one-way ANOVA. (B) Number of multipotential Colony Forming Unit-Granulocyte, Erythrocyte, Monocyte, Megakaryocyte progenitors (CFU-GEMM), *p<0.0001 by one-way ANOVA. (C) Colony Forming Unit-Granulocyte-Macrophage progenitors (CFU-GM), *p=0.0230 by one-way ANOVA. (D) Burst Forming Unit-Erythroid progenitors (BFU-E), *p=0.0030 by one-way ANOVA. Data represent mean ± SEM of 3 independent experiments each of which was plated in triplicate cultures. (E and F) Genome-wide transcriptomal analysis of marrow cells from 3-month-old mice from each of the four indicated FA genotypes was performed. A total of 12 BMC samples from 12 mice (*Fancc-/-;Fancg-/-*, *Fancc-/-*, *Fancg-/-* and WT) were analyzed using 12 AffyMetrix MOE 430 2.0 GeneChip arrays. (E) Unsupervised hierarchical clustering, the scale represents the level of correlation between the groups. (F) Principal component analysis. In this case the multidimensional data were reduced to a new coordinate system such that the greatest variance (20.8%) by any possible projection of the data is plotted on the first coordinate (the first principal component, PCA1). The second greatest variance (14.5%) was plotted on the second coordinate (PCA2) and the third greatest (10.8%) plotted on the third coordinate (PCA3). Results from both analyses confirmed that double mutant cells diverged widely from both the WT samples and both of the single mutant samples.

A Venn diagram provides a visual representation of the differential gene expression by each genotype (Figure 13G), providing evidence that each strain has uniquely expressed genes. The differences did not include variations in lineage specific gene expression (Table III) assuring that the biological differences observed (see below) did not derive from modest differences in the populations present in the bone marrow of the animals.

Since the hallmark of FA cells is hypersensitivity to DNA cross-linking agents, the growth of hematopoietic progenitors from single and double mutant 3-month-old FA mice and syngeneic WT control mice in the presence of MMC was evaluated (Dr. Samantha Ciccone) (Figure 14A). The progenitors from *Fancc-/-*, *Fancg-/-* or *Fancc-/-;Fancg-/-* mice had comparable hypersensitivity to MMC (Figure 14A).

To further evaluate abnormalities in the hematopoietic compartment, cohorts of WT, *Fancc-/-*, *Fancg-/-* and *Fancc-/-;Fancg-/-* mice were observed up to 24 months. These mice were sacrificed and evaluated when they developed signs of illness. A significant decrease in survival of *Fancc-/-;Fancg-/-* mice was observed as compared to other genotypes (Figure 14B).

Table III. Expression of genes relevant to lineage specific differentiation. Expression of genes relevant to lineage specific differentiation did not differ between *Fancc-/-* (n=3), *Fancg-/-* (n=3) and double *Fancc-/-;Fancg-/-* (n=3) mice. The genes listed in column 2 were not differentially expressed when gene expression profiles of the three strains were compared using GeneSifter (Geospiza, Seattle, WA).

Lineage	Genes Expressed
Erythroid	Sfp1, Ank1, Epb4.2, Hba1, Hbb-b1,Eraf1, Spna1
Granulocytes	Cbfa2t3, Sfp1, Ltf, Mpo, Cd11b, Cd15, Csf3r
Eosinophils	Il-25, Il5ra
Monocytes/Macrophages	SpiB, Lif, Cd14
Megakaryocytes	Sp3, Tesc, mpl
T-cells	Tcf7, CD3, CD4, CD8
B-cells	Cd19, Cd20, Cd1d, Rag1, Vpreb1
NK-cells	Il-21, Il-11R, Ikzf1, Stat5b
Lymphoid (general)	IL2rg, Il-7ra, Nfkbid, Syk, Lef1, Il-4r, Il-6, Il-20r, Il-12b, Cd40
Unlimited (relevant to hematopoiesis)	c-kit, Flt1, Flt2, Flt3, Xrcc5, Ercc2, Sox4, Xrcc4, Tal1, Meis1, Irf4, Egr1, Wisp1, CD28, Foxp1, Cd79, Cd79a, Inpp5d,Thpo, Csf2, Csf3

Figure 14

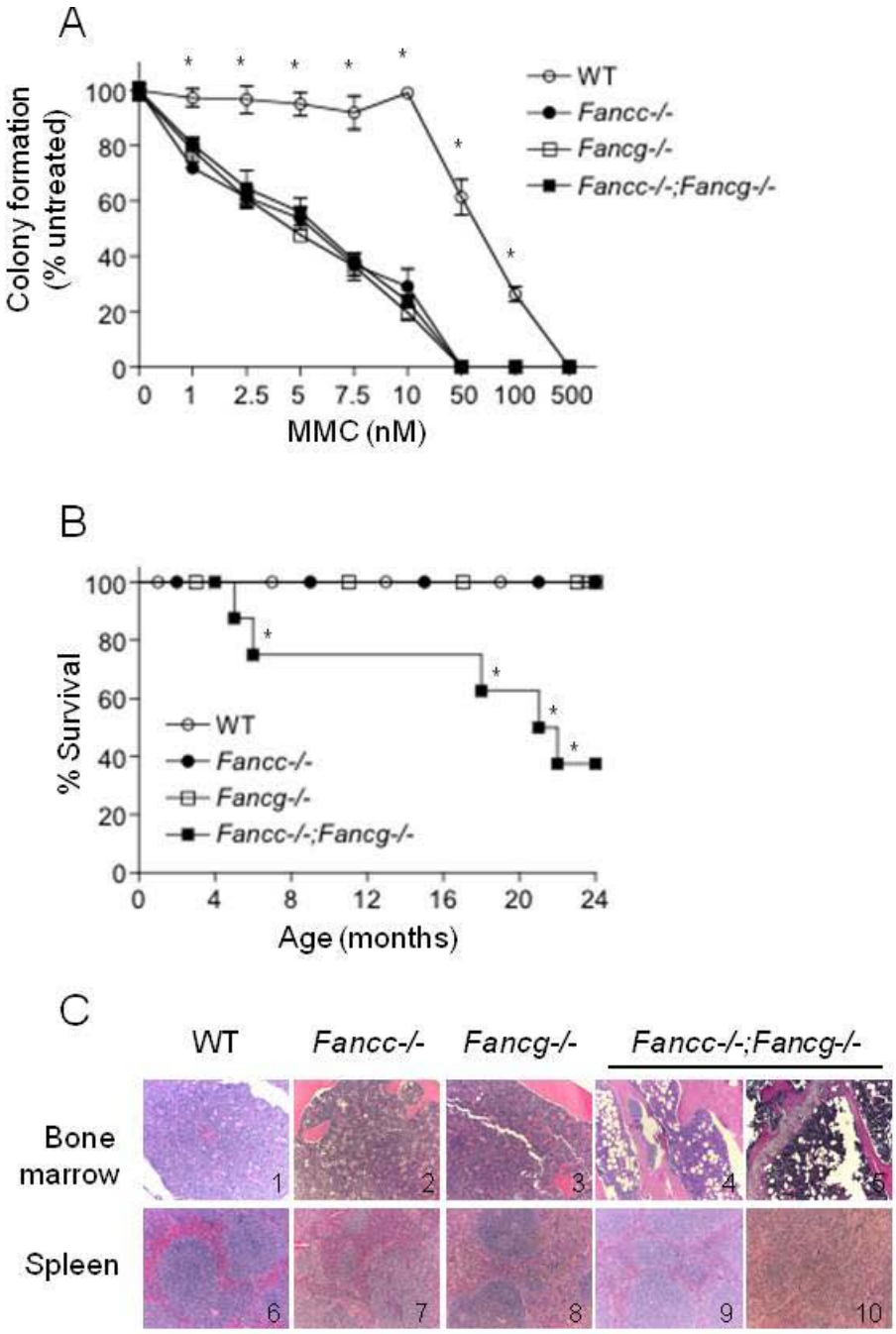
A
Colony formation (% untreated)
100
80
60
40
20
0
0 1 2.5 5 7.5 10 50 100 500
MMC (nM)
WT
Fancc-/-
Fancg-/-
Fancc-/-;Fancg-/-
B
% Survival
100
80
60
40
20
0
0 4 8 12 16 20 24
Age (months)
WT
Fancc-/-
Fancg-/-
Fancc-/-;Fancg-/-
C
WT
Fancc-/-
Fancg-/-
Fancc-/-;Fancg-/-
Bone marrow
Spleen
1
2
3
4
5
6
7
8
9
10

