MUTATION ANALYSIS IN TWINS WITH FANCONI ANEMIA AND MODIFICATIONS TO A GENE MAPPING TECHNIQUE

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Anuj N. Mankad

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CERTIFICATE OF APPROVAL

This is certify that the Ph.D. thesis of

Anuj Mankad

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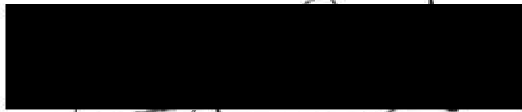

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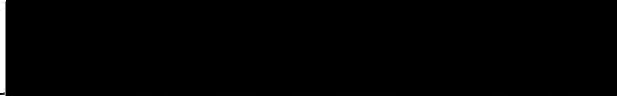

John Belknap

Susan Olson

Matthew Thayer

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ABBREVIATIONS

α-MEM = Minimal Essential Medium, alpha modification
μF = micro-Farad
A. thaliana = Arabidopsis thaliana, the mustard seed plant
a.a. = amino acid
BFU-E = burst forming unit – erythrocyte
BLAPs = BLM (Bloom syndrome protein) associated polypeptides
bp = base pair
C. elegans = Caenorhabditis elegans, a round-worm
cDNA = complementary DNA, DNA reverse-transcribed from mRNA
CFU-GM = colony forming unit – granulocyte macrophage
CHO cells = cells derived from chinese hamster ovary
cM = centiMorgan
Con A = concanavalin A
D. melanogaster = Drosophila melanogaster, the fruit fly
DAPI = 4,6-diamidino-2-phenylindole (a fluorescent stain for DNA)
DEAH-box = protein region containing aspartate, glutamate, alanine, and histidine
DEB = diepoxybutane
DMEM = Dubelco's Modified Eagle's Medium
DNA = deoxyribonucleic acid (the genetic molecule)
DNA-PK = DNA-Dependent Protein Kinase
Dox = Doxycyclin, a tetracycline analog
DSB = double strand break (of DNA)
dsRNA = double stranded RNA activated protein kinase
DTT = dithiothreitol
EBV= Epstein-Barr virus
EDTA = Ethylenediaminetetraacetic acid
ELTCI = extended long term culture initiating cell
FA = Fanconi anemia
FAAPs = Fanconi anemia associated polypeptides
FAH = fumaryl acetoacetate hydrolase
FBS = fetal bovine serum
G1 / S / G2/ M phases = gap 1 / synthesis / gap 2 / mitotic phases of the cell cycle
G-CSF = granulocyte colony stimulating factor
GFP = green fluorescent protein
GM-CSF = granulocyte-macrophage colony stimulating factor
HDR = homology directed repair (of DNA)
HLA = human leukocyte antigen (a means by which to distinguish the body's own cells from invaders)
HPRT = hypoxanthine-guanosine phosphoribosyl transferase
HR = homologous recombination
HRP = horseradish peroxidase
HSC = hematopoietic stem cell
ICL = interstrand (DNA) crosslink
IFN-γ = interferon gamma

IL-3 = interleukine-3
IL-6 = interleukine 6
INOS = inhibitor of nitrogen oxide synthase
IRES = internal ribosome entry site
kb = kilobase
kD = kilo-Dalton
L-Glut = L-glutamine
LTCI cell = long term culture initiating cell
MMC = mitomycin C
MMCT = microcell mediated chromosome transfer
mRNA = messenger RNA (the intermediary between DNA and protein)
MTX = methotrexate
NCBI = National Center for Biotechnology Information
NES = nuclear exclusion signal
NF-κB = neuclear factor kappa B
NHEJ = non-homologous end joining
NLS = nuclear localization signal
OMIM = Online Mendelian Inheritance in Man
P/S = penicillin + streptomycin
PAGE = polyacrylamide gel electrophoresis
PBL = peripheral blood lymphocyte
PBS = phosphate buffered saline solution
PCR = polymerase chain reaction (a way by which to amplify DNA)
PEG = polyethylene glycol
PHA = phytohemagglutinin (type P)
PIC = protease inhibitor cocktail
PKR = double stranded RNA dependent protein kinase
PMSF = phenylmethylsulfonyl fluoride
PVDF = polyvinylidene difluoride
RMN complex = Rad50/MRE11/NBS1 complex
RNA = ribonucleic acid
RT = reverse transcriptase (an enzyme which produces DNA from RNA)
RT-PCR = reverse transcription polymerase chain reaction, or PCR done on cDNA obtained by using a reverse transcriptase on mRNA
S. cerevisiae = Saccharomyces cerevisiae, brewer's yeast
SCE = sister chromatid exchange
SCF = stem cell factor
SDS = sodium dodecylsulfate
SDS-PAGE = SDS-polyacrylamide gel electrophoresis
STAT = signal transduction and activator of transcription
SV40 = simian virus 40
TBS = tris buffered saline
TBS-T = tris buffered saline plus tween-20 detergent
TNF-α = tumor necrosis factor alpha
TRAIL = TNF-related apoptosis-inducing ligand

tTA = tetracycline responsive transactivator / tetracycline repressible transcription activator
UVA = ultraviolet radiation type A
V(D)J recombination = recombination occuring in the variable, diversity, and joining regions of the genes coding for molecules involved in the immune system
VNTR = variable number tandem repeat
wt = wild type (i.e. normal)
XP = xeroderma pigmentosum
XRCC = X-ray cross complementation group

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Abstract

Monozygotic twin sisters, with solely non-hematological symptoms of Fanconi anemia (FA), were discovered to be somatic mosaics. Spontaneous and mitomycin C induced chromosomal radial formation, which could be corrected with wild type *FANCA*, was noted in fibroblasts but not in lymphoblasts. Molecular analysis of *FANCA* revealed a frameshift causing deletion in exon 27; an exon 28 mutation altered arginine 880 to glutamine (R880Q), resulting in primarily cytoplasmic expression and reduced function. Additionally, an acquired, peripheral blood specific, exon 30 missense mutation was found in cis with the exon 28 mutation. It altered glutamate 966 to lysine (E966K), functionally compensated for the primary mutation, and restored nuclear localization. As the same secondary mutation was present in the blood of both sisters, but not in their fibroblasts, nor in either parent, and as both sisters have been free of hematopoietic symptoms for over two decades, this *de novo* mutation likely occurred in a single hematopoietic stem cell (HSC) in one twin. Apparently, descendants of this functionally corrected HSC, via intra-uterine circulation, repopulated the blood lineages of both sisters. This would imply that treating FA patients with gene therapy might require transduction of only a few hematopoietic stem cells.

Specific secondary issues raised by the presence of the mutations were also studied. In addition to the functional studies on the mutant alleles, the possibility of alternate splicing caused by the exon 28 and 30 mutations was investigated. No mutation-specific alternate transcripts were found, although an alternative transcript was noted in wild type cells that had bearing on the studies at hand. This transcript, which lacked exon 30, was found to be unable to rescue FA-A cells from mitomycin C sensitivity. A hypothesis concerning correlation between one of the *FANCA* mutations and pancreatic cancer in members of the kindred was also explored and invalidated.

While the patients studied in the first part of this thesis had mutations in a known FA gene, several FA patients appear to have mutations in yet unidentified genes. The technique of microcell mediated chromosome transfer (MMCT) can be employed to map the defective gene in such cases. The object of MMCT is to form hybrid cells with a single donor cell chromosome in a recipient cell genomic background. The hybrids are assayed for correction of a cellular defect, in order to determine if the desired gene is on the transferred chromosome. While attempting to utilize this technique, several difficulties arose which affected the efficiency of the transfer procedure. Steps were taken to deal with these issues and to increase the yield and usefulness of the technique. Improvements and modifications made to the technique are described, including means by which to reduce the number of whole cell hybrids, use of randomly marked mouse cells as donors, and the development and application of assays for successful chromosome transfer from such donor cells. The modifications made did increase yields, while generating several successful MMCT hybrids.

Introduction

Fanconi anemia (FA) is a bone marrow disorder, with high rates of morbidity and mortality. However, it is difficult to diagnose because of its variable phenotype, and the understanding the actual pathways involved in FA is incomplete. Moreover, not all of the FA genes have been identified. The goal of this work was twofold: first, to study a family in whom monozygotic twin sisters were initially diagnosed as non-FA in order to understand how the mutations they carry affect their phenotype and to gain a better understanding of the renewal capability of functionally corrected stem cells, and second, to explore modifications in a technique that could be used both to discover new FA genes and, potentially, for therapeutic purposes.

The remainder of this chapter lays the foundation and provides background for the work involved in this thesis. It covers the symptoms of FA patients, diagnostic methods, and treatment options, as well as an overview of the defects and abnormalities of FA at the cellular level. Furthermore, since this thesis deals with molecular aspects of *FANCA*, this chapter also reviews the literature on the molecular genetics of FA, with emphasis given to the data on *FANCA* and its interactions. The mouse homolog for this gene is also described, and mouse models of FA are delineated. The topic of somatic mosaicism in FA, which is of fundamental importance to Chapter 2, is discussed at the end of this introduction.

Chapter 2 discusses the case of a pair of twins initially described[1] as having a variant form of FA. Follow up work revealed that the individuals in question were, in fact, true FA patients, and that they belonged to the FA-A complementation group. This finding gave rise to the hypothesis that these sisters had inherited mutations in *FANCA*, but had acquired a compensatory sequence change as well. Indeed, in addition to the two expected detrimental alterations, a third mutation was uncovered in blood cells but not skin cells of these patients. Functional assays demonstrated that the protein expressed off of the maternal allele was partially functional due to a missense mutation, and that the downstream compensatory missense mutation returned function to normal levels. Furthermore, the fact that the twins, now in their late 20's, have no hematological symptoms, and that they both carried the same three mutations, indicated that the

acquired mutation occurred in a single hematopoietic stem cell (HSC), and that the progeny from this HSC effectively repopulated both twins.

Chapter 3 describes a technique called microcell mediated chromosome transfer, which has been used for, among other purposes, mapping genes involved in human diseases, including FA. The objective of this technique is to produce hybrid clones that contain both the genome of the recipient cell and a single chromosome from the donor cell line. An assay for a change in phenotype is then used to determine if the chromosome transferred contains the desired gene. Improvements were made to the technique, and to methods used in follow up to the transfer, in order to improve the efficiency of formation of the hybrid clones, and to extend the usefulness of the technique. The alterations made, and the greater insight into the technical aspect of the procedure that emerged, are detailed in the chapter.

Finally, Chapter 4 presents a discussion of the relationship of these findings to one another and their relevance to the fields of Fanconi anemia research, gene therapy, and gene mapping. Future directions of this work are also explored. In summary, these findings ascertain the capacity of self-renewal of a hematopoietic stem cell to a greater extent than previously determined, detail two novel mutations in *FANCA* and elucidate the mechanism for functional correction and repopulation of twin FA-A patients, and extend and improve upon a long-standing technique. Overall, the work in this dissertation will contribute to a better understanding of *FANCA* and the FA pathway, possibly assist with identification of as yet unmapped FA genes, and potentially provide a means for long-lasting treatment of patients with FA and other hematopoietic diseases.

I. Clinical, Diagnostic, and Treatment Synopsis

Clinical presentation of Fanconi Anemia. Fanconi anemia (FA; OMIM #227650)[2] is a heterogeneous, largely autosomal recessive genetic disorder, with, currently, 12 known complementation groups (A, B, C, D1, D2, E, F, G, I, J, L, and M)[3-6]. It was first described by Guido Fanconi, a Swiss pediatrician[7]. The estimated incidence in the general population is 1 in 100,000 live births worldwide, although certain

ethnicities such as Ashkenazi Jews, Afrikaaners, and specific Turkish and Saudi Arabian populations demonstrate higher incidences[8,9].

Most FA cases are diagnosed between 3 and 10 years of age, with age of onset averaging at 7 years[10,11], and a mean age of survival of 16 years; however, diagnosis can also occur in adulthood in cases where onset occurs later[12]. FA patients usually develop macrocytosis and pancytopenia during the first decade of life[10,13-15]. Progressive bone marrow failure gives rise to aplastic anemia, compounded by thrombocytopenia, and, subsequently, leukocytopenia, resulting in susceptibility to infection.

In addition, patients with FA who survive the anemia are prone to the development of malignancies, including acute myeloblastic leukemia by age 40 in about 52 percent[10]. Patients also have increased incidence of squamous cell carcinomas of the head and neck, including of the tongue and larynx, as well as skin; other solid tissue cancers have also been described, including vulvar squamous cell carcinoma, osteosarcoma, hepatocellular carcinoma, pancreatic carcinoma, stomach and esophageal carcinoma, breast cancer, and even meduloblastoma[16-29]. Breast cancer susceptibility may be connected to the fact that a gene known to cause breast cancer when mutated, BRCA2, is also the gene mutated in FA group D1[30], and that the FA group D2 protein colocalizes with BRCA1[31],which also binds FANCA[32]. Increased incidence of solid malignant tumors has also been noted in patients who have undergone bone marrow transplantation to correct the anemia or pancytopenia[33], since the increase in life span conferred by transplant places patients in the age range for occurrence of FA-related tumors.

These hematological and oncogenic findings are compounded by developmental defects. Approximately 50 percent of patients present with one or more congenital abnormalities[34], although their severity and prevalence are highly variable in FA. Such abnormalities can include growth retardation and short stature, thumb and radial deformities, kidney malformation or absence, skin hyperpigmentation and café au lait spots, microcephaly, cardiac malformations, and mild mental retardation[35-37]. Male patients may also present with underdeveloped testes and abnormal spermatogenesis, and growth hormone deficiency has also been noted[38].

Given the variable phenotype of FA and its overlap with other disorders, a purely clinical diagnosis is difficult[39]. However, FA cells demonstrate spontaneous chromosome

breakage, as well as sensitivity to bi-functional alkylating agents, also known as cross-linking agents, which covalently connect nucleotides on opposite strands of DNA. Treatment of FA cells with such agents induces elevated chromosomal breakage and formation of chromosome radials in dividing cells. Spontaneous chromosomal changes are seen in cells from FA patients, like cells from Bloom syndrome and ataxia telangiectasia patients; but in the latter two diseases, the chromosomal abnormalities are not increased with diepoxybutane (DEB) treatment[40,41], even though DEB is a cross-linking agent. A diagnosis of FA can therefore be made based on cytogenetic analysis following treatment of cells to such agents such as diepoxybutane (DEB) or mitomycin C (MMC)[42,43].

Treatment options: bone marrow transplant. For almost three decades[44], bone marrow transplant has been used to treat and, in most cases, cure patients with Fanconi anemia. However, caveats apply to the use of this treatment, especially with patients with Fanconi anemia. First, optimal outcome requires use of bone marrow from an HLA-matched relative, which is not always available. Secondly, agents used to encourage engraftment by killing or causing growth arrest of recipient marrow, such as cyclophosphamide, often work by causing DNA damage, and, as a result, increase the risk, especially in FA patients, of leukemia or other cancers. In addition, patients with FA are more sensitive to such agents[45,46], which, at least in the case of cyclophosphamide, may be partly due to altered drug metabolism[47]. This requires the use of lower doses of such drugs in the pre-transplant regimen for FA patients. Fanconi anemia patients, however, have a predisposition to myelodysplastic syndrome, and lower doses may be insufficient to kill all of the pre-leukemic cells, so the patient may still present with leukemia after transplant. Indeed, leukemia-free survival rates in cases of transplant into patients with pre-existing leukemic transformation have been quite poor[48], and, in cases where the donor marrow responds to the leukemia by mounting a graft-versus-leukemia effect, fatal graft-versus-host-disease can occur[49]. Therefore, although individuals with both FA and signs of pre-leukemic transformation or myelodysplastic syndrome have been cured using bone marrow transplant[50-52], early intervention is preferable.

FA patients may also develop severe graft-versus-host-disease, either acute or chronic, or, conversely, may become ill from fungal or viral infections due to suppression

of the immune system. Patients have even been described who develop severe oesophagitis after transplant[53]. To deal with many of these problems, various preparative regimens have been tried, with mixed success, including use of low, fractionated, localized, or no irradiation, as well as treatment with agents, alone or in combination, such as busulfan, fludarabine, anti-thymocyte globulin, cyclosporin A, MTX, and low dose cyclophosphamide[46,54-64]. The likelihood of successful outcome for transplant patients has improved over time, and a recent study[65] indicated a 100% success rate with 22 patients treated with stem cell transplantation from HLA-matched related donors.

However, since the optimal transplant outcome requires having an HLA-matched relative present, these rates are applicable to only some patients. Other approaches include androgen therapy, which improves peripheral blood counts[66]. This is only a temporary measure, as patients become resistant to the treatment over time and require erythrocyte and platelet transfusions, as well as granulocyte- and granulocyte-macrophage-colony-stimulating factors (G-CSF and GM-CSF) to improve leukocyte count.

Therefore, alternative methods of repopulating the bone marrow of such patients have been explored. The most obvious option is to find donors who are unrelated but HLA-matched with the recipient, or who share most but not all of the HLA types with the recipient. Transplantation from such donors has had poor results in the past, even for patients with severe aplastic anemia[67], and patients with FA can have unusual complications[68]. Comparisons between transplants done with HLA-matched related donors versus alternative donors demonstrated a stark difference in outcome[69,70], and transplants done with matched unrelated donors alone showed poor outcomes[71-73]. In one study attempting to predict outcomes from such transplants, it was determined that those with male donors fared better than those with female donors, and, more importantly, the use of androgen therapy prior to transplant was contra-indicated[73]. In contrast to the previous cases, one patient has been successfully treated with bone marrow from a haploidentical parent[74], and relatively successful transplants of unrelated donor allografts of bone marrow stem cells have been performed[75].

Treatment with Peripheral or Cord Blood. Other alternatives to bone marrow transplant have also been attempted. Peripheral blood stem cells from an unrelated, HLA-

matched individual[76] and from a haploidentical relative[77,78] have been successfully transplanted; however, transplant of stem cells from peripheral blood of an HLA-mismatched donor in one case failed to protect an FA patient from fungal infection[79]. Another approach is transplantation of umbilical cord blood or of stem cells derived from cord blood. Cord blood from a single pregnancy is sufficient, both in terms of cell number and in terms of quality, for one transplant[80,81]. Cord blood transplantation from HLA-matched siblings has been successfully attempted many times[82-87], and, as has been tried with bone marrow transplants, a modified regimen with fludarabine has also been attempted succesfully[88]. These successes have prompted the formation of an international cord blood transplant registry[89,90] in order to match patients with HLA matched newborns and stored cord blood. Cord (or placental) blood transplant from HLA-mismatched individuals or from HLA-matched unrelated individuals has also been attempted[86,91,92], and, for the most part, is successful, but engraftment failure can occur[78]. One caveat to cord blood transplantation, and perhaps to transplantation in general, is that androgen treatment for FA, prior to transplantation, can result in lethal hepatic adenomas[93]. Overall, however, the survival rate after unrelated donor cord blood transplantation is similar to that for HLA-matched unrelated donor bone marrow transplant[94] and, while some concern exists over possibly increased risk for Epstein-Barr virus-associated lymphoproliferative disorders after unrelated cord blood transplant, it appears to be no greater than that for unrelated bone marrow transplant[95].

Nevertheless, parents of patients with Fanconi anemia who cannot find HLA matched relatives have very few immediate options. Searching for unrelated donors takes time, cord blood transplant requires a pregnancy, and neither of these approaches is guaranteed to yield an HLA match. To ensure that an HLA-matched donor is found before it is too late to help the patient, therefore, some parents have undertaken deliberate conception by *in vitro* fertilization. The first case of *in vitro* fertilization using preimplantation genetic diagnosis to select for HLA-matched, non-FA embryos occurred only a few years ago[96], as did the first successful cord blood transplant from such a pregnancy[97], but several such attempts have occurred since then[98-100]. Selection for FA unaffected HLA-matched embryos, whether using preimplantation genetic diagnosis or

not, does raise some ethical issues[101], but also reduces the waiting time required to find a matched donor.

Gene Therapy. A final alternative for parents of children with Fanconi anemia, gene therapy, provides the option with the least chance of graft-versus-host disease or graft rejection, while avoiding ethical issues related to stem cells, since autologous transplant after genetic correction could be utilized[102]. Gene therapy is promising in part because any number of different viral vectors could be used; for example, i*n vitro* correction of both lymphoblasts and CD34+ hematopoietic progenitor cells from Fanconi anemia group C (FA-C) patients has been demonstrated using recombinant retroviral[103] and adeno-associated[104] viral vectors. A retroviral vector carrying *FANCC* but marked with I-NGFR (a cell surface marker) rather than antibiotic resistance[105] has been used, and FANCC has also been transferred into lymphoblasts from an FA-C patient using lentiviral vectors[106]. Lymphoblast cells from four FA-A patients have also been transduced with *FANCA* using retroviral vectors[107]; viral vectors containing other Fanconi genes are also available or could be easily produced. Use of viral vectors with internal promoters, which must integrate intact, is preferred in order to prevent inadvertent activation of oncogenes and gene mis-expression due to random integration of the vector.

Clinical protocols for transduction of CD34+ hematopoietic progenitor cells, from both cord blood and peripheral blood, with FANCC-containing retroviral vectors, have also been developed, including ex vivo expansion of transduced cells[108,109]. One group has run a clinical trial for treatment of patients with group C Fanconi anemia by retroviral transduction of wild type *FANCC* into CD34+ progenitor cells, and three or four cycles of gene transfer resulted in transient *FANCC* expression in peripheral blood and bone marrow, which corresponded to transient increases in bone marrow cellularity[110,111]. However, stable transduction of hematopoietic stem cells has yet to be demonstrated in a clinical trial. In addition, both because of the relatively low transduction efficiency and because of the number of cells required for effective transplant, this approach often requires *ex vivo* expansion of progenitor cells from patients with Fanconi anemia through rapid expansion or long term culture, the latter of which has proven to be difficult[112]. The use of extended mobilization of patient peripheral blood stem cells with G-CSF and

multiple collections, followed by storage and pooling, might provide enough cells for use in gene therapy[113]. This and other techniques are being tested, in various combinations, in phase I clinical trials for gene therapy of both FA-C and FA-A complementation group patients[114].

However, other technical difficulties still need to be overcome. Possible toxicity of expression from retroviral vectors, which would cause destruction of transduced stem cells[115], is one problem, especially in cases of overexpression, common with many viral vectors, which may need to be avoided with certain FA complementation group genes. In addition, a type of catch-22 is also at issue. On the one hand, long-term expression – required for effective therapy -- has yet to be demonstrated. On the other hand, long-term expression of certain types of viral vectors might elicit an immune response. Ideally, gene therapy would utilize gene targeting to the native locus, such that the endogenous promoter would drive expression, but the efficiency of gene targeting has yet to be improved to the level at which this approach would be feasible.

II. Functional Phenotypes of Fanconi Anemia Cells

The defining cellular characteristic of Fanconi anemia is an elevated level of spontaneous chromosome breakage[116], which is enhanced by exposure to mitomycin C[42], diepoxybutane[43], psoralen plus long wavelength ultraviolet light[117], nitrogen mustard[118], or other interstrand crosslinking agents. Fanconi cells also exhibit radial exchange figures and endoreduplication, especially after treatment with crosslinking agents[119].

Cell cycle delay. Primary fibroblasts from Fanconi anemia patients also display other cellular defects, including decreased growth rates and increased generation times[117]. A slower cell cycle in lymphocytes has also been demonstrated[120]. This elongation of the cell cycle is at least partially caused by cell cycle arrest. Until recently, this arrest was thought to occur at the G2/M boundary or in late G2 phase[118,120-124]. However, an important study found that introducing interstrand crosslinks specifically during G2 phase arrests cells not at the G2/M boundary, but in the following S phase, after partial DNA replication[125]. Interestingly, another group found evidence for impaired S-phase arrest[126], which seems to directly contradict the previous finding. However, the data from this

study can be interpreted as supporting an elongated S-phase. The authors found a higher proportion of S-phase replicating cells in an FA population compared to wild type cells, but BrdU staining used in the study may simply indicate unscheduled DNA synthesis caused by futile attempts to repair the cross-link via translesion synthesis. In this light, their work implies elongated S-phase in FA cells. Earlier works, using BrdU staining and cell cycle analysis, also support a long S-phase, such as a study that describes arrest as occurring in "the first chromosome cycle (S, G2/M phases)"[121]. This cell cycle arrest depends on the p53 pathway, since FA cells upregulate p53 at lower MMC concentrations compared to wild type cells[127,128] and mouse embryonic fibroblasts from *fancd2* (FA-D2 gene) knockout mice underwent arrest after DNA crosslinking, but not those from *fancd2 / trp53* double knockout mice[129]. Yet the arrest is caused not by failure of the normal cell cycle checkpoints, but as a normal response to extensive or difficult-to-repair DNA damage[130,131].

Secretion of and response to growth factors. Another aspect of the FA cellular phenotype that has been extensively studied, largely as an attempt to understand the mechanism of bone marrow failure, is the response to or secretion of various factors. Over a decade ago, co-cultivation of FA cells from one complementation group with certain mouse "FA-like" cell lines partially corrected the MMC sensitivity of these cells[132], indicating that secreted factors may play some part in the severity of the FA phenotype. Lack of response of Fanconi bone marrow to stem cell factor (SCF) was demonstrated in various colony formation assays[133]. Moreover, FA fibroblasts and lymphoblasts have reduced production of interleukine-6 (IL-6), and adding IL-6 to the medium partially corrects MMC sensitivity[134]. IL-6 addition also reduces tumor necrosis factor alpha (TNF-α) overproduction noted in FA lymphoblasts[135]. Additionally, partial correction of MMC sensitivity of FA lymphoblasts by anti-TNF-α antibodies[135], overexpression of TNF-α in human marrow mononuclear cells from FA patients[135], and sensitivity to TNF-α of bone marrow-derived cells from FA patients and from a mouse model of FA[136,137], have all been demonstrated. FA cells are not differentially sensitive to a TNF family member, TRAIL[138], but bone marrow cells from an FA mouse model are sensitive to macrophage inflammatory protein 1 α[136].

These findings are generally accepted; however, sensitivity of FA cells to interferon gamma (IFN-γ), specifically cells from the FA-C complementation group, has been controversial. Initial characterization of an FA-C mouse model (*Fancc* knockout mice) indicated sensitivity of hematopoietic progenitor cells to IFN-γ[139] and this was also demonstrated in other studies[136,140,141]. But contradictory data also exist[142,143]. Sensitivity in mice may be strain-specific. IFN-γ is overexpressed in bone marrow from FA patients[137], and Fanconi group C bone marrow seems to be sensitive to IFN-γ[140]. Several further studies of the IFN-γ signal transduction pathway and possible interaction with the Fanconi pathway have explored IFN-γ inducible genes--in particular, the Jak / STAT pathway--as well as increases in reactive oxygen species, interactions of heat shock protein 70 with FANCC, apoptosis by caspase-3 activation via p38, the dsRNA protein kinase PKR and its interaction with FANCC, and the roles of nitric oxide and iNOS[144-152].

Oxygen sensitivity. Just as the significance of IFN-γ sensitivity has been questioned, the importance of another cellular phenotype of FA cells, sensitivity to oxygen and to oxygen radicals, has been debated. Several indirect lines of evidence for oxygen sensitivity exist, including increased release of oxygen radicals from leukocytes of Fanconi patients[153,154], as well as accumulation of 8-hydroxy-2'-deoxyguanosine and 8-hydroxyguanosine, indicative of oxidative damage to DNA and RNA, respectively, in FA cells[154,155]. Somewhat reduced hydrogen peroxide decomposition and catalase activity in FA cells has also been reported[155], as have reduced oxygen consumption, increased activity of the antioxidant PHGPx, and constitutive expression of NF-κB, which is induced by presence of reactive oxygen species[156]. Also, one report[157] claims that overexpression of the intracellular antioxidant thioreduxin abolishes the DNA damaging effects of MMC and DEB, although it has no effect on chromosome breakage.

In addition to these indirect lines of evidence, Fanconi lymphocytes have been shown to have chromosome breakage under hyperoxic conditions[117,158]; however, while amount of breakage increased with oxygen tension, the response was variable between different patients[159], and normal cells also showed chromosomal aberrations under hyperoxic conditions[160]. Mitomycin C (MMC) can generate free radicals, and in fact, does so more than some of its analogs[161]. Adding MMC to cultures prior to experiments with different levels of oxygen did, in fact, increase the sensitivity of FA cells[159], and, in

a separate study, Fanconi lymphoblasts were more sensitive than wild type cells to MMC under normoxic conditions (20% oxygen), but not under 5% oxygen, the latter of which favor cross-link formation[162]. This does not mean, however, that FA cells are not sensitive to crosslinks, as the authors claim, since they are sensitive, after all, to psoralen plus two doses of UVA radiation, with which one can ensure formation of crosslinks. Rather, these findings imply that hypersensitivity to oxygen in FA lymphocytes and, perhaps, in bone marrow cells, may play a major role in the anemia phenotype. In terms of cell cycle defects, exposure of primary FA fibroblasts to 5% oxygen restores in vitro growth to normal, and exposure to 35% oxygen can cause accumulation in S/G2/M phase[163]. In contrast, while primary FA fibroblasts cultured in 1% oxygen show enhanced growth, and primary fibroblasts from multiple patients are uniformly sensitive to oxygen but variably sensitive to MMC, transformation with SV40 large T antigen abolishes oxygen sensitivity without affecting sensitivity to MMC[164]. Transformed lymphoblasts from a group C Fanconi patient are also not differentially sensitive to oxygen when compared to retrovirally corrected cells[165]. These lines of evidence indicate that oxygen sensitivity is secondary to the molecular defect, even if it plays a role in the clinical progression of the disease.

Mitochondrial function. The issue of oxygen sensitivity raises the question of whether Fanconi cells have normal mitochondria, since mitochondrial function is directly tied to cellular levels of oxygen and oxygen radicals. Unlike normal mitochondria, FA cell mitochondria have a filamentous shape and form an interconnected reticular structure[166]. In an oxygen-dependent, psoralen-induced manner, FA cells undergo an unusual, rapid, and transient mitochondrial matrix densification[167]. However, while FA cells seem to be sensitive to glycolytic metabolism inhibitors, they are resistant to mitochondrial damage-induced apoptosis[168].

Apoptosis. In fact, several studies have concluded that Fanconi cells are deficient in or resistant to typical p53- and caspase-3-dependent apoptosis in response to ionizing radiation[169-171], although one study in *Fancc* (FA-C group) knockout mice seems to contradict this[172]. In contrast, both bone marrow derived hematopoietic progenitor cells and lymphocytes, particularly those from FA-C complementation group patients or *Fancc* knockout mice, have been repeatedly found to be prone to apoptosis when cultured *ex-*

vivo or when exposed to certain inhibitory cytokines, such as INF-γ and TNF-α[136,140,144,146,147,172-181], and cytokine induced apoptosis may be p53 dependent[172]. In fact, FA lymphocytes and lymphoblasts have been shown to have a higher than normal rate of spontaneous apoptosis[169,170], and a model involving knockdown of the FA-D2 complementation group homolog in zebrafish demonstrated an increase in spontaneous, p53-dependent apoptosis[182]. Nevertheless, Fanconi cells do not require p53 in order for DNA interstrand cross-linking agents such as mitomycin C to induce cell death[128], and the dying cells may utilize something other than the canonical apoptosis pathway[170,183]. Instead, MMC-treated FA cells undergo a necrotic or caspase-3 independent apoptosis-like cell death[184]. Fanconi cells may also have an altered reaction to Fas[170,184]. However, the use of the non-apoptotic or apoptosis-like pathway in response to interstrand crosslinking agents is not absolute; whether Fanconi cells treated with cisplatin undergo caspase-mediated or caspase-independent cell death seems to depend on cisplatin concentration[185]. Specific study of FA-C cells by various groups has resulted in some interesting findings, other than the abovementioned increased INF-γ and TNF-α induced apoptosis. Unlike FA-A cells, FA-C cells do not appear to undergo DNA fragmentation upon treatment with crosslinking agents[185,186], and so *FANCC* may act as a switch between apoptosis and necrosis due to MMC exposure, or the various FA proteins, separately or as a complex, may act to suppress apoptosis in favor of cell cycle arrest while the cell attempts to repair the interstrand cross link. It had been demonstrated, for example, that retroviral expression of *FANCC* can suppress growth factor withdrawal-induced apoptosis in factor-dependent FA-C cells[187], block Fas-triggered cell death in *Fancc* knockout mice[188], and, as expected, correct both MMC sensitivity and cell cycle delay[189].

Sensitivity to ionizing radiation. While FA cells have been shown to be resistant to conventional apoptosis specifically induced by ionizing radiation, evidence indicates a possibility of cellular sensitivity to X-rays and gamma rays, and these DNA-damaging agents may still cause generalized cell death . However, the results of studies on this topic have been somewhat contradictory. A study using skin-contact irradiation found that FA patients had increased sensitivity to gamma radiation[190]. An assay for cell killing demonstrated that FA fibroblasts were sensitive to gamma radiation, but not as sensitive

as ataxia telangiectasia cells[191], and another similar study found FA cells were sensitive to radiation, but could recover normally[192]. Similarly, phytohemagglutinin-stimulated FA lymphocytes, but not concanavalin A-stimulated ones, showed reduced ability to divide after irradiation[193]. This ambiguity makes it difficult to determine the extent of sensitivity to gamma irradiation. Likewise, some studies have concluded that FA fibroblasts do not have greater sensitivity to X-rays than normal cells, either in terms of cell or clonal survival or in terms of DNA synthesis after irradiation[194-196]. Peripheral blood lymphocytes from FA patients have also been shown to have normal chromosomal aberration frequency in response to ionizing radiation[42]. A study testing lymphoblasts and fibroblasts from the same patients found that some lymphoblasts are sensitive to X-rays in terms of chromosomal aberrations, but that fibroblasts, even those from the same patients, have the same degree of colony survival as normal fibroblasts, after gamma irradiation[197]. But other studies of FA fibroblasts or peripheral lymphocytes have demonstrated significantly increased sensitivity to X-rays, in terms of chromosomal breaks or aberrations, as measured by G2 phase cell fractions, and by initial and residual tail moment in a single-cell gel electrophoresis assay (also known as a comet assay)[123,198-202]. In addition, FA fibroblasts are moderately sensitive to the radiomimetic drug bleomycin[203], and FA lymphocytes show increased *in vitro* sensitivity to the DNA damaging agent cyclophosphamide[204]. These contradictions in the literature seem to indicate a moderate sensitivity to ionizing radiation does exist in some FA cells, but it is a variable phenotype.

A moderate increase in cellular sensitivity to ionizing radiation, relative to wild type cells, has been noted in mouse models of FA[143,205,206], although cells from an FA-D2 mouse model exhibit a normal phenotype[207,208]. Chinese hamster ovary (CHO) cells show a more mildly increased, almost normal sensitivity[209-212]. FA cells from an FA-C mouse model have been found to upregulate p53 in response to ionizing radiation[172], and cells from FA-C model mice seem to be unable to maintain a G2 phase cell cycle arrest after irradiation, especially if they also lack p53[213]. The latter finding does not necessarily contradict the findings of S-phase cell cycle arrest discussed earlier, since, in those cases in which FA cells have been shown to have extended S-phase arrest, the arrest is either

spontaneous or is induced by interstrand crosslinking agents, not ionizing radiation[121,125,126,129].