Figure 14. *Fancc-/-;Fancg-/-* mice have a shortened life span and an increased risk of BMF despite similar MMC sensitivity of *Fancc-/-, Fancg-/-* and *Fancc-/-;Fancg-/-* cells. (A) Sensitivity of WT, *Fancc-/-*, *Fancg-/-* and *Fancc-/-;Fancg-/-* cells to MMC. Data represent mean ± SEM of 3 independent experiments each of which was plated in triplicate cultures isolated from the bone marrow of 3-month-old syngeneic mice. WT is significantly different than any of the other experimental groups, which had comparable sensitivity, *p<0.001 by two-way ANOVA. (B) Kaplan-Meier curve shows a significant decrease in survival of *Fancc-/-;Fancg-/-* mice compared to WT, *Fancc-/-* or *Fancg-/-* mice, n=8/genotype *p=0.0004 for *Fancc-/-;Fancg-/-* as compared to WT, *Fancc-/-* or *Fancg-/-* by the log rank test for trend. (C) *Fancc-/-;Fancg-/-* mice, but not WT, *Fancc-/-* or *Fancg-/-* mice, develop aplastic anemia and myeloid malignancies. Panels 4 and 9 are from a 20-month-old *Fancc-/-;Fancg-/-* mouse and panels 5 and 10 are from a 23-month-old *Fancc-/-;Fancg-/-* mouse.

In order to determine the cause of the increased mortality in these mice, serial evaluations of complete peripheral blood counts of primary *Fancc-/-;Fancg-/-* mice were performed. At the time of death or sacrifice due to illness, 50% of double knockout mice had a moderate leukocytosis (white blood cell counts >15.9K/µl), dysplastic megakaryocytes, a myeloid maturation arrest and splenomegaly (>200mg) with an invasion of myeloid cells leading to a disruption of normal splenic architecture (Figure 14C, representative samples). Another 25% of the *Fancc-/-;Fancg-/-* mice had anemia (hemoglobin level <7.0g/dL), decreased bone marrow cellularity (<50% of WT) and hypoplastic marrow containing large numbers of adipocytes (Figure 14C, representative samples). The remaining mice did not have signs of illness at 24 months of age.

Our lab also examined the phenotype of the *Fancc-/-;Fancg-/-* hematopoietic stem cells in greater detail by utilizing a competitive repopulation assay. In this assay, stem cell activity is quantified by comparing the ability of mutant and WT BMC to establish long-term hematopoiesis in competition with isogenic WT cells (Abramson, Miller et al. 1977; Harrison 1980; Jordan and Lemischka 1990). Upon transplantation of mixed test and competitor cells (two cohorts were performed, one with a ratio of 5:1(shown), one with a ratio of 3:1)

into lethally irradiated recipients, PB chimerism was assessed monthly for 1 year by flow cytometry to determine test cell contribution to hematopoiesis (Figure 15) (transplantations and chimerism performed by Dr. Samantha Ciccone, histological analysis performed by Anna C. Leath). The test cell repopulating activity was decreased in recipients reconstituted with either *Fancc-/-* or *Fancg-/-* BMC as compared to WT BMC (Figure 15B).

Interestingly, the peripheral blood chimerism of recipients reconstituted with *Fancc-/-;Fancg-/-* BMC showed widely variable chimerism over time (Figure 15B, 16A) in contrast to stable chimerism in recipients reconstituted with all the other test cell genotypes (Figure 15B). The *Fancc-/-;Fancg-/-* chimerism data is shown in Figure 15B to allow for comparison with the other genotypes and then again in Figure 16A which shows the change in chimerism of each individual recipient mouse. In some *Fancc-/-;Fancg-/-* BMC recipients, the test cell population progressively out-competed the competitor cells while in other recipients the repopulating activity of the test cells was progressively reduced (Figure 16A).