Double strand break repair. A related phenotype of FA cells to the sensitivity to ionizing radiation involves a defect in the repair of DNA double strand breaks. Evidence for this phenotype first arose as a result of studies of the HPRT locus in FA cells. While initial examination of the HPRT locus suggested that the frequency of point mutations after psoralen treatment was reduced in FA cells[214,215], it was later determined that, in fact, the frequency of deletions is increased in Fanconi cells relative to wild type cells[216-219], and this finding was confirmed in a study of the glycophorin A locus[220]. This suggested that FA cells are deficient in error-free repair of blunt-ended DNA double-strand breaks (DSB). Indeed, FA cells had a lower fidelity of blunt end joining, independently of overall ligation efficiency, and this lower fidelity could be corrected by reintroduction of a wild type copy of the missing FA gene[221,222].

Despite findings of decreased fidelity of V(D)J coding joint formation in FA lymphoblasts[223], the activity of the DNA-PK complex and XRCC4 are normal in FA cells[222]. And, while one study suggests that cells from an FA-A complementation group mouse model have defects in non-homologous end joining (NHEJ) repair of DNA double strand breaks produced by ionizing radiation[205], another study persuasively demonstrated independence of the NHEJ and FA pathways, particularly downstream of the FA-A complementation group gene product[207]. This may, therefore, imply a defect in homologous recombination repair in FA cells. In both V(D)J recombination and the formation of a double strand break as a repair intermediate after interstrand crosslink, extensive degradation of DNA can occur, requiring a high fidelity repair process to prevent significant deletions. Elevated homologous recombination activity has been reported in Fanconi fibroblasts[224]. Related to this, *Fancc*, *Fancg*, and *Fancd2* deficient DT40 chicken B cells have each been shown to be defective in homologous recombination repair of DSB and to have increased sister chromatid exchange (SCE), and it has been claimed that FA cells display increased SCE[225-228], although the latter finding is still controversial. Recent evidence also suggests that the FA pathway is involved in upregulating both homology-directed repair and single-strand annealing, but not non-homologous end joining[229]. This evidence, however, does not rule out the hypothesis that

the FA pathway is involved in regulating error-free homology-directed repair and controlling error-prone illegitimate recombination repair.

Crosslink repair. The first steps in interstrand crosslink repair by homologous recombination involve excising the crosslink, via incision of the DNA on each side of the crosslink[230]. One study reported that combining the protein complexes of two cell lines from different Fanconi anemia complementation groups complemented the unscheduled DNA synthesis and apparent incision defects[231], suggesting that FA cells are defective in the excision step itself, as well as in the steps required for DNA-repair related DNA synthesis. In support of this, the first one-sided incision of a crosslink was shown to be defective in FA cells but normal in well-spaced wild type cells; however, this defect also existed in confluent wild type and xeroderma pigmentosum (XP) cells, suggesting that DNA synthesis was required before the assay used in this study could reveal incisions[232]. Defective incision on both 3' and 5' sides of both furan and pyrone sides of an interstrand crosslink, as well as of 3' and 5' sides of the pyrone side monoadduct, was reported in a study involving chromatin associated protein extracts and crosslink- or monoadduct-containing 140 bp DNA substrate[233].

In contrast, the production of excision breaks after UV irradiation was normal in FA cells in one study[194]. Further more, in recent work, use of a modified comet assay to test FA cells individually demonstrated that, in fact, incision of the crosslink by ERCC1 is normal in FA cells and does not require DNA replication[234]. Instead, DNA replication is required for further processing of the incision into a full double strand break, and the Fanconi complex is activated after the formation of the DSB[234,235], perhaps to regulate the rate and fidelity of homologous recombination. The FA complex may thus be involved in stabilization or repair of stalled replication forks[236], but such a model is not necessarily mutually exclusive with either the crosslink or DSB repair model, since the collapse of stalled replication forks can give rise to structures similar to DSB.

In addition to finding normal excision and double-strand break formation in FA cells, one group demonstrated that the RAD50/MRE11/NBS1 (RMN) complex, theorized to be involved in both homologous recombination and non-homologous end joining, forms nuclear foci in wild-type cells treated with MMC, but that this focus formation is defective in MMC-treated FA-A, FA-G, and FA-C cells[235]. Furthermore, normal NBS1

phosphorylation is lacking in MMC-treated FA cells, and RAD51 nuclear foci in FA cells are both mildly reduced in frequency, and reduced, delayed, and more prolonged in formation[235]. For all three phenotypes, the authors showed that these deficiencies in FA-C cells could be corrected by ectopic overexpression of FANCC. Their study also indicated that FA cells are normal in their response to ionizing radiation. Additionally, the authors described a lack of expected increase in BRCA1 foci in MMC-treated FA cells compared to wild-type cells. These findings support the hypothesis of a role for FA in DSB repair by HR, as well as of the RMN complex in ICL repair and, possibly, in cell cycle arrest in response to ICL damage, via NBS1 phosphorylation[235].

Telomere maintenance. The elevated level of chromosomal rearrangements and aberrations in FA cells suggests that the FA pathway may be involved in the fidelity of telomere maintenance. Shorter telomeres have been noted in cells from Fanconi patients, including in peripheral blood leukocytes[237-239]. In addition, in both peripheral blood mononuclear cells and fibroblasts from FA patients, accelerated telomere shortening has been noted, along with, paradoxically, increased telomerase activity, indicating that even increased telomerase activity is insufficient to maintain telomere length[240-242]. Furthermore, reduced telomere length maybe due to an increased shortening per cell division, rather than because of increased cell divisions required of hematopoietic stem cells to replace rapidly dying peripheral blood cells[241]. A higher rate of telomere breakage and an increase in chromosome end fusions has also been reported; the former may explain the increased rate of telomere shortening per cell cycle[243], but the latter is controversial. A correlation between the rate of telomere shortening and onset of severe aplastic anemia or malignancy has been observed[244], and it has been claimed that significantly shortened telomeres in patients with bone marrow failure syndromes were only found in those patients who had progressed to aplastic anemia[245]. Work on mouse *Fancg* knockouts, both with and without telomerase, as well as on primary cells from FA complementation group G patients, suggests that the accelerated telomere shortening is not due to deficiency of telomere maintenance per se, and that FANCG is not involved in telomere maintence[246].

As further work on known cellular phenotypes of FA is done, a better understanding of some of the phenotypes may emerge; in other cases, new, more subtle

phenotypes may be found, as predicted by the fact that yeast two hybrid screen results implicate FA proteins in transcription regulation, cell signaling, oxidative metabolism, and cellular transport[247]. The FA pathway may include aspects of these other cellular pathways as a means by which to coordinate cellular functions while ensuring accurate and timely repair of DNA damage.

III. The Genetic and Molecular Aspects of Fanconi Anemia

Currently, there are 12 known FA complementation groups (A, B, C, D1, D2, E, F, G, I, J, L and M)[3-6]. Each of these complementation groups corresponds to a separate gene. A brief summary of information regarding each gene can be found in Table 1. Since none of these genes have homologs in prokaryotes or in single-celled eukaryotes, but a *FANCD2* homolog does exist in *A. thaliana*, the FA genes seem to have arisen during the evolution of multicellular eukaryotes. This, in turn, implies that the evolutionarily defined function of the gene products lies in protecting the organism from the consequences of damage to DNA other than simple cell death, such as systemic cell loss or cancer. As detailed above, these are precisely the consequences that comprise the greatest risks to FA patients, in whom a defect in any one gene can lead to bone marrow failure or leukemia. The patients studied in Chapter 2 belong to the first FA complementation group defined[248], FA-A, since they have a defect in *FANCA*.

Table 1: Essential data on FA genes.

Gene	Location	cDNA length	ORF length	Protein (a.a.; kD)	# exons
FANCA	16q24.3[249,250]	5.5kb[251,252]	4368bp[251,252]	1455a.a.; 163kD[251,252]	43[253,254]
FANCB / FAAP95	Xp22.31[255]	3.0kb[256]	2580bp[256]	859a.a.[256]; 90/95kD[255]	10[255]
FANCC	9q22.3[257,258]	4566bp[257]	1,674bp[257]	557a.a.; 60kD[259-261]	14 [262]
FANCD1 / BRCA2[263]	13q12.3[263,264]	11,385bp[265]	10257bp[265]	3418a.a.[265]; 380kD[30]	27 [265]

FANCD2 FANCD2-44 FANCD2-43 FANCD2-S FANCD2-L	3p25.3[266]	 5189bp[256] 4455bp[256]	 4416bp[256] 4356bp[256]	 1471a.a.[256] 1451a.a.[256] 155kD[267] 162kD[267]	 44 [267] 43 [256]
FANCE	6p21.2-21.3[268]	2.5kb[268]	1,611bp[268]	536a.a.[268]; 58kD[269]	10 [268]
FANCF	11p15[270]	1.3kb[270]	1,125bp[270]	374a.a.; 40kD[270]	1 [270]
FANCG / XRCC9[212]	9p13[212]	2.5kb[212]	1869bp[271]	622a.a.; 68kD[271]	14 [271]
FANCJ / BRIP1 / BACH1[272]	17q23[273,274]	4563bp[256]	3750bp[256]	1249a.a. [256] 130[272]/150kD[274]	19 [275]
FANCL / PHF9 / FAAP43	2p16.1[256]	1750bp[256]	1128bp[256]	375a.a. [256]; 43kD[4]	14 [275]
FANCM / FAAP250	14q21.3[256]	7143bp[256]	6147bp[256]	250 kD[6]	23 [6]

***FANCA* general information.**

Two groups simultaneously and independently cloned the gene responsible for the FA-A group, one using a functional complementation assay to narrow down a cDNA pool and identify the gene[251], and the other using a combination of positional cloning and exon trapping[252]. The *FANCA* cDNA is about 5.5 kb long, with a 4368 bp open reading frame, which codes for a 1455 a.a., approximately 163 kD protein[251,252]. The protein contains an N-terminal bipartite nuclear localization signal (NLS), consisting of amino acids 18 to 34 and 19 to 35, as well as a partial leucine zipper consensus sequence from amino acids 1069 to 1090[251]. Mutation studies of the latter region indicate that the leucines themselves are not necessary for FANCA function, but the alpha-helical structure of this region seems to be required[276]. In genomic DNA, *FANCA* localizes to chromosome 16q24.3[249,250], and spans 43 exons over approximately 80kb, with a GC-rich promoter region typical of housekeeping genes[253,254]. By Northern blot, *FANCA* is expressed in all tissues analyzed, including pancreas, kidney, liver, skeletal muscle, lung, brain, heart, and placenta, and the most prominent of several alternate transcripts is about 4.7 kb in length[252].

FANCA: Subcellular localization.

Work with N-terminal GFP fusion constructs has demonstrated predominantly nuclear localization of FANCA protein[277,278], yet, by immunofluorescence, FANCA localizes to both nuclear and cytoplasmic compartments and even associates with intracellular membranes[261]. Also, the observation of some cell-to-cell variation in the ratio of cytoplasmic to nuclear FANCA levels implies a shuttling of FANCA between the different cellular compartments[261].

Nonetheless, nuclear localization of *FANCA* is required for function. *FANCA* constructs with C-terminal appended nuclear exclusion signals fail to correct MMC sensitivity[261], and FA-A cell lines with H1110P and delF1263 mutations exhibit neither MMC resistance nor nuclear localization[279,280]. This also implies that C-terminal amino acids may play a role in nuclear retention of the protein. Unfortunately, this hypothesis has not been confirmed with C-terminal deletion constructs, as both cytoplasmic expression[278,281] and nuclear expression of such constructs has been noted[277].

The first 42 amino acids of FANCA (NLS) have been shown to be necessary for nuclear localization[277], yet, surprisingly, according to one report, mutation of either portion of the bipartite NLS does not entirely prevent nuclear localization, and even deletion of the NLS altogether only serves to equalize cytoplasmic and nuclear expression levels[278]. Purely cytoplasmic localization requires deletion of the first N-terminal 250 amino acids[278], implying a role for amino acids 43 to 250 in assisting with nuclear entry. Within the NLS region, amino acids 16 through 20 have been predicted to form a nucleolar localization signal, and nucleolar localization has been noted, as has restriction of EGFP fluorescence to chromatin in mitotic cells[277], although these sub-nuclear localizations have been disputed[278].

Mutations in other FA genes have varying effects on FANCA localization. By immunofluorescence, FANCA has predominantly nuclear and lesser cytoplasmic expression in wild type and FA-D2 cells, but, in FA-C, E, F, and G cell lines, expression levels in are essentially equal between cytoplasm and nucleus, while FA-B cells show mostly cytoplasmic expression of FANCA[280].

FANCA phosphorylation.

Nuclear fractions from FA group A, B, C, and E lymphoblasts have either very low levels of FANCA or altogether lack FANCA[282]. Similarly, while FANCA is phosphorylated in wild type cells, this phosphorylation does not occur in cell lines from FA groups A, B, C, E, F, or G[282]. Hence, a correlation exists between phosphorylation and nuclear localization[282]. A wortmanin-inhibited serine-threonine protein kinase (tentatively named FANCA-PK) binds and phosphorylates FANCA in the cytoplasm, specifically at serine residues[283]. FANCA is predicted to have a binding site for the serine / threonine protein kinase Akt, and Akt can phosphorylate the serine at this site, S1149, *in vitro*[284]. Nevertheless, mutation of the serine has no effect on ability of the protein to correct MMC sensitivity, and, in fact, Akt inhibits FANCA phosphorylation *in vivo*[284].

Interaction of FANCA with FANCG

In addition to phosphorylation by a kinase, FANCA interacts with FA proteins. Specifically, FANCA interacts with FANCC and FANCG, the gene products deficient in FA-C and FA-G cells, respectively. Expression of FANCG, FANCA, or FANCC has been repeatedly demonstrated to increase expression levels of the other two proteins in respective mutant cell lines[285-287]. This is due to increased protein stability; in pulse-chase experiments, FANCA and FANCG each significantly increase the half-life of the other[287]. FANCG directly binds FANCA, as evidenced by multiple assays, including co-immunoprecipitation after *in vitro* translation[285,286,288,289]. This binding requires amino acids 400 to 428 of FANCG[289,290], and the NLS region of FANCA, specifically amino acids 18-29[285,290]. Binding is augmented by amino acids 585 to 622 of FANCG[290], but is unaffected by either large C-terminal deletions or mutations, such as H1110P or delF1263, of FANCA[286,289,290]. Therefore, since the H1110P mutant, but not an NLS-deleted form of FANCA, stabilizes FANCG expression levels, the increase in protein stability is a consequence of the direct binding and not of functional complementation[285]. On the contrary, FANCA-FANCG binding is necessary for FANCA function. A version of FANCA in which the FANCG binding domain is replaced with a SV40-NLS fails to complement an FA-A cell line, even though FANCA enters the nucleus[281,290].

FANCA and FANCG both have primarily nuclear localization, but also appear in the cytoplasm, and the complex between these two proteins can also be immunoprecipitated from both cellular compartments[286]. Furthermore, localization is

concordant. If FANCG is coexpressed with a version of FANCA fused to either an SV40-NLS or an NES, it appears wherever the FANCA fusion protein is directed[290]. Moreover, cytoplasmically expressed H1110P and R1116G mutants of FANCA, which can still bind FANCG, prevent its entry into the nucleus, and non-functional C-terminal deleted forms of FANCA that bind FANCG act in a dominant negative fashion in wild type cells[287,290]. Thus, normal binding between FANCA and FANCG occurs in the cytoplasm, and translocation of the oligomer to the nucleus is required for conferral of MMC resistance[287,290]. Of note, the level of FANCA-FANCG complex, while normal in wild type and FA-E cell lines, is reduced in FA-B, FA-C, and FA-F cell lines, indicating that expression of other Fanconi genes is required to stabilize the interaction[286]. In particular, expression of FANCC in an FA-C cell line increases nuclear levels of FANCA and FANCG[287].

Interactions with FANCC.

The previous finding correlates with the fact that interaction between FANCA and FANCC, via both N-terminal amino acids and leucine 554 of FANCC, allows translocation of a complex containing FANCA and FANCC to the nucleus[291]. Retroviral correction increases nuclear levels of both FANCA and FANCC proteins in FA-A and FA-C cells[281], supporting the idea that nuclear localization of either protein is augmented by translocation of the complex. While once disputed[261], the interaction between FANCA and FANCC proteins does occur[279,281,282], but, in contrast to strong, direct FANCA-FANCG binding, FANCA and FANCC interact weakly, indicating indirect or transient binding[282,285,288]. Other Fanconi proteins are likely mediators of binding, since FANCA and FANCC do not interact in FA group B, E, and F cell lines, whereas FANCA and FANCG do, albeit at reduced levels[285].

Specifically, while FANCA and FANCG can bind each other in FA-C cell lines[285], expression of FANCG is required for FANCA-FANCC interaction and enhanced nuclear accumulation of the FANCA-FANCC oligomer[285]. Amino terminal deleted forms of FANCA may fail to bind FANCC at least partially because they can not bind FANCG[290]. In agreement with this hypothesis, replacement of the FANCA NLS, to which FANCG binds, with that of SV40 Large T antigen allows nuclear localization, yet

abolishes not only the conferral of MMC resistance, but also FANCC interaction and translocation of the FANCA - FANCC complex to the nucleus[281].

This does not, however, mean that FANCA binds FANCC specifically through FANCG, since FANCC and FANCG do not bind each other directly[288,290]. In fact, deletion of the carboxy terminal two thirds of FANCA not only significantly reduces nuclear localization of FANCA, but also abolishes FANCC interaction and translocation, and fails to complement MMC sensitivity[281]. Note that this region of FANCA is not required for FANCG binding. Thus, FANCA function also depends on its interaction with FANCC, which is dependent on more than FANCG co-expression or FANCA-FANCG binding.

Even without stable, direct binding of FANCC to either FANCA or FANCG, immunoprecipitation of each of the three proteins does pull down the other two, indicating that, by a combination of direct and indirect binding, they form part of the Fanconi nuclear complex[285,292]. Expression of FANCG not only increases the nuclear to cytoplasmic ratio of FANCA (and vice versa), but also it increases nuclear levels of FANCC[287]. Furthermore, proteins expressed off of C-terminal deletion constructs of FANCG bind FANCA and enter the nucleus, but fail to complement FA-G cell lines[289]. Since they also fail to even indirectly bind FANCC, FANCA-FANCG binding alone is necessary but not sufficient for FANCG function[289].

The equivalent finding for FANCA function can be inferred from work done with non-complementing FANCA mutations and C-terminal deletions that do not affect the FANCG binding region[281,285,286]. Furthermore, both FANCA phosphorylation and FANCA nuclear localization correlate with FANCC-FANCA coimmunoprecipitation, none of which are detected in various FA cell lines[282]. Of note, neither delF1263 nor H110P mutant alleles of FANCA demonstrate FANCA nuclear localization, phosphorylation, interaction with FANCC, nor co-translocation with FANCC[279,282], implying that central and C-terminal amino acids, such as those mutated in the patients discussed in Chapter 2, can also influence these activities.

To summarize, FANCA and FANCG bind directly via the N-terminal portion of FANCA, while FANCC seems to interact indirectly with both of these two proteins. The three proteins stabilize each other, form part of the Fanconi core complex, and promote

nuclear localization of the complex, such that mutation in any one of the three genes can lead to a reduction in both general cellular levels and nuclear levels of the other two gene products. FANCA – FANCG binding is required for interaction with FANCC, which, in turn, has been correlated with both the cytoplasmic phosphorylation and the nuclear localization of FANCA, and all of these steps are required for FANCA function.

Interaction of FANCA with non-FA proteins.

FANCA has been reported to interact with several non-FA proteins, including PKR[173], sorting nexin 5 (SNX5)[293] and human non-erythroid alpha spectrin II[294], although these findings are controversial and not universally accepted. According to the reports, the latter protein has been shown, in a FANCA dependent manner, to bind the FANCA-FANCG-FANCC complex, to facilitate binding of the complex to DNA at interstrand crosslinks, and to colocalize to nuclear foci with both FANCA and XPF[294-296]. Lack of FANCA (as well as of FANCC or FANCG) seems to destabilize nonerythroid alpha spectrin II, and levels of nonerythroid alpha spectrin II are reduced in FA-A, FA-B, and FA-C cell lines[294,297,298]. FANCA also interacts with BRG1, a subunit of the SWI/SNF chromatin remodeling complex, via exon 43 of FANCA[299]. While FANCA is not required for SWI/SNF general function, FANCA and BRG1 do co-localize in the nucleus, and FANCA might recruit the SWI/SNF complex to sites of DNA damage by means of this interaction[299]. While the FANCA-BRG1 interaction appears to occur without the presence of FANCG or FANCC[299], FANCC may recruit DNA conformation altering machinery via its interaction with the POZ transcriptional repressor protein FAZF[300]. Furthermore, a direct, DNA-damage independent interaction has been demonstrated between FANCA and BRCA1, in which the first 589 a.a of FANCA and amino acids 740 to 1083 of BRCA1 are required[32].

General review of FANCA mutations.

The use of specific mutant *FANCA* alleles to study nuclear localization, phosphorylation, and biochemical interactions of FANCA protein, underscores the importance of mutation analysis. Unfortunately, although some mutations have been found repeatedly, *FANCA* mutations are extremely heterogeneous, and vary from patient to patient as well as between different ethnic groups[301-309], making both clinical genotype-phenotype correlations and classification for molecular analysis difficult[310].

These mutations have been found across the gene, although roughly two-thirds are in the 3' half of the gene[309]. Mutation types include nonsense mutations, splicing mutations, missense mutations, small deletions, insertions, or duplications, and even large deletions spanning several exons or the entire gene[309]. Microdeletions and microinsertions have been shown to be associated with apparent errors in replication at short direct repeats or homonucleotide tracts[301], or other mutation hotspots such as TGAGGC or CCTG sequences, or CG rich regions[311]. Large deletions have been associated with Alu repeats[254,312,313]. FANCA is rich in Alu repeats[253], and it has been suggested that a 26 nucleotide core sequence in the Alu repeat stimulates illegitimate recombination, resulting in gene rearrangements[314]. The presence of these hypermutable regions in FANCA may partially explain the fact that roughly 60-65% of FA patients have mutations in FANCA[197,315,316]. In fact, mutations are so variable that two cell lines with different mutations in FANCA can complement each other in a whole cell fusion, even thought retroviral infection with wild type FANCA corrects both cell lines. Correction for such an occurrence resulted in the reassignment of the single FA-H cell line into the FA-A complementation group[317].

Other complementation groups.

Even with the removal of a now defunct complementation group, FA-H, there are still 11 other complementation groups besides FA-A. As FANCA functions as part of a complex of proteins, a review of other members of the complex and of the FA-related DNA crosslink response pathway provides a greater context for studies of *FANCA*. Therefore, a summary of the literature regarding other members of this pathway is given below, starting with *FANCC*, the first FA gene cloned.

FANCC.

FANCC, cloned using functional complementation of MMC / DEB sensitivity[257] is expressed in a wide variety of tissues and organs, including liver, spleen, skin, blood, brain, muscle, and peripheral nerves[257]. FANCC protein seemed to localize primarily to the cytoplasm[259-261], and enforced exclusive expression in the nucleus failed to correct the MMC sensitivity phenotype, which, in FA-C cells, seemed to predominantly involve induction of crosslinks rather than repair[318]. This was at odds with the DNA repair function attributed to the Fanconi pathway, and, in fact, it has more recently been shown

that about 10% of FANCC localizes to the nucleus[319]. *FANCC* has been proposed to have both a regulatory role in growth, differentiation, or survival of hematopoietic progenitor cells, as well as a function related to MMC resistance[320], although these two functions may overlap. The FANCC protein, which is upregulated in S phase and maximally expressed at the G2/M boundary[321], has been reported to bind many other proteins, including p53[322], the cyclin-dependent kinase cdc2[321], and the double stranded RNA-dependent protein kinase PKR, which, in turn, binds and inactivates eIF2-alpha[147]. These interactions may be involved in the apparently p53-dependent cell cycle arrest occurring in Fanconi cells after exposure to crosslinker[125,127,128]. In addition, FANCC protein binds to STAT1 and Hsp70[145,146], and, directly or indirectly, to other members of the Fanconi complex[269,285,291,323].

FANCG.

The gene associated with the FA-G complementation group was, like *FANCC*, cloned by functional complementation, but was initially identified as *XRCC9*[212]. *XRCC9* is the gene that complements the MMC sensitivity of the X-ray cross complementation group (XRCC) Chinese hamster ovary (CHO) cell line UV40[212]. This fits with the finding that UV40 cells are sensitive not only to ionizing radiation, but also to UV radiation, alkylating agents, and DNA crosslinking agents, and exhibits high spontaneous chromosomal breakage[324]. *FANCG* is identical to XRCC9[271]. The mRNA is most highly expressed in testis and thymus in humans[212]. FANCG protein has an N-terminal leucine zipper motif[212], and localizes predominantly to the nucleus, but also shows variable cytoplasmic expression[286]. As mentioned above, FANCG co-localizes with its binding partner, FANCA, and is required for FANCA-FANCC interaction[285,289,290]. FANCG undergoes phosphorylation of the serine at position 7. Mutation of this serine to alanine abolishes the ability of the protein to correct for MMC sensitivity, but does not effect interaction with FANCA or FANCC[325]. In addition, like FANCA and FANCC, FANCG protein interacts with PKR[173], the dsRNA dependent protein (serine) kinase. Also, in line with findings of TNF-α overexpression and sensitivity of FA cells, TNF-α treatment of a wild-type cell line appears to result in both phosphorylation and upregulation of FANCG, as well as concurrent upregulation of FANCA, and an increase of FANCA-FANCG complex in the nucleus[326]. Furthermore, specific inhibition of IKK2 attenuates FANCG expression[326].

FANCF.

After FANCG, cloning of several FA genes occurred in reverse alphanumeric order; the next gene cloned was for the complementation group, FA-F. The FANCF gene, consisting of a single exon, was discovered by functional complementation cloning[270]. While the FANCF protein was inferred by alignment to share homology with the N-terminal portion of the RNA binding protein ROM[270], which implied that the FANCF protein may directly bind DNA, this homologous region is not necessary for FANCF function, nor is it conserved in the Xenopus homolog[327]. FANCF protein is small enough to allow passive diffusion, in both directions, through the nuclear pores. Subcellular localization studies, however, indicate almost exclusively nuclear localization in wild type cells, as well as in FA- A, B, C, and E cell lines[280]. FANCF restores nuclear FANCA levels in an FA-F cell line, and interacts with FANCA, FANCC, and FANCG in the FA nuclear complex[280]. In addition, FANCF stabilizes interaction between FANCA and FANCC[280], reinforces FANCA-FANCG binding[280,327], and assists in the binding of FANCC and FANCE to the complex[327]. The combination of these findings suggests that FANCF may act as a regulatory or adaptor protein[327]. Biallelic silencing of *FANCF*, via hypermethylation of the promoter region, has been shown to occur in acute myeloid leukemia cell lines[328], ovarian cancer[329-331], and cervical cancer[332], lending support to the idea of a regulatory function for *FANCF*. However, FANCF interaction with the complex is strongly reduced in FA-B, C, E, and G lymphoblasts, and FANCF does not co-precipitate with either FANCA, FANCC, or FANCG in an *in vitro* translation system[280]. Yeast two hybrid analyses have demonstrated that the C-terminal of FANCF binds FANCG[333], and that FANCG can act as a bridge between FANCF and FANCA[334]. This implies that FANCF may bind only to complexed FANCG after the complex is imported into the nucleus, and, perhaps, may play a role in nuclear retention of the complex, contingent on the presence of the other FA proteins.

FANCE.

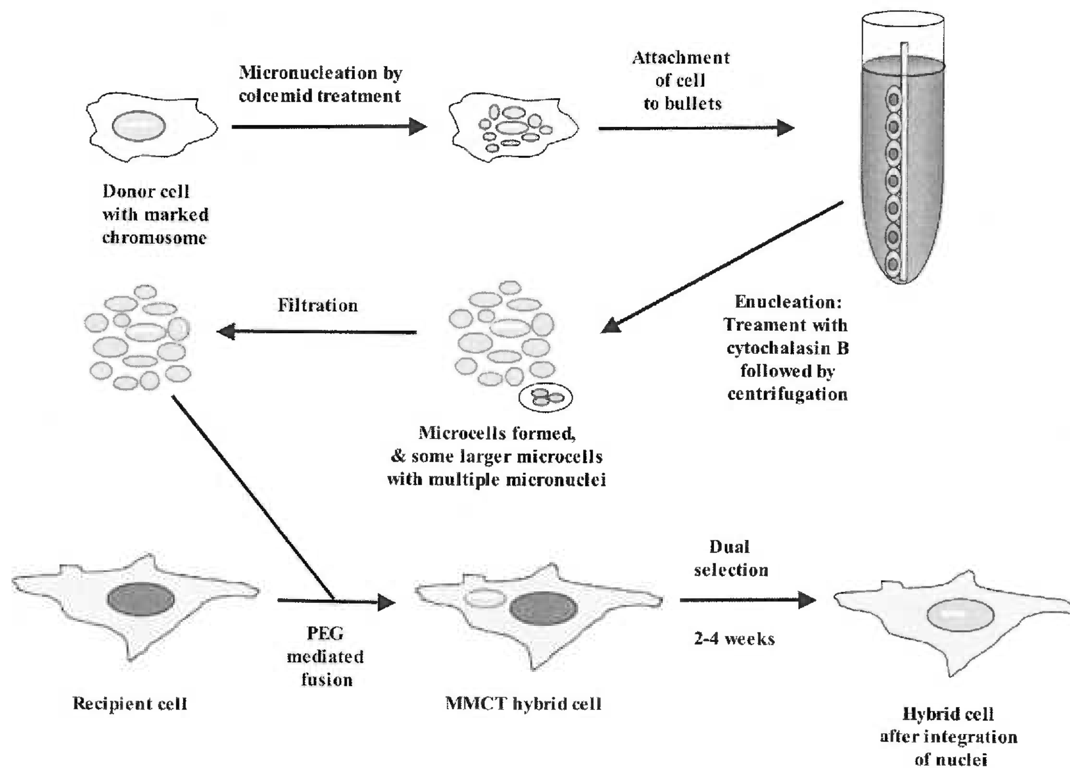
The gene for the fifth Fanconi complementation group, FA-E, was also the fifth gene cloned. Like previous Fanconi genes, *FANCE* was cloned by functional complementation[268]. *FANCE* mRNA spans about 15 kb[268]. FANCE protein directly binds both FANCC[269,333] and FANCD2[333,334], is required for nuclear localization of

FANCC[269,335], and acts as a bridge for FANCA-FANCD2 interaction[269], suggesting a role for FANCE in bringing FANCD2 to the complex for monoubiquitination. Yeast-two-hybrid experiments also indicate a weak interaction between FANCE and FANCA[333], as well as between FANCE and FANCG[333], perhaps implying a transient or regulated interaction between the FANCA-FANCG oligomer and the FANCE-FANCC oligomer in the nuclear complex.

FANCD2.

Cloning of FANCD2 proved somewhat more difficult than that of previously described FA genes. Initial mapping of the gene for what was then considered the FA-D complementation group was complicated by a lack of large families required for linkage analysis of recessive disorders and positional cloning techniques. In addition, functional complementation cDNA cloning was hampered by overexpression-related toxicity. Therefore, preliminary localization of FANCD2 was carried out using a technique called microcell mediated chromosome transfer (MMCT). Figure 1 summarizes the MMCT protocol. A complete description of the MMCT technique, including recent modifications and improvements, can be found in Chapter 3.

Figure 1: Depiction of the microcell mediated chromosome transfer protocol.



By MMCT, the gene in question was found to lie on a 50 cM region of chromosome 3[336], and then the region was further narrowed by deletion mapping of non-complemented hybrids[266]. The gene was isolated, and mutations were found in the PD20 cell line. Since this gene complemented three Fanconi cell lines, including PD20, by retroviral infection, but not the reference FA D cell line, HSC62, it was named *FANCD2* instead of *FANCD*[267].

Like other Fanconi genes, *FANCD2* displays fairly ubiquitous, low level expression, but, unlike other Fanconi genes, *FANCD2* has homologs in lower eukaryotes, including D. melanogaster, A. thaliana, and C. elegans, but not S. cerevisiae[267]. *FANCD2* spans 44 exons[267], although alternative splicing yields two transcripts. One (FANCD2-44; NCBI Nucleotide database accession #AF230336)[256] is a 5189 bp mRNA with a 4416 bp open reading frame, coding for a 1471 a.a. protein. The other (FANCD2-43; accession

#AF340183)[256] is a 4455 bp mRNA with a 4356 bp open reading frame, coding for a 1451 a.a. protein.

In addition to two transcripts, Western blot for FANCD2 protein reveals two isoforms, FANCD2-S and FANCD2-L[267], which are a non-ubiquitinated and monoubiquitinated form, respectively[31]. The modification, which activates the protein in its DNA repair function[31], requires formation of the Fanconi complex[31], as well as the presence of the proteins ATR and RPA1[337], and occurs only in S-phase[338]. Moreover, while both FANCD2-43 and FANCD2-44 are monoubiquitinated, only monoubiquitinated FANCD2-44 translocates to chromatin-bound nuclear foci and corrects MMC sensitivity[339]. FANCD2 is also phosphorylated at serine 222 by ATM, independently of the lysine 561 monoubiquitination[340]. However, monoubiquitination, but not phosphorylation, of FANCD2 is required to promote homology directed repair (HDR) in FA cells, which are apparently HDR deficient, implying a role for HDR in ICL repair[229].

FANCD2 interacts with NBS1[341]. NBS1, ATM, and MRE11 are all required for radiation-induced S-phase checkpoint-related FANCD2 phosphorylation, and the formation of nuclear foci consisting of NBS1, MRE11, RAD50, and FANCD2 is required for resistance to MMC[341]. Furthermore, FANCD2, BRCA1, and RAD51 exhibit an S-phase specific interaction[338], and monoubiquitinated FANCD2 and BRCA1 show nuclear co-localization in a DNA-damage dependent, S-phase specific manner[31].

FANCD1.

As mentioned above, two FA-D cell lines were not corrected by, nor had mutations in, *FANCD2*, and new complementation group, FA-D1, was formed to encapsulate these[267]. Just as *FANCG* was equated with a known gene, *FANCD1* was revealed to be the breast cancer related gene *BRCA2*[30]. Cell lines from FA-D1 patients were demonstrated to have biallelic C-terminal truncating mutations, and wild type BRCA2 functionally corrected these cell lines[30].

Cloned about 8 years prior[263] to the recognition of its role in FA, *FANCD1 / BRCA2* is a huge gene, spanning around 70 kb of genomic DNA[265]. The cDNA alone is 11,385 bp long, without polyadenylation signal or poly(A) tail, and includes an especially large exon 11[265]. Expression is highest in breast and thymus, as well as testis, with lower

levels in spleen, lung, and ovary[265]. BRCA2 protein has two C-terminal nuclear localization signals, which are required for nuclear localization[342]. BRCA2 protein forms chromatin bound foci with BRCA1 and RAD51. It also relocates to replication forks following exposure of cells in S phase to hydroxyurea or UV irradiation. This implies a DNA repair and / or homologous recombination function for *BRCA2*[343,344]. Several groups have provided evidence of a role for BRCA2 in homologous recombination repair of DNA breaks, partly via regulation of RAD51 activity, and in stabilization of stalled replication forks[345-348]. Once it was determined that BRCA2 was also FANCD1, the idea that the Fanconi genes may be involved in the same pathways became generally accepted[349,350].