Figure 15

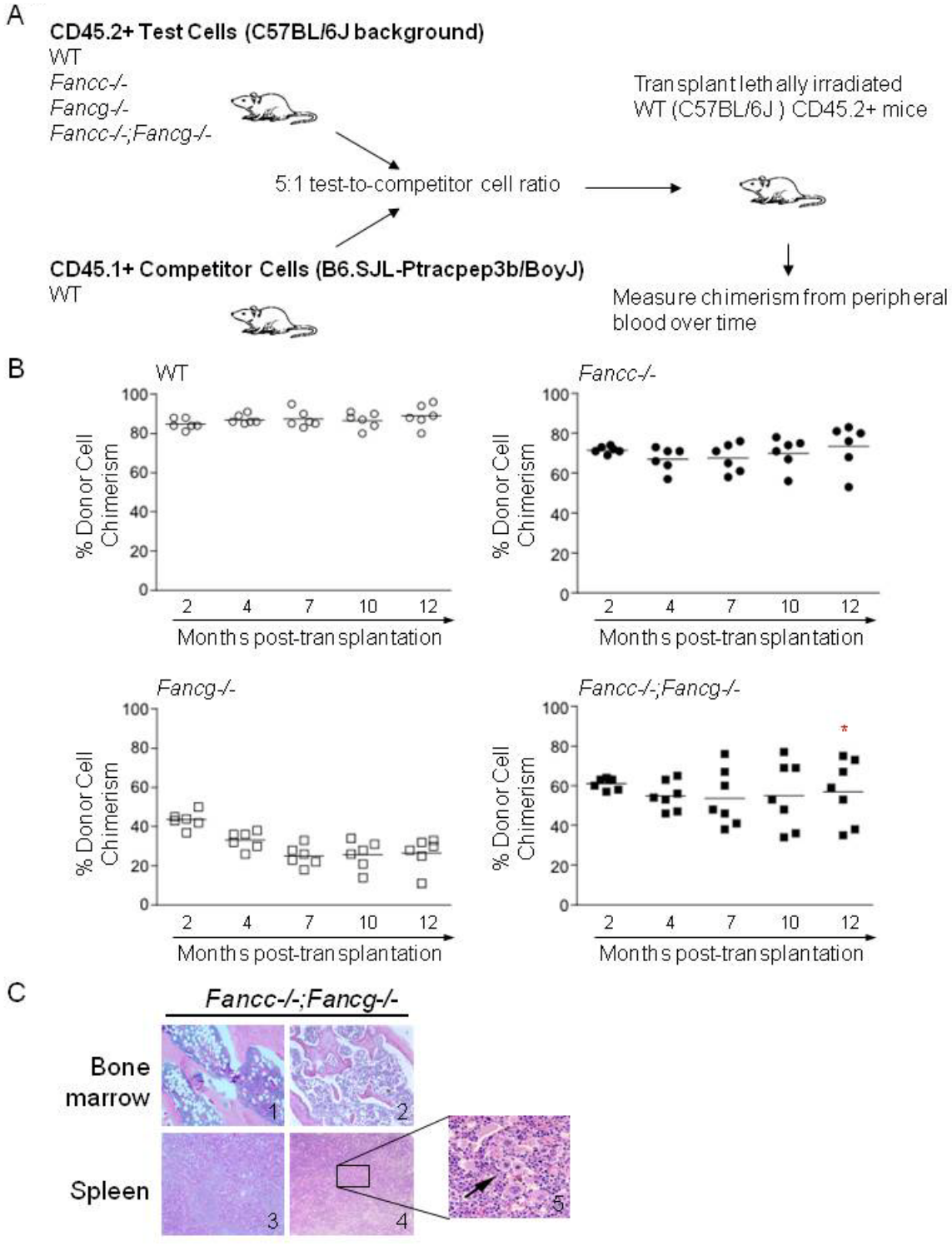
A
CD45.2+ Test Cells (C57BL/6J background)
WT
Fancc-/-
Fancg-/-
Fancc-/-;Fancg-/-
Transplant lethally irradiated
WT (C57BL/6J) CD45.2+ mice
5:1 test-to-competitor cell ratio
CD45.1+ Competitor Cells (B6.SJL-Ptracpep3b/BoyJ)
WT
Measure chimerism from peripheral blood over time
B
WT
Fancc-/-
Fancg-/-
Fancc-/-;Fancg-/-
% Donor Cell Chimerism
Months post-transplantation
C
Fancc-/-;Fancg-/-
Bone marrow
Spleen
1
2
3
4
5

Figure 15. *Fancc-/-;Fancg-/-* hematopoietic stem cells undergo clonal evolution and malignant transformation in vivo. (A) Experimental design: Test BMC and isogenic WT competitor cells were co-transplanted into lethally irradiated recipient mice at a 5:1 ratio (2.5 X 10^6 test cells and 0.5 X 10^6 competitor cells). (B) The contribution of transplanted test cells to hematopoiesis was sequentially monitored in the peripheral blood of individual mice. *Fancc-/-* and *Fancg-/-* stem cells establish hematopoiesis less efficiently than WT hematopoietic stem cells. Increasing variability was observed in the peripheral blood chimerism in mice transplanted with *Fancc-/-;Fancg-/-* BMC, but not *Fancc-/-, Fancg-/-* or WT BMC. There was significant increase in the variability of chimerism in recipients receiving the *Fancc-/-;Fancg-/-* cells when comparing 4 months to 12 months, ANOVA with Bartlett's test to compare variances, *p=0.0149. (C) Mice reconstituted with BMC from *Fancc-/-;Fancg-/-* mice showed abnormal bone marrow and spleen architecture 12 months post-transplantation. Panels 1 and 3 are from mouse 5 in Figure 16A, with a chimerism of 38% at 12 months post-transplantation. Panels 2, 4 and 5 are from mouse 2 in Figure 16A. Panel 5 shows megakaryocyte invasion in the spleen.