BRCA2 / FANCD1 is also classified as a caretaker gene[351]. When inherited in the heterozygote state, mutations in *BRCA2* have been associated with early onset breast cancer[263], as well as male breast cancer[352], and, to a lesser extent, ovarian cancer[353], prostate cancer[354,355], and pancreatic cancer[356]. Therefore it is not surprising that, as other Fanconi patients and families with biallelic BRCA2 mutations were encountered, they also demonstrated predisposition to various cancers. In one case, this involved genetic susceptibility to breast cancers and brain tumors,[357], in another case susceptibility to breast cancers and early onset leukemia[28], and, in a third case, onset of meduloblastoma or Wilms tumor in the patients but no bone marrow failure[358]. Also, genetic reversion of one allele has been discovered in an acute myelogenous leukemia cell line derived from an FA patient relapsed after bone marrow transplant[359]. Furthermore, BRCA2 directly interacts with FANCG[360] and with FANCD2[361], which would imply that the Fanconi genes might have tumor suppressor function. Indeed, mutations in both FANCC and FANCG have been found in non-FA patients with pancreatic cancers[27,29,362,363], and, as mentioned above, inactivation of FANCF occurs in ovarian tumors. A possible correlation between a mutation in *FANCA* and the occurrence of pancreatic cancer in a kindred is tested in Chapter 2.

FANCL.

The *FANCL* gene was discovered prior to the designation of the FA-L complementation group. A recently immunoisolated protein complex contains the five known FA core complex proteins (FANCA, FANCC, FANCE, FANCF, and FANCG), as

well as the Bloom syndrome related helicase BLM, topoisomerase III alpha, replication protein A (RPA) and several BLM-associated or Fanconi anemia associated polypeptides (BLAPs and FAAPs, respectively)[364]. Under 0.7M salt, four polypeptides of 43, 90, 100, and 250 kD mass remain bound to the subcomplex containing some known core complex FA proteins. The 43 kD protein (FAAP43) was identified by mass spectrometry as PHD finger protein 9 (PHF9)[4]. An FA cell line of unassigned complementation group which lacks expression of this protein was found to carry a homozygous or hemizygous mutation in the gene, and was complemented for MMC sensitivity by wild type *PHF9*. Hence, the gene was also named *FANCL*[4].

Experimental evidence, including reciprocal coimmunoprecipitation data suggests that this protein is part of the FA core complex[4]. The level of PHF9 / FANCL protein in nuclear extracts from FA-A, FA-B, and FA-E cell lines is lower that in normal cells, and nuclear accumulation of PHF9 depends on FANCA expression[4]. FANCL contains E3 type ubiquitin ligase like activity, and it is required for monoubiquitination of FANCD2[4]. Thus, it is likely to be the core complex subunit which directly catalyzes the monoubiquitination of FANCD2[4,365]. As such, it is both the first protein in the FA pathway to be cloned via a biochemical approach, and the first to have a defined enzymatic activity[366].

FANCB.

Like *FANCL*, the *FANCB* gene was discovered via the biochemical approach. While it has been generally believed that FA is an autosomal recessive disorder, patients from the FA-B complementation group are almost exclusively male, indicative of an X-linked disease. When the gene for the 90kD FAAP protein, FAAP90, also known as FAAP95, was mapped to Xp22.31[255], this gene became a candidate for *FANCB*. FA-B cell lines were found to have mutations in the gene, such that the FA-B cells assayed did not express *FAAP95*, and FA-B lymphoblasts were corrected for MMC sensitivity with enforced expression of wild type *FAAP95*[255]. Hence, the gene for FAAP95 was named *FANCB*[255], and data that had been previously interpreted to mean that *FANCB* was *BRCA2*[30] were refuted[255,367].

FANCB undergoes X-inactivation, and, in female carriers, inactivation is often skewed towards the mutant allele, such that only the wild type allele is active[255].

Nevertheless, somatic mutation of FANCB, especially in males, could lead to tumor formation with a greater frequency than for autosomal genes, and these tumors should be treatable with cisplatin or other crosslinking chemotherapeutic agents[255].

As demonstrated by co-immunoprecipitation, FAAP95 is an FA core complex protein[255]. Therefore, deficiency results in lack of FANCD2 monoubiquitination, as confirmed by siRNA experiments[255]. Despite having a putative C-terminal bipartite nuclear localization signal, FANCB requires FANCA expression for nuclear localization[255], and, in turn, FANCB is required for FANCL stability[255] as well as for FANCA nuclear localization[280].

FANCJ.

Two more complementation groups, FA-I and FA-J, have recently been defined in the same paper[5]. The gene for the FA-I group has not yet been cloned, but seems to function upstream of FANCD2 monoubiquitination, yet downstream of Fanconi complex formation.

The *FANCJ* gene has only recently been identified, and was determined, virtually simultaneously by three groups[273,274,368], to be the DEAH-box DNA helicase BRIP1, also known as BACH1. It is interesting to note that each contributing group identified the gene by a different approach. One group noted the similarity in phenotype between BACH1 deficient cells and FA-J cells, and searched for mutations in the BACH1 gene in the FA-J cells[368], while another used linkage analysis[273]. A third group[274] utilized a combination of positional cloning and microcell mediated chromosome transfer, the same technique used to map *FANCD2*.

FANCJ / BRIP1 is a member of the RecQ-like helicases, a family that includes *BLM*, *WRN*, and *RECQL4*, as well as *XPD*[274]. Like, XPD, FANCJ unwinds DNA in a 5' to 3' direction[274]. BRIP1 seems to prefer forked duplexes as unwinding substrates[369,370], and BRIP1 has been shown to interact with BRCA[272]. Therefore, it is not surprising that BRIP1 has been found to be necessary for homologous recombination[368]. Also, FANCD2 monoubiquitination is normal in FA-J cells[5]. FANCJ therefore seems to be involved in helping the Fanconi complex to repair DNA damage at a point downstream of monoubiquitination.

FANCM.

The third FAAP, FAAP250, was recently identified as another FA core complex protein, and the gene that encodes for this protein was named *FANCM*[6]. This identification was so recent, that the only major source for information on this protein is the original paper[6]. Cells depleted for FAAP250 have significantly reduced levels of monoubiquitinated FANCD2 and increased sensitivity to MMC, as would be expected for a member of the FA core complex. Additionally, cells from patients who were found to have mutations in *FANCM* also have distinct reductions in FANCA and FANCG levels, and somewhat reduced levels of FANCL protein. In these cells, nuclear localization of FANCA and FANCL is deficient, similar to FA-B cells.

FAAP250 is modified, apparently via phosphorylation, in response to MMC, cisplatin, or hydroxyurea treatment. This modification is independent of FANCD2 monoubiquitination or the presence of FANCA, FANCB, FANCE, or FANCL. Like FANCJ, FAAP250 is a member of the DEAH-box helicase family, but, in this case, shares strongest homology to ERCC4 / XPF, in that both FAAP250 and XPF have an N-terminal helicase-ATPase domain and a C-terminal endonuclease domain. Yet, the divergence in sequence of the endonuclease domain from that of XPF likely renders this domain non-functional in FAAP250. Similarly, mutations in the helicase domains of both proteins probably prevent actual helicase function, such that only the ATPase domain is active. Nevertheless, FAAP250 appears to have translocase function, in that it can displace DNA triplexes, such as those that can form during the homologous recombination repair of DNA double strand breaks. It has therefore been hypothesized that FAAP250 / FANCM may act to translocate the FA core complex along DNA as part of the cross-link detection or repair process. The details of the actual function of the core complex are still uncertain.

Temporally coordinated subcellular localization of the FA complex.

While the exact function of the FA complex is yet unknown, the subcellular localization of the complex has been tracked. FANCA, FANCC, and FANCG co-localize to chromatin in wild type cells, but not in FA cells[371]. The three proteins also coimmunoprecipitate from chromatin extracts[371], implying that the co-localization is observed because the proteins are acting as a complex. From 4 hours after treatment to a peak of 24 hours after treatment, the FA complex shifts to chromatin and nuclear

fractions in MMC treated wild type cells, but not in ionizing radiation treated cells[371]. Exit of the complex from chromatin during mitosis can also be observed by both immunofluorescence and via immunoblots of subnuclear fractions from synchronized cells at various phases of the cell cycle[371]. Related to this, FANCA displays a granular appearance in the nucleus[371], but, in contrast to an older study[277], is excluded from both the nucleolus and from condensed mitotic chromosomes[371], implying nuclear exclusion during mitosis. Correspondingly, FANCG is doubly phosphorylated at or just prior to mitosis, which is significant because similar findings of chromatin and nuclear matrix localization, followed by phosphorylation and exit from chromatin during mitosis, have been described for BRG1[372-375].

Fluorescence microscopy of fluorescently FANCA, FANCG, and FANCC has confirmed co-localization in the nucleus and chromatin in whole cells and chromatin preps[376]. Notably, missense mutant alleles of the three proteins localize solely to the cytoplasm, confirming that interaction is required for nuclear co-localization[376]. Complexes of these three proteins form chromatin foci at the G1-S border and during S phase, then diffuse to the nuclear periphery by G2[376]. Nuclear exclusion of these FA complex proteins in pre-condensation stage mitotic cells has also been specifically observed[376].

Careful analysis of chromatin fibers demonstrates that the FANCA-FANCG-FANCC complexes form irregularly spaced foci along the fiber in asynchronized cells, show more prominent yet diffuse fluorescence in G1-S and S phase, and re-form foci in G2, before becoming absent during mitosis[376]. Increased, but more spread out, expression of the complex also occurs in MMC treated cells, but this increase is no greater for MMC treated asynchronized or S-phase cells than for untreated cells which enter S-phase[376].

BRAFT-1 complex

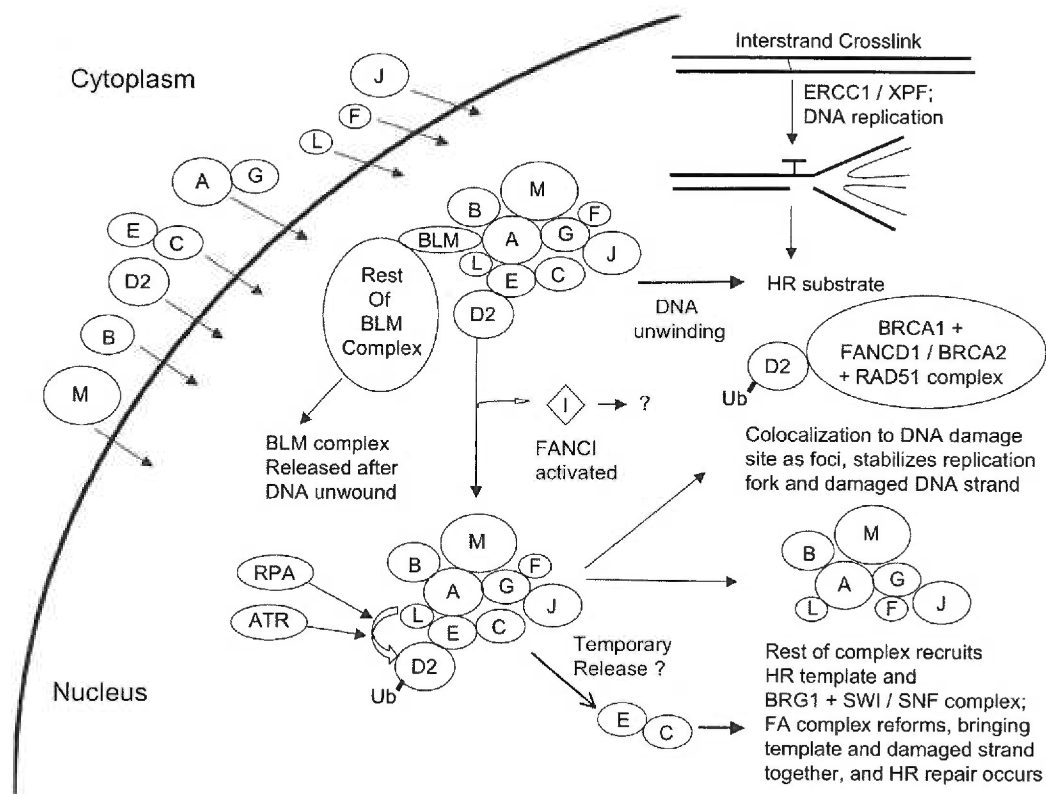
The Fanconi complex has also been demonstrated to interact with a BLM, topoisomerase III alpha, and replication protein A containing complex. This combination complex, referred to as the BRAFT-1 complex, is involved in DNA unwinding, but not in FANCD2 monoubiquitination[364]. It is via the isolation of this complex that the FAAP components of the FA complex have been discovered. The BRAFT complex, which

seems to require FANCA-BLM binding, does not incorporate the entire pool of either complex, allowing for independent interactions of either complex with other proteins[364].

Model of FA complex

The number of identified components of the FA complex has increased dramatically over the last few years, as has the amount of information regarding the complex as a whole. It is becoming obvious that the FA pathway is very dynamic, and involves proteins from other pathways. Hence, it is becoming more and more difficult to represent the FA pathway in a single model. However, a simplified model of the FA complex and aspects of the pathway can be attempted. Such a hypothetical model is given in Figure 2.

Figure 2: Model of the known FA complex proteins and simplified FA pathway.



IV. Mouse *Fanca* and mouse models

Analyses of mutations in human *FANCA*, such as the study of two patients described in Chapter 2, can benefit from a review of work done on the mouse homolog, *Fanca*. Similarly, mouse models of FA are useful to studying phenomenon such as repopulation in patients with corrected cells; a brief overview of these mouse models is, therefore, also given here.

Mouse homolog to *FANCA*.

Mouse *Fanca* was cloned by two groups[377,378], and expression patterns during embryogenesis[379], in adult tissues[377], or in a combination of the two[378] were determined. Mouse *Fanca* cDNA is 4503 bp long, with a 4320 bp open reading frame, encoding a 1439 a.a., 161 kD protein[377,378]. Mouse and human nucleotide sequences share 74% nucleotide identity[377,378], and the proteins are predicted to share about 65% identity[377,378] and 70%[378] to 81%[377] similarity. A partial leucine zipper motif is conserved, as is the nuclear localization signal[377,378], although the arginine at position 18 is not[378]. Mouse *Fanca* can complement MMC sensitivity in an FA-A lymphoblast cell line[377]. In addition, the predicted secondary structure and the hydrophobicity plot of mouse protein are similar to those for human FANCA, and the mouse gene is located in the region of mouse chromosome 8 syntenic to human chromosome 16q[378].

Mouse models of FA.

Since the study of the FA pathway and its function is furthered by a greater understanding of each component of the pathway, several mouse models for FA have been produced, including *Fancc* knockouts[139,380], *Fancg* knockouts[206,381], *Fanca* knockouts[143,382-384], and even *Fanca* + *Fancc* double mutant mice[143]. In all cases, FA mouse models do not exhibit anemia, morphological defects, or embryonic lethality, but do exhibit impaired fertility, due to hypogonadism and primordial germ cell defects, as well as hypersensitivity, in all tissues examined, to MMC or other crosslinkers. In fact, *pog* mice, which were specifically named for a defect in proliferation of germ cells[385], were later found to be a model for loss of the FA-L complementation group gene[4], and bone marrow cells from these mice have also been found to be hypersensitive to MMC[4]. Hence, the finding of high levels of *Fanca* expression in testes[377] is significant. In at least two cases[381,383], *in vitro* culture of hematopoietic progenitors from bone marrow under

stimulation or MMC treatment revealed a deficiency of clonogenic survival, especially of granulocyte colonies.

Two exceptions to the lack of morphological phenotype involve *Fanca* knockout mice with targeted disruption of exons 1 to 6[384], and *Fancd2* knockout mice[208]. Both of these have, in addition to the common phenotypes, increased in incidence of solid tumors -- specifically, in the *Fancd2* null mice, epithelial cancers including mammary adenocarcinomas -- as well as mispairing of meiotic chromosomes, implying a role for the FA pathway in meiotic recombination[208,384]. Micro-ophthalmia has also been observed in a C57 Black6 background in both gene knockout mice[208,384]. Perinatal lethality has also been noted in *Fancd2* null mice[208]; similarly, *Brca2* knockout mice generally display embryonic lethality[386,387], or, in *Brca2* knockout models in which mice are born, a tumor formation phenotype has been noted[388,389].

V. Somatic Mosaicism in Fanconi Anemia

The twins described in Chapter 2 exhibit somatic mosaicism, a condition characterized by subpopulations of cells within a tissue or organ that differ in genotype at one or more loci in the genome. Indeed, in approximately 25% of FA patients[390], MMC or DEB treatment of both fibroblasts and lymphocytes reveals a form of somatic mosaicism in which fibroblasts may be sensitive to MMC and DEB, while lymphocytes appear resistant. The mechanism of somatic mosaicism in such situations involves a direct reversion, compensatory mutation, or intragenic crossover or gene conversion in a single hematopoietic progenitor. This change results in a functional FA allele, phenotypic correction, and outgrowth of a clonal subpopulation of peripheral blood cells. In cross-linker sensitivity assays, the existence of a corrected subpopulation in the peripheral blood reduces the percent of MMC / DEB sensitive lymphocytes in the total population of cells tested. At the clinical level, the occurrence of somatic mosaicism for functionally corrected peripheral blood may rescue a patient from onset of anemia, thrombocytopenia, or pancytopenia, at least temporarily. This rescue appears to have occurred in the patients described in this thesis.