Figure 16

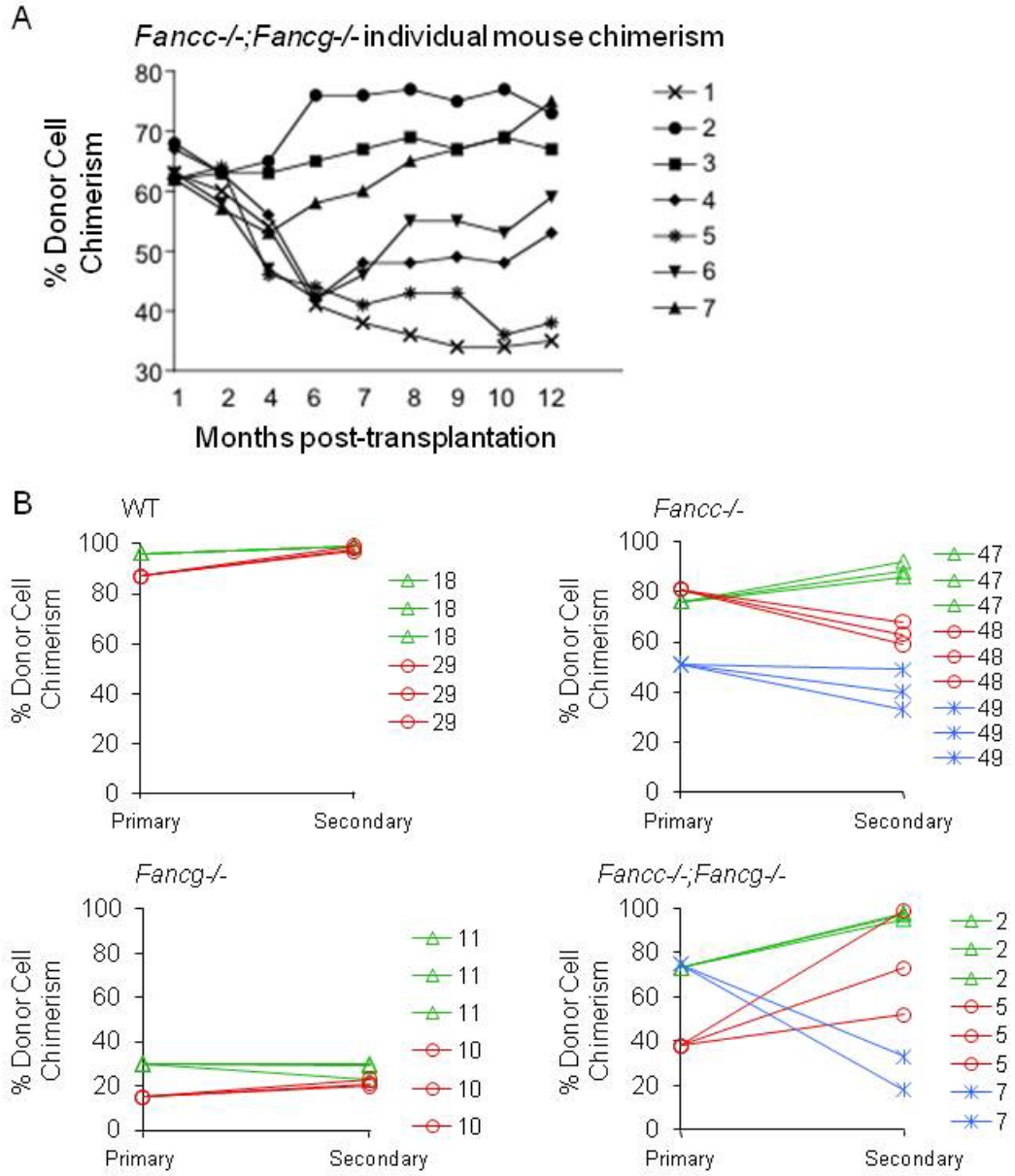
A
Fancc-/-;Fancg-/- individual mouse chimerism
% Donor Cell Chimerism
80
70
60
50
40
30
1 2 4 6 7 8 9 10 12
Months post-transplantation
1
2
3
4
5
6
7
B
WT
% Donor Cell Chimerism
100
80
60
40
20
0
Primary
Secondary
18
18
18
29
29
29
Fancc-/-
47
47
47
48
48
48
49
49
49
Fancg-/-
11
11
11
10
10
10
Fancc-/-;Fancg-/-
2
2
2
5
5
5
7
7

Figure 16. *Fancc-/-;Fancg-/-* hematopoietic stem cells have malignant potential in vivo, while *Fancc-/-* or *Fancg-/-* cells do not. (A) Fluctuations of donor cell chimerism were sequentially examined in peripheral blood of individual recipients, each symbol represents an individual recipient mouse (n=7). (B) Secondary transplantations were performed to further evaluate stem cell repopulating ability. Two or three primary recipients from each genotype were selected and 3 X 10^6 bone marrow LDMC were transplanted into each lethally irradiated recipient (2-3 recipients per primary mouse). Each symbol represents an individual recipient mouse. For the *Fancc-/-;Fancg-/-* genotype, the mouse numbers correspond with those shown in Figure 16A. There were 3 secondary recipients transplanted with cells from mouse #2, 3 from mouse #5 and 2 from mouse #7. This shows the continuous increase in variability of the chimerism for the *Fancc-/-;Fancg-/-* donor cells.

To further evaluate this unstable chimerism and the stem cell functionality, secondary transplantations were performed for all genotypes and only those recipients transplanted with the *Fancc-/-;Fancg-/-* donor cells had a continued increase in chimerism variability (Figure 16B) (Secondary transplantations performed by Dr. Samantha Ciccone, chimerism analysis performed by both Dr. Samantha Ciccone and Anna C. Leath, histological analysis performed by Anna C. Leath). The dramatic increase in test cell chimerism in some of the recipients suggests the development of clonal evolution of repopulating cells.

To evaluate whether the progressive increase in test cell chimerism in *Fancc-/-;Fancg-/-* BMC recipients was associated with the development of hematologic malignancies, spleen and bone marrow were harvested from the recipient mice. Recipients reconstituted with either WT, *Fancc-/-* or *Fancg-/-* cells all had normal splenic architecture and histology (data not shown). In contrast, recipient mice reconstituted with *Fancc-/-;Fancg-/-* BMC showed a range of hematologic abnormalities including myeloid malignancies and aplastic anemia (Figure 15C, Table IV). The spleens from these recipients were enlarged and displayed complete disruption of normal splenic architecture and abnormal cell histology (Figure 15C).

Table IV. Mice reconstituted with *Fancc-/-;Fancg-/-* donor cells have an increased risk of myeloid malignancy. This summary represents all of the data from two cohorts of competitive repopulation transplantations and one cohort of non-competitive transplantations.

Donor Cell Genotype	Malignancy
WT	0/16
Fancc-/-	0/15
Fancg-/-	0/16
Fancc-/-;Fancg-/-	9/17

To confirm that these phenotypes were indeed due to reconstitution of the hematopoietic system by the donor cells and not the recipient's endogenous BMC, $CD45.2^{+}$ cells were sorted, cultured for growth of progenitors in semisolid medium and individual colonies were genotyped. Less than 10% of the colonies were from the recipient's residual BMC consistent with previous results (data not shown) (Si, Pulliam et al. 2008).

Cytogenetic evaluation showed that only the transplanted *Fancc-/-;Fancg-/-* BMC accumulated genetic abnormalities such as aneuploidy and multiple chromosomal translocations (Figure 17, Tables V and VI) (G-banding and SKY performed by Dr. Gail Vance's lab). Collectively, the spectra of hematologic abnormalities observed are consistent with phenotypes found in FA patients such as hypoplastic bone marrow and hematopoietic malignancies (Butturini, Gale et al. 1994).

In an assay comparable to that used to identify a patient's complementation group, only complementation of both *Fancc* and *Fancg* in *Fancc-/-;Fancg-/-* myeloid progenitors resulted in resistance to MMC. Cells that contain a transgene that complements only one gene were still hypersensitive to MMC (Figure 18).

Figure 17

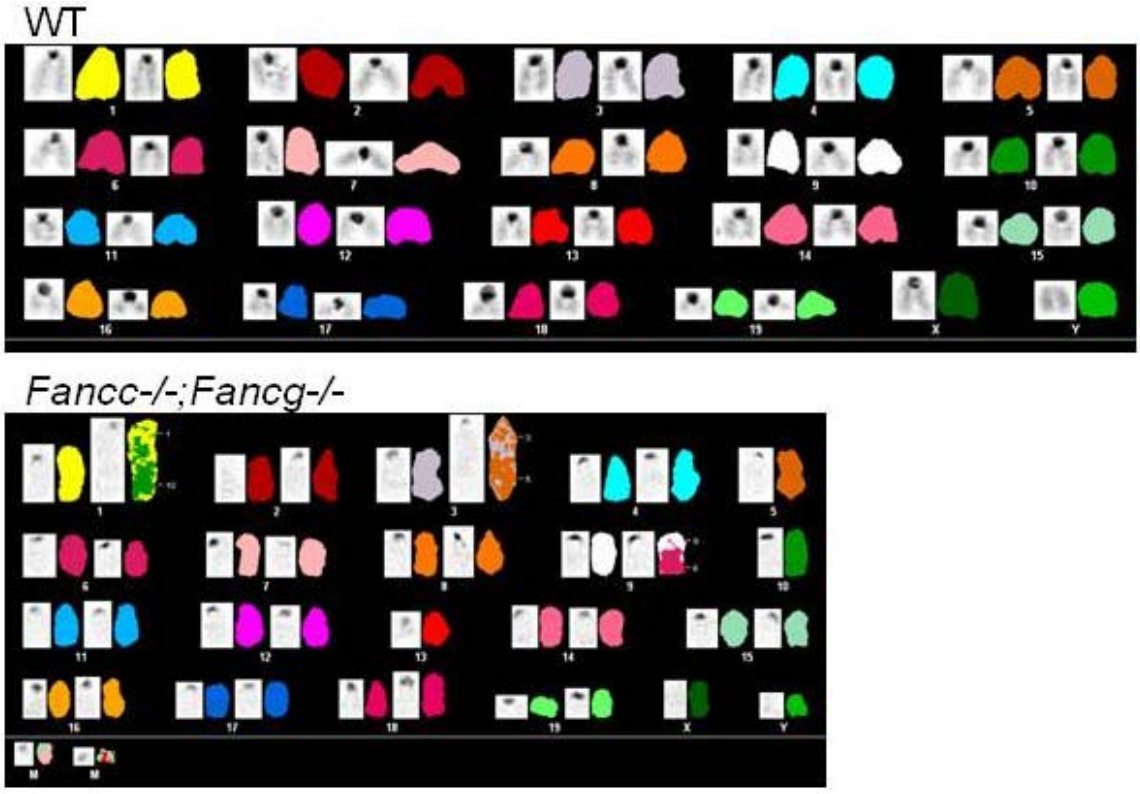

Figure 17. Spectral karyotyping shows aneuploidy and chromosomal translocations in *Fancc-/-;Fancg-/-* cells from the bone marrow of non-competitive recipients. WT shows a normal spectral karyogram. See Tables V and VI for detailed descriptions of the chromosomal abnormalities found.

Table V. G-banding analysis confirms chromosomal abnormalities in *Fancc-/-;Fancg-/-* cells.

Genotype	Cytogenetic Results	# of cells analyzed*
WT	40,XY [5]	5
Fancc-/-;Fancg-/-	41~43,XX,+mar1,+mar2,+mar3 [cp 6]/ 40,XX [5]	12
Fancc-/-;Fancg-/-	40,XY,der(1)t(1;10),dic(3;5),-5, der(6)t(6;18), der(9)t(6;9),-10, +mar1,+mar2 [cp 5]/ 40,XY [6]	15
Fancc-/-;Fancg-/-	40,XX,-1,?del(2),+der(2)t(2;6),t(3;13), +del(4),-6, del(11),+der(11)t(1;11),del(14), ?15,-16 [5]/ 40, XX[6]	11
Fancc-/-	40,XX[9]/Nonclonal[1]	10

*Cells not included in the nomenclature include nonclonal cells and normal cells of the opposite sex.

Table VI. Spectral karyotyping (SKY) analysis confirms chromosomal abnormalities in *Fancc-/-;Fancg-/-* cells.

Genotype	SKY Results	# of cells analyzed*
WT	40,XY [4]	5
Fancc-/-;Fancg-/-	41~45,XX,+mar1,+mar2,+mar3 [cp 2]/ 40,XX [7]	12
Fancc-/-;Fancg-/-	40,XY,der(1)t(1;10),dic(3;5),-5,der(6)t(6;18), der(9)t(6;9),-10,+mar1,+mar2 [2]/ 40,XY [7]	12
Fancc-/-;Fancg-/-	40, XX,-1,?del(2), +der(2)t(2;6), t(3;13), +del(4),-6,del(11),+der(11)t(1;11),del(14), ?15,-16 [1]/ 40,XX [2]	3

*Cells not included in the nomenclature include nonclonal cells and normal cells of the opposite sex.

Figure 18

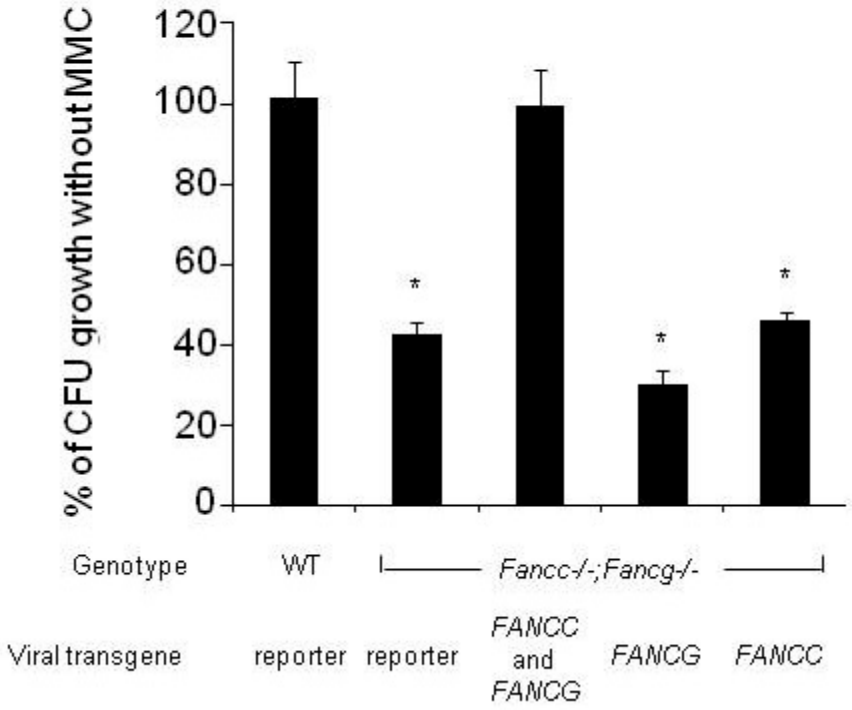
% of CFU growth without MMC
120
100
80
60
40
20
0
*
*
*
Genotype
WT
Fancc-/-;Fancg-/-
Viral transgene
reporter
reporter
FANCC and FANCG
FANCG
FANCC