Other cases of somatic mosaicism in FA patients have also been described. In one series, somatic mosaicism in FA was described in 8 patients between 9 and 30 years of

age, 6 of whom displayed only mild hematological symptoms[390]. Of these 8 patients, 3 were compound heterozygotes for mutations in *FANCC*, and intragenic crossover or gene conversion appears to have been the mechanism of correction[390]. Another report[391] described two cases of acquired compensatory mutations for frameshifts in *FANCA*. In one case, two independently acquired single nucleotide deletions in cis counteracted a single nucleotide deletion, while the second case involved initial and compensatory insertions. In addition, the authors reported the case of two brothers, originally homozygotes for a missense mutation in *FANCC*, who independently acquired the same secondary point mutation in the same codon as the original mutation, resulting in expression of non-wild type, yet functionally corrected, protein in peripheral blood[391].

The first demonstration of somatic mosaicism caused by reversion in a lymphohematopoietic progenitor cell[392] was a report of a patient with an insertion in *FANCA* in his fibroblasts. Deletion of the inserted nucleotides was demonstrated in cultured myeloid (CFU-GM and BFU-E) and lymphoid (peripheral blood mononuclear cells and EBV-transformed B-cells) lineage cells. However, the use of relatively short-term multilineage clonal growth for these assays implied that the reversion occurred in a long-term culture-initiating cell (LTCI) rather than in an *extended* long-term culture-initiating cells (ELTCI)—cells with growth characteristics better representative of hematopoietic stem cells than LTCI cells[393]. Moreover, in this patient, pancytopenia was progressive, indicating that the reversion did not occur in a hematopoietic stem cell in the bone marrow, but, rather, in a multipotent progenitor.

Yet another report[394] described five patients, four of whom had various mutations in *FANCA*. These four patients showed direct reversions of initial mutations in the blood, and all four of them improved, over four to nine years, in cell counts of multiple hematological cell types. Further study of the fifth patient, a compound heterozygote for mutations in *FANCC*, indicated intragenic crossover, which, as it unfortunately failed to rescue the patient, must have occurred in a later stage leukocyte progenitor.

Finally, in one paper, patients were diagnosed based on a combination of FANCD2 monoubiquitination status and cytogenetic breakage analysis following treatment with nitrogen mustard[395]. Of the 8 patients described as somatic mosaics, 5 were placed in complementation group A, one in D2, and two were unidentified but had

normal FANCD2 monoubiquitination, which might imply that they belong to group J or have mutations in another gene downstream of the Fanconi protein complex. Of the group A patients, two had mild pancytopenia or thrombocytopenia, positive breakage results in peripheral blood lymphocytes (PBL), and only the non-ubiquitinated form of FANCD2 (FANCD2-S) in fibroblasts, but showed FANCD2 monoubiquitination in peripheral blood lymphocytes. Another group A patient, 21 years of age, had normal blood counts for 13 years but positive breakage in peripheral blood lymphocytes (PBL). The fourth patient, at 23 years of age, had negative breakage in PBL but mild pancytopenia; and the fifth, 34 years of age, was negative for PBL breaks, with improvement from anemia and severe thrombocytopenia to sub-normal blood counts during the course of low dose androgen therapy. The patient placed in complementation group D2, based on lack of FAND2 protein in fibroblasts, regained expression of both isoforms of FANCD2 in PBL, but was positive for PBL breaks, and, at 4 years of age, was below the mean age of onset of anemia. The two unidentified complementation group patients were either negative or ambiguous for PBL breaks, but suffered either mild anemia or mild pancytopenia. In short, while many of these patients showed improvement, none of these patients were negative for both PBL breaks and hematological symptoms, except for the 34 year old, who had undergone androgen therapy.

In cases of somatic mosaicism leading to hematopoietic improvement such as those above, it is likely that selection occurs in which functionally reverted hematopoietic cells repopulate a hematologic system gradually depleted of uncorrected cells. Indeed, in mouse models of Fanconi anemia, selection has been demonstrated under various conditions. Wild-type bone marrow was selected over *Fancc* knockout mouse bone marrow in radio-ablated wild-type mice and in MMC treated *Fancc* knockout mice[396]. Both wild-type and retrovirally corrected *Fancc* knockout mouse bone marrow cells were selected over recipient mouse bone marrow in cyclophosphamide-treated and gamma-irradiated *Fancc* knockout mice[397]. *In vivo* selection of lentivirally corrected *Fancc* and *Fanca* bone marrow, transplanted into lethally irradiated wild-type mice or into cyclophosphamide-treated *Fancc* and *Fanca* knockout mice, was also demonstrated[398].

While reports of somatic mosaicism in Fanconi anemia are numerous, somatic mosaicism caused by functional reversion at the level of the hematopoietic stem cell has yet to be demonstrated. Rigorous criteria for a finding of genotypic correction of a hematopoietic stem cell should include both resolution of cross-linker sensitivity in hematopoietic cells and complete lack of hematological symptoms for at least two decades, as well as a molecular description and functional assay of the initial mutation and of the genotypic correction. The patients described in Chapter 2, and the data analyzed therein, seem to fulfill these criteria. The patients were previously described[1] monozygotic twin sisters, who were initially thought to be Fanconi variants, because they did not exhibit the anemia phenotype at age 13. Chapter 2 describes the molecular follow up and findings on these patients.

Chapter 2:

Mutation analysis of monozygotic twins with Fanconi Anemia

Introduction:

This chapter discusses the case of 2 patients who were originally diagnosed[1] as FA variants since they had the physical features of FA patients, including short stature and thumb defects, as well as skin hyperpigmentation and kidney malformation in one twin, but had no hematological symptoms. These monozygotic twin sisters were, surprisingly, found to have mutations in the *FANCA* gene, which elicited the question of why they were still hematologically symptom free at age 28. The finding of an acquired mutation in the whole blood of both twins, which compensated for an inherited mutation, led to the conclusion that this correction occurred at the level of the stem cell and that these patients became somatic mosaics by repopulation.

In approximately 25% of Fanconi anemia patients[390], clastogen treatment of both fibroblasts and lymphoblasts reveals somatic mosaicism in the hematopoietic system, such that the blood contains clastogen-sensitive mutant cells as well as cross-linker resistant, revertant cells. Multiple molecular mechanisms for restoring normal function in mutant FA alleles have been demonstrated, including compensatory mutations in cis, intragenic crossovers, and gene conversion[390-392,394]. Some cases of somatic mosaicism result in hematopoietic improvement; in these cases, reversions are thought to occur in a single hematopoietic progenitor, leading to outgrowth of a clonal subpopulation of corrected peripheral blood cells via an endogenous selection process and growth expansion of the cells with a functional FA allele. Indeed, in murine models of FA, selection of functionally corrected hematopoietic cells has been clearly demonstrated under various conditions[396-398].

Clinically, the occurrence of somatic mosaicism for functionally corrected peripheral blood could rescue a patient from onset of anemia, thrombocytopenia, or pancytopenia, or at least delay onset. Case reports of somatic mosaicism have involved reversions in the *FANCA*, *FANCC* and *FANCD2* genes and have demonstrated correction

of multiple blood lineages, suggesting growth selection of corrected progenitors[390-392,394,395]. Some of the patients had milder than expected anemia.

However, to date, functional reversion at the level of the hematopoietic stem cell (HSC), followed by repopulation via unassisted natural selection, has yet to be demonstrated to be a source for somatic mosaicism. One test of direct reversion in a lymphohematopoietic stem cell of one FA patient used relatively short-term multilineage clonal growth assays [392], which do not, by themselves, demonstrate a stem cell origin for mosaic cells. In fact, this patient had progressive pancytopenia, indicating that the reversion occurred in a in a multipotent progenitor rather than in a bone marrow HSC. In another series, all somatic mosaic patients, except one, had mild forms of anemia, pancytopenia or thrombocytopenia, or positive breakage results in peripheral blood lymphocytes (PBL), or both; the exception, a 34 year old FA-A patient, was negative for PBL breaks and had significant hematological improvement, but underwent repopulation of the blood during treatment with low dose androgen therapy[395]. The use of this therapy called into question whether the improvement would have been as significant without treatment.

Hence, somatic mosaicism resulting from unassisted repopulation by a functionally reverted hematopoietic stem cell has yet to be demonstrated. Rigorous criteria for a finding of genotypic correction of a hematopoietic stem cell should include resolution of cross-linker sensitivity in hematopoietic cells and lack of hematological symptoms for at least two decades, as well as a molecular description and functional assay of the initial mutation and of the genotypic correction. The patients and data described in this chapter meet these criteria. This chapter describes the molecular events in monozygotic twin sisters[1] who have mutations in the *FANCA* gene but, at age 28, are free of any hematological symptoms. As such, it is the first report of somatic mosaicism in FA in which an acquired downstream missense mutation functionally corrects for an inherited mutation *in vivo*—presumably in a hematopoietic stem cell—and in which unassisted endogenous selection leads to repopulation and a clinically normal hematopoietic system in both twins. The analysis of these molecular changes provides evidence that molecular reversion can occur at the level of the HSC and result in long-term restoration of hematopoiesis.

Materials and Methods:

Patient samples, cell lines, and culture conditions

Primary fibroblasts (.F) were obtained from skin punch biopsy, while lymphoblasts (.L), were obtained via Ficoll-Paque extraction from whole blood and EBV-immortalized, in accordance with IRB protocol #3582, O.H.S.U. These cells were cultured in appropriate standard media. Patients' cells were labeled as follows: PD845 and PD839 cell lines were derived from the affected twins, PD846 from the mother, and PD852 from the father. The *FANCA* functionally-null GM6914 cell line has been previously described[399]. Retroviral packaging cell lines AM12[400] and PA317[401], were grown in DMEM medium supplemented with 5% bovine calf serum, 2mM L-Glut., and 1% P/S. A wild-type (normal adult) lymphoblast line (PD7) and FA lymphoblast lines (FA-A [HSC72], FA-C [PD-4], FA-D1 [HSC62], FA-D2 [PD-20], FA-E [EUFA130], FA-F [EUFA121], corrected FA-F [EUFA121+FANCF] and FA-G [EUFA316]) have been previously described[335]. PD751.F cells were primary fibroblasts obtained from the Fanconi Anemia Cell Repository, but from a patient with a disorder other than FA, and PD751.T + Telo cells were transformed with SV40 large T antigen and immortalized with telomerase. All cells were grown in a humidified 5% CO2-containing atmosphere at 37°C. Bone marrow was obtained for Wright's-Giemsa stained smears and for in vitro assays from the patients and volunteers according to OHSU IRB protocol # ise823.

Cytogenetic analysis

Cytogenetic analysis of mitomycin C (MMC; Sigma-Aldrich) and diepoxybutane (DEB; Sigma-Aldrich) sensitivity by chromosomal breakage (DEB) and radial formation (MMC) assay was done as previously reported[131,267,402,403]. At least 35 G-banded metaphase cells from each culture were scored for DNA breaks and radial formations by microscopic analysis. For complementation analysis, primary PD839 fibroblasts (PD839.F) were infected with retroviral supernatant representing various FA genes (A, C, D2, F, and G) as previously described[404]. Puromycin selected cells were assayed for

correction of MMC sensitivity. Karyotyping of bone marrow was done according to standard procedures, similar to those previously described[403]

PCR

All PCR were done on a Stratagene RoboCycler Gradient 96 machine, and used the following mix, unless otherwise stated: 2.5 μl 10X PCR Buffer with 1.5 mM $MgCl_2$ (Roche) + 0.2mM each dNTP mix + 0.2μl Taq polymerase (Roche) + 100 ng each primer + 100 ng genomic DNA + PCR dH_2O to 25μl

Mutation confirmation in genomic DNA

Mutations discovered by sequencing were confirmed in genomic DNA, obtained from patient cells as previously described[405], by 2% agarose gel electrophoresis of restriction digested PCR products, as follows

PCR: 95°C for 5 min., then 37 cycles of 95°C for 30 sec., X°C for 30 sec., and 72°C for 30 sec., followed by final extension at 72°C for 1 min.

X = 60°C for exon 26 assay, 51°C for exon 27 assay, 54°C for exon 28 assay, and 52°C for exon 30 assay

Exon 26 assay for G2457A polymorphism; 10% DMSO was required in the PCR mix:

forward primer: 5' TGCTCTGCCACCCTCATTC 3'

reverse primer: 5' CGAGAGAGAGGAGAGAAGACGC 3'

259bp product; BstNI cleavage:

wild type (2457 G) allele: 124, 71, 41, 12, and 11 bp products

polymorphism (2457 A) allele: 165, 71, 12, and 11 bp products

Exon 27 assay for the 2555ΔT mutation:

forward primer: 5' CCATCCAGTTCGGAATGC 3',

reverse primer: 5' AAGAAAACTTGCAGAGAGAGCAA 3'

155 bp product; mutant allele specific cleavage to 131 & 23 bp products with BstAPI

Exon 28 assay for the G2670A mutation:

forward primer: 5' GGTCTGTTTCCACCTGAGCATTTGC 3',

reverse primer: 5' CTGGCTACGTCCTCCTCACCAAGAG 3'

153 bp product; mutant allele specific cleavage to 130 + 23 bp products with XcmI.

Exon 30 assay for the G2927A mutation; 10% DMSO was required in the PCR mix:
forward primer: 5' AGTGCTGTGTGTCCCTTACTATG 3',
reverse primer 5' CTGTCCCTCCAGAGAACCC 3'
269 bp product; wild type allele specific cleavage to 143 and 126 bp products with Hinf I.

RNA extraction

RNA was extracted from patient fibroblasts and lymphoblasts, as well as wild-type control (PD751.F) fibroblasts, using RNA-Zol (Tel-Test Inc. via Fisher Scientific), in a manner similar to that previously described[406].

RT-PCR assay for alternate splicing

RNA from patient and wild type fibroblasts and/or lymphoblasts was used to make cDNA. The Fermentas First Strand cDNA Synthesis Kit protocol was followed, and murine MLV-RT and oligo$(dT)_{18}$ primer were employed to produce cDNA from 1μg total RNA per reaction; RT- control reactions were also carried out. PCR was performed using the following primer pairs:
5' AATGAGGATGACAGCAGCG 3', and 5' GACAGACGAAGGCAGGCG 3';
5' GGAACATCCCAGTCACCG 3' and 5' GACAGACGAAGGCAGGCG 3';
5' CTCAGCATCAACACGCCG 3' and 5' ATCTTCCTCTTCTCTAAACACTCG 3'
The first primer pair was used to amplify from within exon 23 to within exon 33, the second pair from within exon 18 to within exon 33, and the third pair from within exon 23 to within exon 39. For all three primer pairs, the temperature program was: 95°C, 5 min., then 40 cycles of 95°C for 30 sec., 58°C for 30 sec., and 72°C for 2 min., followed by 72°C for 10 min. All three primer pairs amplified over a kilobase of sequence, and were employed to detect alternate transcripts that skipped multiple exons, changing the transcript size by over 100 bp.

To detect potential alternative splicing which skipped only parts of exons 28 and 30, resulting in smaller transcript length changes, 440 bp of this region was amplified, and products were run out on 2% agarose gel. The following primer pair was used:
5' GCAAGTTTTCTTCCCAGTCACG 3' and 5' GTGGAAATCCATCAGTGCGTTG 3'

The temperature program for this PCR was: 95 for 5min, then 37 cycles of 95°C for 30 sec., 57°C for 30 sec., and 72°C for 30 sec., followed by final extension at 72°C for 1 min.

Plasmids and retroviral infection

The retroviral expression vectors, pMMP-puro[404] and pMMPpuroFANCA[285], were described previously, and include an IRES driven Puromycin resistance cassette[285].

Site directed mutagenesis pMMPpuroFANCA derived constructs:

The R880Q, E966K, and R880Q-E966K (double) mutant FANCA cDNAs were produced with the QuikChange site-directed mutagenesis kit (Stratagene). The cDNA inserts were verified by direct DNA sequencing. Production of pMMP retroviral supernatants and infection of fibroblasts were performed as previously described[281,407]. Cells were grown under 1 μg/ml Puromycin selection.

RNA derived constructs in pMMPpuroFANCA vector:

Products from PCR using the primer pair which amplified from within exon 23 to within exon 33 were cloned and sent for sequencing; suitable inserts were cut out with Bpu1102 I and Bam HI (New England Biolabs), gel purified, and used to replace the equivalent fragment of pMMPpuroFANCA. The resulting constructs were electroporated into AM12 cells, and retroviral supernatant from each Puromycin selected AM12 cell line was prepared and used to infect GM6914 cells as described previously[281,407]. The resulting GM6914 cell lines were cultured under 1 μg/ml Puromycin selection.

RNA derived constructs in Tetracycline / Doxycyclin (Dox) responsive vector:

To compensate for restriction site incompatibility, a linker consisting of two annealed oligos, 5'GATCCttttACCGGTttttttCCTAGGttttAT 3' and 5'CGATaaaaCCTAGGaaaaaaACCGGTaaaaG 3', was used to replace the BamHI and ClaI sites of pREV-TRE (Clontech Laboratories, catalog# 6137-1) at a 100:1 ligation ratio.

Then, this construct was cloned, and the AgeI to AvrII fragment (containing FANCA), from each pMMPpuroFANCA-based, RNA-derived construct, was ligated into the matching sites in the inserted linker; the resulting constructs were cloned and checked by restriction mapping. In this way, four pREV-TRE-FANCA constructs, expressing the

wild type, paternal, maternal, or lymphoblast allele of FANCA, were produced.

Immunofluorescence microscopy

Cells were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) for 20 minutes, followed by permeabilization with 0.2% Triton X-100 in PBS (3 minutes). They were then incubated in anti-FANCA (N terminal) antibody[291], diluted 1:200 in 3% bovine serum albumin/0.05% Triton X-100/0.04% sodium azide/PBS, for 1 hour at room temperature. Cells were subsequently washed 3 times in PBS, then incubated in fluorescein-conjugated anti-rabbit IgG secondary antibody (Jackson Immunoresearch, West Grove, PA), diluted 1:500 in 3% bovine serum albumin/0.05% Triton X-100/0.04% sodium azide/PBS, for 30 minutes at room temperature. Three more washes were applied, and the nuclei were counterstained with DAPI (4,6 diamidino-2-phenylindole) diluted in PBS at 1 μg/mL for 5 minutes. Three more washes were applied, and the slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA). Images were captured on a Zeiss Axioplan 2 Imaging microscope with a 40X oil immersion lens and Zeiss AxioCam HRc camera, using OpenLab Improvision 3.1.5 software. Images were trimmed and overall brightness and contrast were adjusted with Adobe Photoshop software (Adobe, San Jose, CA).

Subcellular fractionation

1-3 X 10^7 fibroblasts were resuspended in 0.4 ml Hypotonic Buffer (10 mM HEPES, pH 7.4 to 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 μM phenylmethylsulfonylfluoride [PMSF], 1X Complete, EDTA free Protease Inhibitor Cocktail [PIC; Roche]), then incubated on ice for 15 minutes. Cells were disrupted with 40 rapid strokes of a 1.5ml Dounce homogenizer, and the homogenate was incubated on ice for 10 minutes after addition of 2.5 μl NP-40 (a.k.a. IGEPAL). The homogenate was vortexed briefly at low speed, then microcentrifuged at 7000 rpm (4000-4500g) for 5 minutes. Supernatant was transferred to a fresh 1.5 ml tube as the cytoplasmic fraction. The nuclear pellet was washed in Hypotonic Buffer and repelleted, thrice, then resuspended in 200 μl Lysis Buffer 1 (20 mM HEPES, pH 7.4 to 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2% Triton X-100, 1 mM DTT, 1 μM PMSF, 1X PIC) and

sonicated for 10 seconds at medium power. After 30 seconds on ice, sonication was repeated. The sonicate was microcentrifuged at maximum speed (14,000 rpm) for 5 minutes, and the supernatant was transferred to a fresh tube as the nuclear fraction. The cytoplasmic and nuclear fractions were mixed 1:1 with Lysis Buffer 1 and Hypotonic Buffer, respectively, to ensure the same final solution for both fractions. Protein concentrations were determined using the BioRad DC Protein Assay.

Subcellular localization immunoblot

Fractions (100 ug protein per sample) were subjected to 5.1% SDS-PAGE to probe for the 163 kDa FANCA protein, as well as to 6.0% SDS-PAGE to probe for topoisomerase I (a ~90 kDa nuclear protein), and to 9.0% SDS-PAGE to probe for β-tubulin (a 55 kDa cytoplasmic protein). The latter two blots were used to detect cytoplasmic contamination in the nuclear fraction and to verify retention of nuclear proteins in the nuclear fraction. Proteins were transferred to PVDF membranes (Millipore, Billerica, MA) using the Panther Semi-dry Electroblotter (Owl Separation Systems, Portsmouth, NH; Model HEP-1), according to product protocol. Membranes were blocked in 5% milk in PBS for 1 hour at room temperature (anti-Topoisomerase I and anti-FANCA Westerns) or overnight at 4°C (anti-β-tubulin Western), then incubated (with shaking) overnight at 4°C, in one of the following primary antibodies, diluted in 5% milk in PBS: rabbit polyclonal anti-β-tubulin antibody (Santa-Cruz Biotech, Santa Cruz, CA, catalog # sc-9104), diluted 1:5000; rabbit polyclonal anti-topoisomerase I antibody (Santa-Cruz Biotech, catalog # sc-10783), diluted 1:1000; or mouse monoclonal anti-FANCA (human) 5G9 antibody[280], diluted 1:250. Membranes were rinsed, at least thrice, for 5 to 10 minutes each, at room temperature, in TBS-T (137mM NaCl, 20mM Tris-Cl pH 7.5-7.6, 0.05% Tween-20). Membranes were blocked in 5% milk for 1 hour, then incubated, at room temperature, for 30 minutes to an hour, in one of two secondary antibodies, both diluted 1:3000 in 5% milk. Anti-topoisomerase I and anti-β-tubulin Westerns required goat anti-rabbit IgG secondary antibody, conjugated to horseradish peroxidase (HRP) (Bio-Rad, Hercules, CA, catalog # 172-1013). Anti-FANCA Western required goat anti-mouse IgG HRP-conjugated secondary antibody (Santa Cruz Biotech, catalog # sc-2005). After rinsing membranes in TBS-T as above, Enhanced

Chemiluminescence (ECL; Amersham Biosciences, Piscataway, NJ) was used for detection.

Production of Tet-responsive transactivator (tTA) expressing GM6914 clones

GM6914 cells, electroporated (350V and 960µF, in cytomix[408], using the BioRad GenePulser with Capacitance Extender) with pREV-Tet-Off plasmid (Clontech Laboratories, Mountain View, CA, catalog# 6140-1) were placed under 400µg/ml G418 selection. G418 resistant cells were seeded, and clones were expanded to 100 mm plates.

Tet-responsive transactivator (tTA) Immunoblotting

Cells were trypsinized, washed with PBS, and resuspended in Lysis Buffer 2 (50 mM Tris, 150 mM NaCl, and 1% Triton X-100, with 1X PIC and 1mM PMSF). Lysis involved 1/2 hour room temperature agitation, pulse-vortexing, and centrifugation for 10 minutes at 16,000 g. Supernatants were flash frozen in liquid nitrogen and stored frozen at -80°C. Lysates were subjected to 12% SDS-PAGE, 50µg protein per sample. After 2-hour block in 5% milk in PBS, the PVDF membrane was incubated in anti-VP16(1-21) monoclonal IgG_1 antibody (Santa Cruz Biotech; catalog # sc-7545), diluted 1:1000 in 1% milk in PBS, overnight at 4°C. After thrice washing in TBS + 0.1% Tween-20, the membrane was incubated at room temperature for 30 minutes in goat anti-mouse IgG_1 HRP conjugated secondary antibody (Santa Cruz; catalog # sc-2969), diluted 1:3000 in 1% milk in PBS, and thrice washed again. ECL was used for detection. The three GM6914 e:pREV-Tet-Off clones expressing the highest levels of tTA (D3, E1, and I15) were retained.

Production of Dox regulated FANCA expressing GM6914 cell lines

PA317 cells were transfected with the various pREV-TRE-FANCA constructs using Fugene lipofection reagent (Roche) as per protocol, then cultured under 200 µg/ml Hygromycin B selection. Retroviral supernatant was produced from each resulting cell line, and each of the three GM6914 e:pREV-Tet-Off clones were infected, in parallel, with each supernatant, as previously described[281,407]. Infected cell lines were placed under 250µg/ml Hygromycin B and 400µg/ml G418 selection, to generate a set of cell lines

expressing pREV-TRE-FANCA with the wild type, paternal, maternal, or lymphoblast allele of *FANCA* for each clone.

Immunoblotting for Dox regulated FANCA protein expression

Equal numbers of cells from the 4 GM6914 e:pREV-Tet-Off clone D3 derived cell lines were each cultured for 3 days under 0, 7.8, 31, 125, 500, or 2000 ng/ml Dox. Cells were then trypsinized, washed with PBS, and resuspended in 50μl Lysis Buffer 2. Lysis involved three cycles of vortexing, flash-freezing in liquid nitrogen, and thawing, followed by 10 minute, 16,000 g centrifugation. Supernatants were flash-frozen and stored at -80°C. Equal volumes of each lysate were subjected to 5.1% SDS-PAGE. Immortalized non-Fanconi fibroblasts (PD751.T +Telo), treated as above but without Dox, were used as endogenous level controls, and 50μg of lysate from D3 clone cells electroporated with pREV-TRE+linker was used as negative control. Transfer, blocking, blotting, washing, and detection was done as for tTA westerns, except with antibodies used for FANCA subcellular immunoblot, with primary antibody diluted 1:500, and secondary diluted 1:3000, both in 5% milk..

FANCD2 monoubiquitination immunoblot

Immunoblotting for FANCD2 westerns and related immunoblots, as well as for westerns involving site-directed-mutagenesis constructs was performed as previously described[335]. Briefly, cells were lysed with 1× sample buffer (50 mM Tris-HCl, pH 6.8, 86 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate [SDS]), boiled for 5 minutes, and subjected to 7.5% (10.0% for FANCF detection) SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were transferred to nitrocellulose membranes using a submerged transfer apparatus (BioRad, Hercules, CA) filled with 25 mM Tris base, 200 mM glycine, and 20% methanol. After blocking with 5% nonfat dried milk in TBS-T (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween20), the membrane was incubated with the primary antibody diluted in TBS-T (1:1000 dilution), washed extensively, and incubated with the appropriate horseradish peroxidase-linked secondary antibody (Amersham, Piscataway, NJ). Chemiluminescence was used for detection.

MMC resistance assays

Growth assay for GM6914 cell lines with pMMPpuroFANCA based constructs

Cell lines expressing pMMPpuroFANCA with one of 4 RNA derived *FANCA* alleles, or pMMPpuro vector only, were seeded, 1000 cells per well, in triplicate, to 96 well plates. Media was changed the next day, to add 0, 10, 25, 50, 75 , or 100 nM MMC, plus 1μg/ml Puromycin, and cells were cultured for 7 days. Cell number per well, as measured by fluorescence of a DNA-activated dye, was quantitated using the CyQUANT system (Molecular Probes, Eugene, OR) and FLUOstar 403 fluorescence plate reader (BMG LabTechnologies, Durham, NC), set to 485 nm excitation and 535 nm emission as previously described[208]. Gain was set based on cells expressing wild type allele in 0nM MMC wells. Relative average % fluorescence was calculated and graphed for each cell line for each MMC concentration. This experiment was repeated without Puromycin, and with 5 day culturing, without substantial difference in overall outcome.

Fresh retrovirus was also produced, using AM12 cells, from site-directed-mutagenesis constructs, in parallel with retrovirus produced from RNA-derived constructs, and GM6914 cells were infected with these retroviral supernatants, independently of cells used in immunofluorescence experiments. The resulting cell lines were also assayed for growth under MMC selection in a similar manner as cell lines expressing RNA-derived pMMPpuroFANCA based constructs.

Colony forming ability (CFA) assay

GM6914 cell lines expressing pMMPpuroFANCA constructs with wild type allele, maternal allele, and lymphoblast allele, or vector only (pMMPpuro), were seeded to 18 100 mm plates each, 500 cells per plate. Puromycin selection was removed, and 0, 5, 10, 20, 40, or 80 nM MMC was added, 3 plates per cell line per MMC concentration. Media was changed ~64 hours later, without Puromycin or MMC. Cells were allowed to grow for 7 more days (~11 days after addition of MMC) and then plates were stained with methylene blue stain (Fisher Scientific, Hampton, NH; 1-2% dissolved in 50% methanol). Colonies were counted and averaged for each cell line for each concentration of MMC, and the % CFA relative to 0nM MMC was graphed for each cell line.

Growth assay for GM6914 cell lines expressing pREV-TRE-FANCA constructs

For each clone (GM6914 e:pREV-Tet-Off D3, E1, and I15), cells from each of the 4

FANCA allele expressing cell lines were seeded to 96 well plates, 1000 cells per well. G418 and Hygromycin B selection was removed around 12 hours later, and replaced with media containing 0, 7.8, 31, 125, 500, or 2000 ng/ml Dox. About 24 hours later, media was replaced again, this time containing appropriate Dox levels and, additionally, 0, 20, 40, or 80 nM MMC. Thus, for each cell line, cells were exposed, in triplicate, to each of the four MMC concentrations at each of the six Dox concentrations, in a Dox by MMC matrix. Cells were cultured for between 5 to 7 days (depending on experiment) and then were analyzed using the CyQUANT assay and FLUOstar reader as described above. CFU-GM and BFU-E. Low-density bone marrow cells were obtained as previously described[140], except for the use of the EasySep kit (Stem Cell Technologies, Vancouver, B.C.). These cells were exposed to various doses of Mitomycin C in suspension culture for only 24 hours, after which they were washed in complete medium and plated in methylcellulose with complete medium, Steel factor (50 ng/ml), IL-3 (10 ng/ml), and erythropoietin (2 Units/ml). Colony forming units granulocyte-macrophage (CFU-GM) and burst forming units erythrocyte (BFU-E) were counted 14 days after plating.

Multilineage progenitor assays

Multilineage progenitor assays were undertaken as previously described[392]; DNA obtained from individually plucked CFU-GM and BFU-E from both twins was analyzed via the abovementioned PCR–digest assay for the acquired exon 30 sequence change (2927G>A).

X-inactivation assay

An X chromosome inactivation assay was applied to genomic DNA from patients' whole blood and fibroblasts, as well as from the parents and with DNA from a randomly chosen normal female sample. The assay was performed essentially as previously described[409]. DNA samples were digested with either Hpa II or Hha I prior to PCR, and results were effectively independent of enzyme used.

Assays for alternate transcript lacking exon 30

PCR products from either wild type fibroblast or PD839.F cell cDNA, containing a region from within exon 23 to within exon 33, but excluding exon 30 due to an in-frame deletion at the mRNA level, were cloned into pMMPpuroFANCA using the same methods as described for other transcripts. GM6914 cells were infected with retrovirus made from these two constructs in the same way as was used for other constructs. Cyquant-based MMC survival curves for the resulting cell lines, as well as subcellular localization Westerns, were executed in parallel with cell lines infected with other constructs.

Extraction of DNA from paraffin sections

For each sample, a 500 mg paraffin embedded tissue section, in a sterile 15 ml polypropylene centrifuge tube, was allowed to melt in a 65°C water bath. 1 ml of mixed xylenes was added, and the sample was vortexed, then centrifuged at 7500 rpm for 10 minutes at 37°C; the supernatant was then aspirated. This process was repeated with fresh xylenes, then with 100% ethanol, xylenes again, twice, 100% ethanol again, and, finally, with 70% ethanol. Centrifugation for ethanol steps was done at room temperature. The sample was allowed to air-dry overnight. 3 ml Nuclei Lysis Buffer (10 mM Tris-HCl pH 8.0 + 400 mM NaCl + 2mM EDTA) was added and the sample was homogenized, using a PowerGen Model 125 homogenizer (Fisher Scientific) using 7mm X 195mm sawtooth generator, at highest setting for 1 minute or more, until no tissue chunks remained. DNA extraction was carried out as previously described[405]. The DNA was RNAse treated, reprecipitated with sodium acetate and ethanol, washed with 70% ethanol, and resuspended in 100µl Tris-EDTA pH 8.0.

Results:

Clinical description of patients.

The patients were previously described monozygotic twin sisters[1], with normal hematological parameters from initial diagnosis at age 6 months through follow-up at age 13. One twin had initially elevated chromosome breakage in DEB-treated peripheral blood lymphocytes (PBL), which resolved by age 13. The other twin demonstrated

normal breakage even at six months. Both twins exhibited short stature and thumb defects, and one had abdominal skin hyperpigmentation and surgically corrected malformed kidneys. The twins are now 28 years old and free of hematological symptoms, and they have not shown signs of leukemia or solid tumors. Their complete blood counts are normal, the bone marrows are not hypocellular and show no evidence of myeloid leukemia or myelodysplasia, and neither twin has developed a solid tumor.

Determination of complementation group.

Fibroblasts from one twin (proband) were cytogenetically analyzed for chromosomal radials under mitomycin C (MMC) and diepoxybutane (DEB) treatment and were found to be sensitive to both DNA crosslinking agents; however, lymphocytes from the same twin were found to be MMC-resistant (Table 1A, courtesy of Dr. Yassmine Akkari). Sensitivity to mitomycin C was corrected in fibroblasts by infection with retrovirus containing wild type FANCA (Table 1B, courtesy of Dr. Yassmine Akkari).

Table 1: Cytogenetic analyses of PD839 fibroblasts and lymphoblasts.
A) Fibroblasts and lymphoblasts were treated with MMC and DEB, then scored for number of chromosomal breaks and radials formed. B) PD839 fibroblasts can be corrected for MMC and DEB sensitivity in a chromosome breakage and radial formation assay by introduction of wild-type FANCA.