Figure 18. Correction of both the *Fancc* and *Fancg* defect results in a correction of MMC hypersensitivity. Bone marrow LDMC from WT or *Fancc-/-;Fancg-/-* mice were transduced overnight, as published previously (Si, Pulliam et al. 2008), with either a foamy virus EGFP reporter construct, a single foamy virus with *FANCC/EGFP* or *FANCG/EGFP* or with both viruses to correct both genes. Shown here are WT and *Fancc-/-;Fancg-/-* cells with reporter only, the *Fancc-/-;Fancg-/-* cells with correction of both genes and *Fancc-/-;Fancg-/-* cells with correction of only one gene. Colony assays in the presence of MMC were performed as described previously (Si, Pulliam et al. 2008). The percentage shown was determined by the (# of CFU in MMC)/(# of CFU without MMC)*100. Mean ± SEM is shown, n=6 for *Fancc-/-;Fancg-/-*, n=3 for WT. *$p<0.05$ as compared to WT or *Fancc-/-;Fancg-/-* corrected with both genes by ANOVA.

To rule out the possibility that the observed hematopoietic phenotypes occur following loss of any two core complex proteins, our lab intercrossed the *Fanca-/-* and *Fancc-/-* mice (Dr. Yue Si). The *Fanca-/-;Fancc-/-* mice did not develop bone marrow aplasia or myeloid malignancies although they failed to monoubiquitinate FANCD2 (Y.S. and D.W.C., unpublished data, 28 June 2005). These data are consistent with a previous study utilizing an intercross of *Fancc-/-* mice with *Fanca* hypomorphic mice where *Fancc-/-* and *Fanca-/-* mice had similar numbers of myeloid progenitors and comparable MMC hypersensitivity to each other and *Fanca-/-;Fancc-/-* mice (Noll, Battaile et al. 2002).

Discussion

Numerous elegant studies have demonstrated the seminal role of ID complex monoubiquitination and phosphorylation in the activation of downstream effectors of DNA repair (Bagby and Alter 2006; Taniguchi and D'Andrea 2006; Ishiai, Kitao et al. 2008; Wang 2008; de Winter and Joenje 2009). Additionally, the FA core complex proteins are collectively required for activation of the ID complex and the loss of any single FA core complex protein is sufficient to prevent ID complex monoubiquitination (Taniguchi and D'Andrea 2006; de Winter and Joenje 2009). However, recent studies have provided cellular and biochemical evidence that the FA core complex proteins have functions that are independent of their respective roles in activating FANCD2 (Pang, Christianson et al. 2001; Pang, Christianson et al. 2002; Zhang, Li et al. 2004; Gordon, Alon et al. 2005; Hussain, Wilson et al. 2006; Leveille, Ferrer et al. 2006; Mukhopadhyay, Leung et al. 2006; Wilson, Yamamoto et al. 2008).

Specifically, *FANCC* and *FANCG*, the human homologues of the genes intercrossed here, have been shown to participate in molecular networks outside of the FA core complex. For example, FANCG has recently been shown to have a critical role in supporting the function of mitochondrial periredoxin-3 that is

implicated in rendering FA cells a characteristic hypersensitivity to oxidative stress (Mukhopadhyay, Leung et al. 2006). Also, FANCG but not FANCC has been shown to form a complex with FANCD1, FANCD2 and XRCC3, and this complex is thought to function in homologous recombination repair (Wilson, Yamamoto et al. 2008). Similarly, FANCC is known to participate in the PKR signaling pathway, while the other FA core complex proteins are not (Pang, Christianson et al. 2002; Zhang, Li et al. 2004).

Whether mechanistically these interactions contribute to BMF and myeloid malignancies independent of the ID complex is unclear. However, given the distinct hematopoietic phenotypes observed in syngeneic *Fancc* and *Fancg* deficient mice and observations that *Fancc-/-* and *Fancg-/-* mice do not develop overt BMF, the hypothesis that *Fancc* and *Fancg* may cooperate in stem cell control by conducting a genetic intercross was tested. The prediction was that if these two FA core complex proteins are multifunctional and influence hematopoietic cell survival independent of their role in the FA nuclear core complex, then the *Fancc/Fancg* double knockout mice would display distinct pathological phenotypes from those found in mice with inactivation of *Fancc* or *Fancg* only. Evidence that the phenotypes observed in *Fancc-/-;Fancg-/-* BMC

were specific to disruption of both the *Fancc* and *Fancg* genes was confirmed by the observation that *Fancc-/-;Fancg-/-* myeloid progenitors expressing transgenes that complement both *Fancc* and *Fancg* were resistant to MMC while *Fancc-/-;Fancg-/-* cells that contain a transgene that complements *Fancc* or *Fancg* only were hypersensitive to MMC. While this result was hypothesized, the apparent correction to levels comparable to WT raises further questions. Since the transduction efficiency is not 100%, but there was still a correction to near WT levels, it is then reasonable to hypothesize that the correction is even better than 100%. Future experiments may address this by sorting for transduced cells before plating progenitor assays.

Interestingly, the *Fancc-/-;Fancg-/-* mice developed BMF, myelodysplasia and complex cytogenetic abnormalities that are characteristic of bone marrow phenotypes observed in individuals with Fanconi anemia yet not observed in the single knockout mice. Prior in vitro data supporting cooperation of FANCC and FANCG in the human system include a report in an adenocarcinoma cell line that genetic disruption of *FANCC* and *FANCG* increased cytogenetic abnormalities (Gallmeier, Calhoun et al. 2006). The model described here provides an in vivo

platform that may be useful for questions regarding the pathogenesis of MDS and BMF.

Cross breeding of Fanconi mutant mice with other mice with inactivating gene mutations of non-FA genes has been reported and resulted in increased tumor formation and bone marrow hypocellularity (Hadjur, Ung et al. 2001; Freie, Li et al. 2003; Houghtaling, Granville et al. 2005) though the majority of malignancies observed in those crosses were not representative of those commonly observed in FA patients. The development of the model outlined here provides interesting opportunities for modeling of therapeutic protocols. The failure of established single knockout mice to develop BMF has been a major limitation in translational preclinical applications to test questions surrounding stem cell engraftment, homing and gene transfer. One such hypothesis is that FA patients with hypoplasia or overt BMF will not require myelopreparation prior to transplantation of autologous genetically corrected stem/progenitor cells but the lack of a spontaneous BMF model has limited effectively testing this question prior to phase 1 trials. The *Fancc-/-;Fancg-/-* mice now provide a model that can more accurately allow testing of this experimental question.

Given the phenotypes observed in the *Fancc-/-;Fancg-/-* mice, it was pertinent to ask whether these phenotypes would be broadly applicable to any intercross of FA core complex proteins. Current experimental data suggests that this is not the case. While consistent pathological phenotypes were observed in the *Fancc-/-;Fancg-/-* mice, using a similar experimental design as outlined in studies shown in this manuscript, intercrossed mice that are deficient in both *Fanca* and *Fancc* have no observable cooperative defects in stem cell function. These data are consistent with previous studies (Noll, Battaile et al. 2002) that did not report biological changes in hematopoietic progenitor cells exposed to alkylating agents in vitro. This suggests that there is something unique in the function of *Fancg,* since *Fancc* is common to both intercrosses and studies to assess this function of *Fancg* are an important area of future investigation.

In summary, these data provide the first genetic and in vivo evidence that the combined inactivation of *Fancc* and *Fancg* lead to a cooperative impairment in hematopoietic stem cell function, supporting the hypothesis that FANCC and FANCG function in divergent molecular pathways of relevance to hematopoiesis in addition to their roles in ID complex monoubiquitination. Our observations also reveal that the *Fancc-/-;Fancg-/-* mouse is a model that best recapitulates the

spontaneous clinical hematopoietic phenotypes of human FA, including malignancies and bone marrow aplasia. Therefore, the *Fancc-/-;Fancg-/-* double knockout mouse may be useful in studies of pathogenesis and experimental treatment of FA-related hematopoietic diseases.

CHAPTER 6

Future directions

This project has focused on the evaluation of a novel gene transfer vector and protocol for FA as well as on developing a genetic model that mimics the disease phenotype observed in patients. This genetic model will hopefully lead to a more complete understanding of the FA gene products' effects on hematopoiesis.

Our data show that the single knockout *Fanca-/-* and *Fancc-/-* mice display the same G-CSF hematopoietic mobilization defect as FA patients. Therefore, these mouse models are useful for testing new mobilizing agents such as AMD3100. Our data show that AMD3100 is effective at augmenting G-CSF mobilization in these mice and future studies may address this in FA patients.

The mechanism of AMD3100 and/or G-CSF mobilization is not fully understood. Additionally, the FA pathway is not fully understood. The *Fanca-/-* and *Fancc-/-* mouse models provide a unique platform for investigation of the mobilization pathways in the FA setting, where one mobilization pathway is inherently deficient (G-CSF) and the other is intact (AMD3100). Additionally, it

will be interesting to see if other FA mouse models (and patient's with the homologous complementation group) have the same phenotype in response to the different mobilizing agents or the combination. If they do not then that would point to a common function of the proteins (FANCA, FANCC and any others with a similar phenotype) related to mobilization outside of the traditional FA pathway. Recently there have been many papers published on the interactions of FA proteins outside of the pathway, but none directly related to mobilization.

From the multi-lineage analysis of the transplanted mobilized cells a lymphoid bias is observed for the *Fancc-/-* mobilized cells, which is not present in the other genotypes. Normally as mice age there is a shift toward the myeloid lineage (Waterstrat and Van Zant 2009). One hypothesis to explain this involves Notch signaling. It has been shown that Notch activity is required for lymphoid differentiation but not myeloid; however, in a mouse model that fails to glycosylate Notch the mice develop MPD, so Notch does play some role in the regulation of myeloid maintenance (Bigas, Robert-Moreno et al. 2010). FA patients typically get AML and not acute lymphoblastic leukemia (Kutler, Singh et al. 2003), which may be explained by an error in Notch regulation. Further

evaluation of Notch signaling in both this and the double knockout model may provide a new explanation for the development of BMF in FA.

While the benefits of transplantation using mobilized donor cells as compared to BMC are well established (Rosenbeck, Srivastava et al. 2010), determining whether or not mobilized cells respond to transduction in a manner similar to aspirated BMC is of utmost importance before these protocols move to clinical trials. Recent evidence has shown that G-CSF mobilized $CD34^+$ cells collected from the PB of healthy patients is efficiently transduced by a lentiviral vector with a short transduction protocol (Millington, Arndt et al. 2009). While our previous studies show our FV vector is capable of efficiently transducing BMC with a single overnight transduction (Si, Pulliam et al. 2008) and the studies here show that the FV vector is able to transduce and correct mobilized murine long-term repopulating cells, further studies are needed before progressing to clinical trials.

Initial experiments should focus on competitive repopulation experiments with secondary transplantations to demonstrate the self-renewal capacity of the transduced mobilized cells. Experiments will then be performed with cord blood $CD34^+$ cells from normal patients that will provide a baseline level of transduction

and repopulating ability in NOD/SCID2 mice. Further experiments will expand to include mobilized $CD34^+$ cells from normal patients. Ideally the transduction would then be tested on $CD34^+$ cells from FA patients and the correction of MMC hypersensitivity would be determined in progenitor assays.

Experiments here have utilized a novel FV vector. The construct used contains the SFFV promoter, which has been associated with clonal outgrowth in clinical trials for chronic granulomatous disease (Stein, Ott et al. 2010). Future experiments will include evaluation of other promoters that will ideally still be effective but will not result in adverse events such as transactivation leading to malignancy. Additionally, investigation of ways to enhance transgene expression such as codon optimization and the addition of insulator elements will be pursued.

The development of a murine model that recapitulates the patient disease phenotype opens multiple new avenues of investigation not available before. This model provides genetic evidence that the core complex gene products (FANCC and FANCG) have functions independent of the core complex and separate from each other. Within the FA scientific community there has been debate for many years on whether core complex proteins have functions independent of the core

complex. Now that there is a genetic model illustrating these independent roles of core complex proteins, future lines of investigation may focus on not only the FA pathway, but also the other functions of the FA proteins. Knowing these functions may help in recognition or discovery of novel therapeutic treatments for FA.

Since the first FA mouse model was developed there has been an interesting question left unanswered: why do the single knockout mice not recapitulate the patient disease phenotype? One hypothesis involves the environment in which the mice live. While children with FA are living in the world where they are exposed to viruses, bacteria and radiation daily, the mice are kept in a sterile facility. It is known that the FA cells are hypersensitive to inhibitory cytokines, which are released by the immune system especially during infections. So a child with FA who acquires an infection may experience an associated loss of HSC. The mice in our facility would not experience this naturally. Additionally, children are exposed to various DNA damaging agents in their environment that the mice are not. The difference in environment may be compounded by the difference in lifespan for the mice versus human patients. Future experiments could involve challenging the mice with various infectious agents.

The follow up question to why do the single knockout mice not display the patient disease phenotype is why do the double knockout mice display the phenotype? One hypothesis is that the result of the abrogation of the separate functions of FANCC and FANCG independent of the core complex is a mutator phenotype in the *Fancc-/-;Fancg-/-* cells which is not present in the single knockout cells. Our data from the cytogenetic analysis would support this hypothesis as only the double knockout cells had cytogenetic abnormalities. Why then do patients only need one gene to be mutated to display the same disease phenotype? In addition to the environmental factors and lifespan difference mentioned in the previous paragraph, another hypothesis is that in FA patients the HSC have experienced a second hit where a second gene has been knocked out or is no longer being expressed. Typically patients are identified when they come to the clinic with some sort of BMF. The patient's skin or peripheral blood cells are tested for hypersensitivity to DNA cross-linking agents and further complementation analysis is done to determine the specific complementation group. If any of the HSC have had a second hit it may not be detected by this procedure. Therefore, it is possible that there may indeed be an acquired double knockout in the bone marrow of an FA patient. Another possibility is that although

a gene may be intact, it may be down regulated through various mechanisms. Work with our collaborator has allowed us to compare expression profiles from our DKO model with those from patients before and after the onset of BMF. This data has led to selection of specific targets that will be investigated.

When considering gene transfer therapy or bone marrow transplantation for FA, whether or not any myelopreparative regimen is necessary has been a long-standing question. It has been shown that the agents typically used for myelopreparation can cause adverse events in FA patients (MacMillan and Wagner 2010) and also that the non-corrected FA murine cells are hypersensitive to DNA cross-linking agents and may transform into malignant clones (Li, Le Beau et al. 2005). However, if no conditioning regimen is used, there may be little to no long-term engraftment as seen in previous clinical trials (Liu, Kim et al. 1999; Kelly, Radtke et al. 2007) and FA single knockout models not experiencing BMF (Si, Ciccone et al. 2006). It has been shown that WT cells and the corrected FA cells do have a competitive advantage over the non-corrected FA cells in repopulation of a lethally irradiated mouse (Haneline, Li et al. 2003; Si, Pulliam et al. 2008). Therefore, the WT cells or corrected FA cells may have an advantage over the endogenous hematopoietic cells in an FA

murine model exhibiting BMF. It has been difficult to thoroughly address this question in the clinic and previously there was not a murine model of FA that displayed spontaneous BMF. The novel murine model described here provides an important platform for evaluation of myelopreparative conditioning regimens.

Initial experiments will involve transplantation of WT cells transduced with a GFP reporter construct or corrected *Fancc-/-* cells into mice displaying BMF, at a dose equivalent to that acceptable for patients. These transplantation experiments will determine whether the mice displaying BMF can be rescued with transplantation in the absence of myelopreparation.

Our lab has shown previously that administration of IFN-γ allows for engraftment of WT cells in *Fanca-/-*, *Fancc-/-* and *Fancg-/-* mice (Li, Yang et al. 2004; Si, Ciccone et al. 2006). Therefore, IFN-γ may be useful as a myelopreparative agent during HSC transplantation for FA patients. Future experiments will address the possibility of using IFN-γ to precondition the bone marrow of FA double knockout mice. *Fancc-/-;Fancg-/-* mice will be treated with IFN-γ and then transplanted with corrected *Fancc-/-* cells, or cells transduced with the reporter construct only, either *Fancc-/-* or WT. If successful, limiting

dilutions may be done to determine the minimum number of corrected cells necessary for engraftment and reconstitution of the hematopoietic system.

In the *Fancc-/-;Fancg-/-* model, a portion of the mice also present with malignancies. This malignant transformation allows us to address another interesting question. It is possible that a malignant clone may either directly or indirectly inhibit the engraftment of transplanted HSC, which would have significant implications for bone marrow transplantation procedures. The novel model presented here will allow for evaluation of whether or not such an event is occurring and an investigation of the mechanism, which could lead to identification of potential therapeutics.

Another important aspect of this model is the opportunity to compare expression profiles before and after the progression to BMF and also to MDS. Microarray data was collected for these different groups and analysis of the data has led to specific gene targets that are elevated in BMF samples as compared to normal samples and then decreased in MDS samples. This was compared to data from FA patients before and after the onset of BMF and/or MDS provided by our collaborator, Dr. Grover Bagby. Some of the genes identified are known to be involved in spindle checkpoint control, which could provide an explanation for the

aneuploidy observed in the mouse model. Further exploration of these gene targets as instigators of cancer will be performed.

This project has evaluated an alternative mobilization protocol for FA HSC/HPC; currently there is a clinical trial of this protocol recruiting FA patients. Data presented here also demonstrate successful transduction of these mobilized cells with a novel FV gene transfer vector. And lastly, this project resulted in the development of a novel murine model that recapitulates the hematopoietic disease phenotype observed in patients, which will allow for assessment of future treatments, including gene transfer therapy.

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Honors, Awards, Fellowships

August 2008 - July 2009, IUSM Gene Therapy Training Grant T32 HL007910-09

May 2009, IUSM Travel Grant

August 2006 - July 2007, IUSM Translational Research Fellowship

August 2005 - May 2006, IUPUI Graduate Student Fellowship

2003, Victor H. and Elizabeth A. Barnett Research Award

Fall 2002, Research Innovation Scholarship Creativity (RISC) Grant

Conferences Attended

Oral Presentations

May 2009, Pediatric Academic Societies Annual Meeting

October 2006, Fanconi Anemia Research Fund 18th Annual Scientific Symposium

Poster Presentations

May 2009, American Society for Gene Therapy

May 2003, British Society for Developmental Biology

Abstracts

December 2006, Annual Meeting of the American Society of Hematology