A)

PD839 cells	Clastogen	Conc. ng/ml	# Rad.	% Rad.	FA range	Normal range
Fibroblasts	None	0	0	0%	0-8%	0%
Fibroblasts	MMC	12	11	22%	16-100%	0%
Fibroblasts	MMC	20	25	50%	47-67%	0%
Fibroblasts	DEB	75	18	35%	20-36%	0%
Lymphocytes	MMC	40	3	6%	30-100%	0-4%
Lymphocytes	DEB	100	0	0%	0-32%	0%

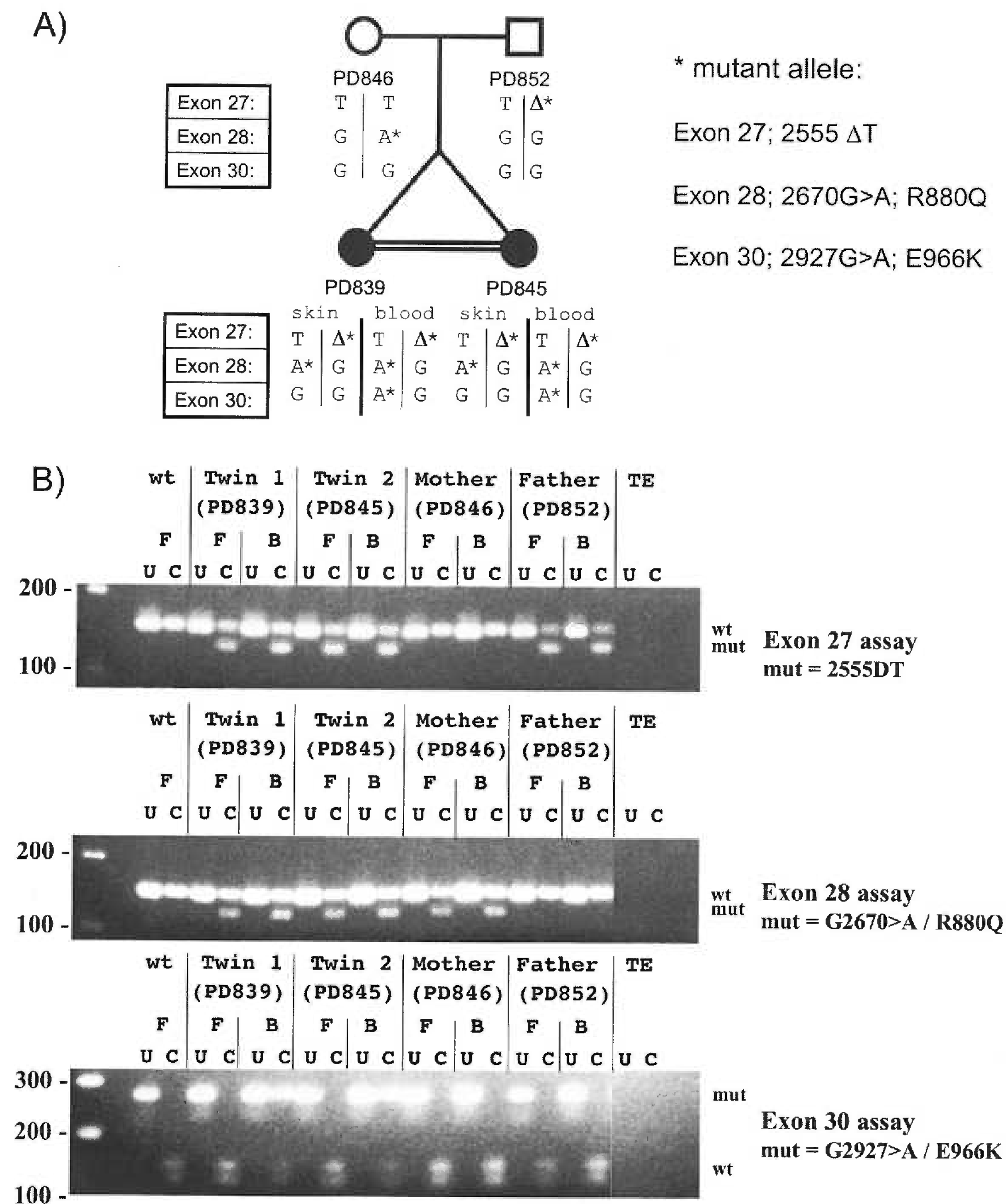
B)

PD839 fibroblasts	Clastogen	Conc ng/ml	# Rad.	% Rad.	1 Rad./cell	More than 1 Rad./cell
No vector	None	0	0	0%	0	0
No vector	MMC	15	23	46%	9	14
No vector	DEB	150	17	47%	7	10
RV:FANCA	None	0	0	0%	0	0
RV:FANCA	MMC	15	3	6%	2	1
RV:FANCA	DEB	150	0	0%	0	0

Mutation detection.

Exon-by-exon sequencing of FANCA from genomic DNA revealed sequence changes as follows, with numbering referring to NCBI Nucleotide Database #NM_000135[256], with start codon at position 32. The twins were found to be homozygous for a previously described[301,302] exon 26 polymorphism (G2457A / G809D). A single thymidine was deleted at position 2555 (2555delT) in exon 27, which should lead to a frameshift and premature termination codon in exon 28. This previously reported[301] mutation was determined, in this case, to be inherited from the father. In exon 28, a nucleotide change of G2670A was found, resulting in an arginine-to-glutamine missense change at amino acid 880 (R880Q). This alteration was maternally inherited, as it also appeared in the mother's genomic DNA. Both the published paternal mutation and the novel maternal mutation described here were found in fibroblasts from the parent of origin as well as in the twins' fibroblasts, lymphoblasts, and whole blood DNA. In contrast, a sequence change present in both twins was detected in genomic DNA from both lymphoblasts and whole blood, but not in fibroblast genomic DNA. This change, in exon 30, was a G2927A nucleotide change, resulting in a glutamate-to-lysine change at amino acid 966 (E966K). By sequencing of RT-PCR products, this alteration was found be *in cis* with the exon 28 change (data not shown). All results were confirmed by PCR-digest assays, and the summary of the putative mutations can be found in Figure 1.

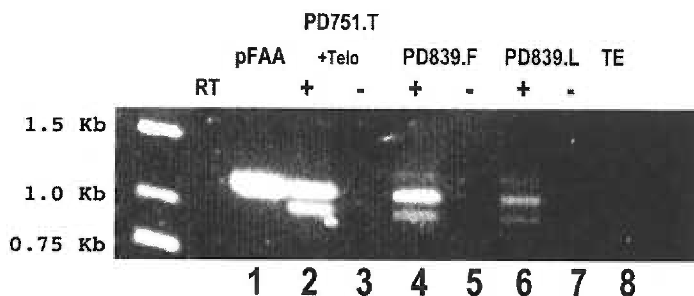
Figure 1: Mutation summary.
A) Pedigree-displaying members of the immediate family of monozygotic twin patients, with haplotypes for each locus listed. An acquired mutation in whole blood was found downstream of the maternal mutation in both patients. B) PCR-digest assay results for exon 27, exon 28, and exon 30 mutation assays. "wt" above each gel signifies wild type genomic DNA, from a non-FA patient. F = fibroblast genomic DNA, B = genomic DNA from whole blood, U = uncut PCR product, C = cut / digested PCR product.



Detection of alternate splice forms.

The existence of two sequence changes *in cis* within the lymphoblast allele gave rise to the hypothesis that the changes affected splicing. The G2670A change might produce an alternate splice donor, such that only part of exon 28 might end up in the mature mRNA. Not only could this lead to loss of amino acids, but, depending on the location of the splice acceptor site for this splice donor, the reading frame could end up shifted. Similarly, the G2927A sequence change could produce a novel splice acceptor site, allowing splicing in from the putative alternate exon 28 splice donor. According to Shapiro-Senapathy splice score analysis[410,411], the G2927A change could give rise to such a splice acceptor, resulting in an in-frame deletion of 243 bp (data not shown). To test this hypothesis, an RT-PCR assay was used to amplify the region of FANCA from within exon 23 to within exon 33 from both wild type and patient fibroblasts and patient lymphoblasts . When the PCR products were run on a gel, a smaller alternate transcript was visible, but it was determined to exist in both wild-type and patient cells in equal amounts (Figure 2). When this smaller transcript was sequenced, it was found to be missing exon 30 (data not shown) amounting to an in-frame loss of 129 bp, or 43 a.a. This alternate transcript was reported previously[303].

Figure 2: PCR for alternative transcript detection.
The plasmid pFAA[107] was used as amplification control, while PD751.T+Telo cDNA was used as wild type control. Reverse transcriptase (RT) + and – reaction products for each RNA were used as PCR template.



To confirm that the sequence changes did not remove the exons to which the original primers bound, a second pair of primers was used to amplify from within exon 18 to within exon 33, and a third pair from within exon 23 to within exon 39. PCR

products from the second two sets of reactions were treated with Sau3AI, which digests the PCR product at a site in exon 30, to determine whether alternate transcripts amplified by these primer pairs were identical to sequenced product from the first reaction. No alternate splicing specifically due to the sequence changes was noted in these assays (data not shown).

It was also possible that the G2670A sequence change could produce a novel splice acceptor, which would splice out the first several nucleotides of exon 28, possibly leading to a frame shift, and this shift could be compensated for by a novel splice acceptor produced by the G2927A change. In such a case, the alternate transcript produced by both changes might lack as little as 50 nucleotides. To test this, a 440bp fragment spanning the region was also amplified, but no such alternate transcripts were detected (data not shown).

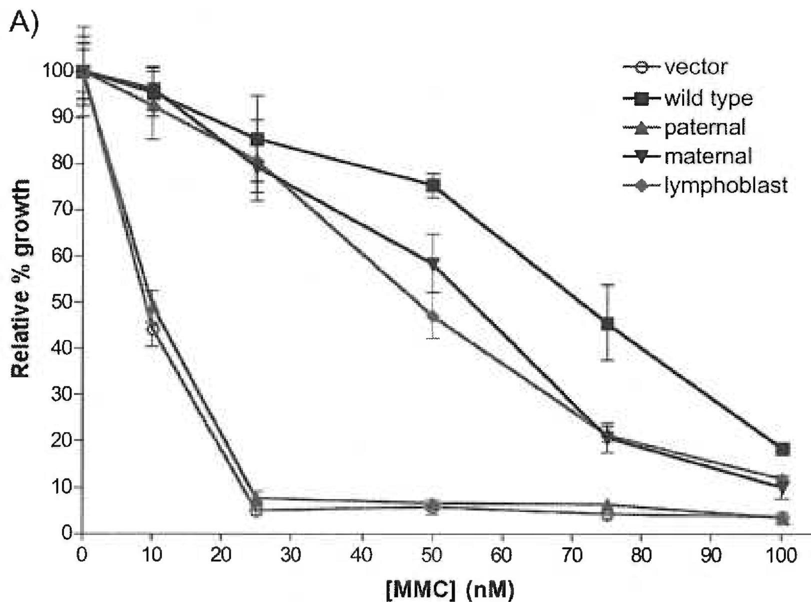
We also found an alternate transcript that was missing exon 38 (data not shown). We found this in the early stages of mutation detection, which were undertaken using cDNA template, prior to the discovery of the sequence changes via exon-by-exon sequencing This transcript was not studied further, as it was found using RNA from wild type cells; however, a mutation leading to complete exclusion of exon 38 from all transcripts has been reported[303], implying the possibility that this apparently rare alternate transcript would be unable to complement MMC sensitivity.

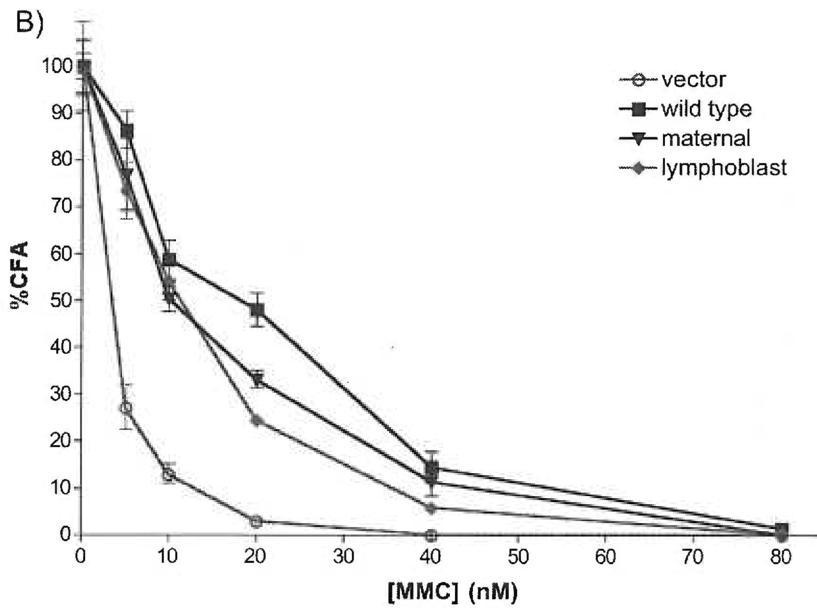
Constitutive expression of patient-derived alleles.

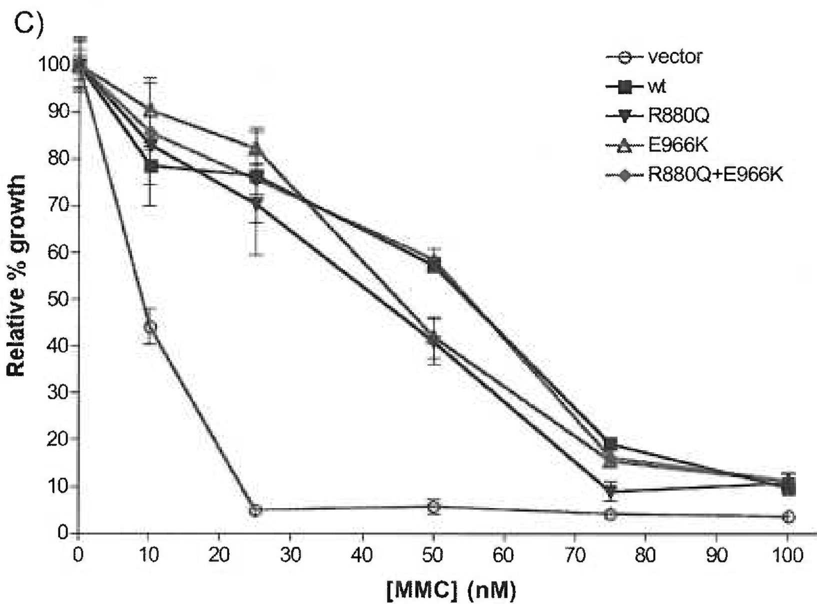
The lack of a splicing phenotype lead us to investigate the effects of amino acid substitutions predicted by the sequence changes. To confirm that these substitutions had functional effects, the region of *FANCA* cDNA spanning these changes was amplified by RT-PCR from RNA extracted from patient fibroblasts and lymphoblasts and from wild type control cells. The region was then used to replace the equivalent region in the pMMPpuroFANCA plasmid. Wild type controls also had the G809D polymorphism, so differences from wild type in all experiments were independent of the polymorphism. GM6914 cell lines produced by infection with the each of the corresponding retroviral supernatants were tested, both by growth assay and by colony-forming ability assay, for mitomycin C sensitivity.

Cell growth, assayed using the CyQUANT fluorimetric DNA quantitation system, demonstrated a clearly mutant phenotype for the paternal allele (2555delT), whereas cells expressing the maternal allele, containing the exon 28 mutation (G2670A), or the lymphoblast allele, containing both the exon 28 mutation and the acquired exon 30 mutation (G2927A), demonstrated intermediate phenotypes (Figure 3A). The colony forming assay results paralleled this outcome. Under increasing concentrations of MMC, the colony-forming ability of cell lines expressing either the maternal allele or lymphoblast allele construct fell between that of cell lines expressing wild type allele and those expressing vector-only (Figure 3B). The outcome of the Cyquant assay was replicated with site-directed mutagenesis-derived constructs. The R880Q change, the E966K change, and the two changes in combination all partially corrected MMC sensitivity (Figure 3C).

Figure 3: MMC resistance assays of constitutively expressing cells.
GM6914 cells expressing various constructs, were assayed for complementation of MMC sensitivity. A) patient-derived alleles; cell growth assayed by CyQUANT. B) patient-derived alleles; colony-forming ability under increasing MMC concentrations. C) site directed mutagenesis alleles, cell growth assayed by CyQUANT. Intermediate phenotypes for both maternal and lymphoblast alleles were noted under each of these conditions.





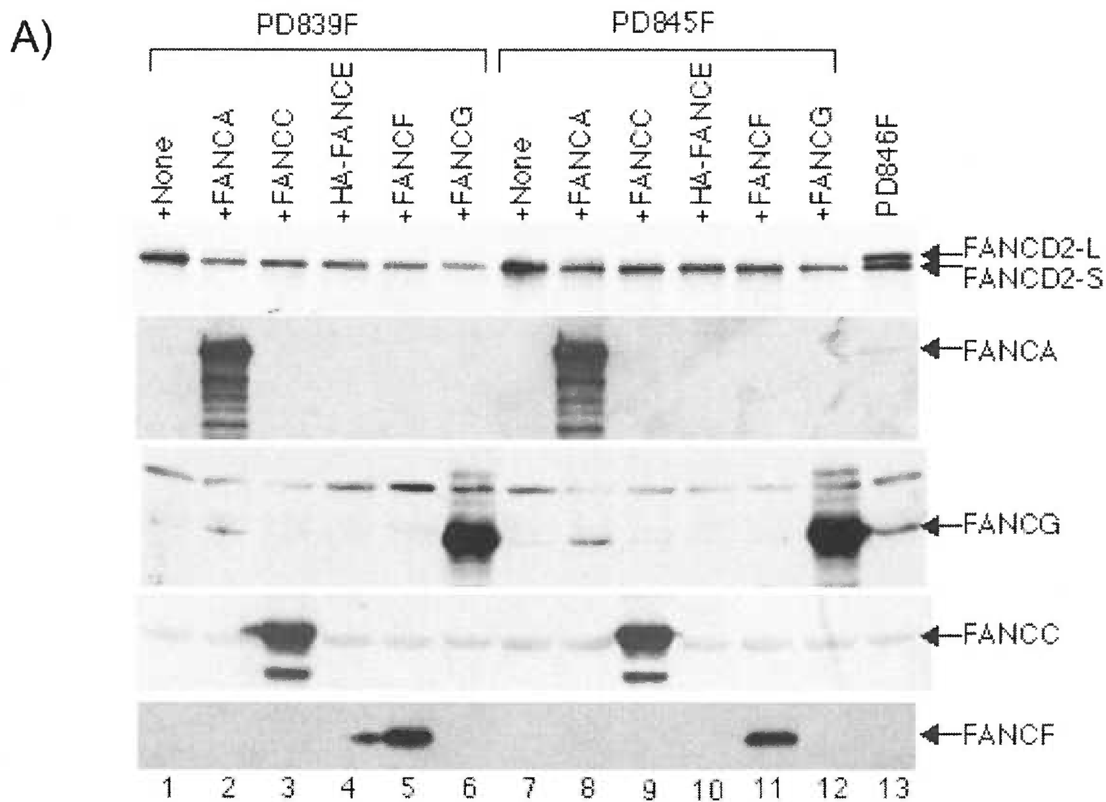


FANCD2 immunoblot of site-directed mutagenesis construct expressing cell lines.

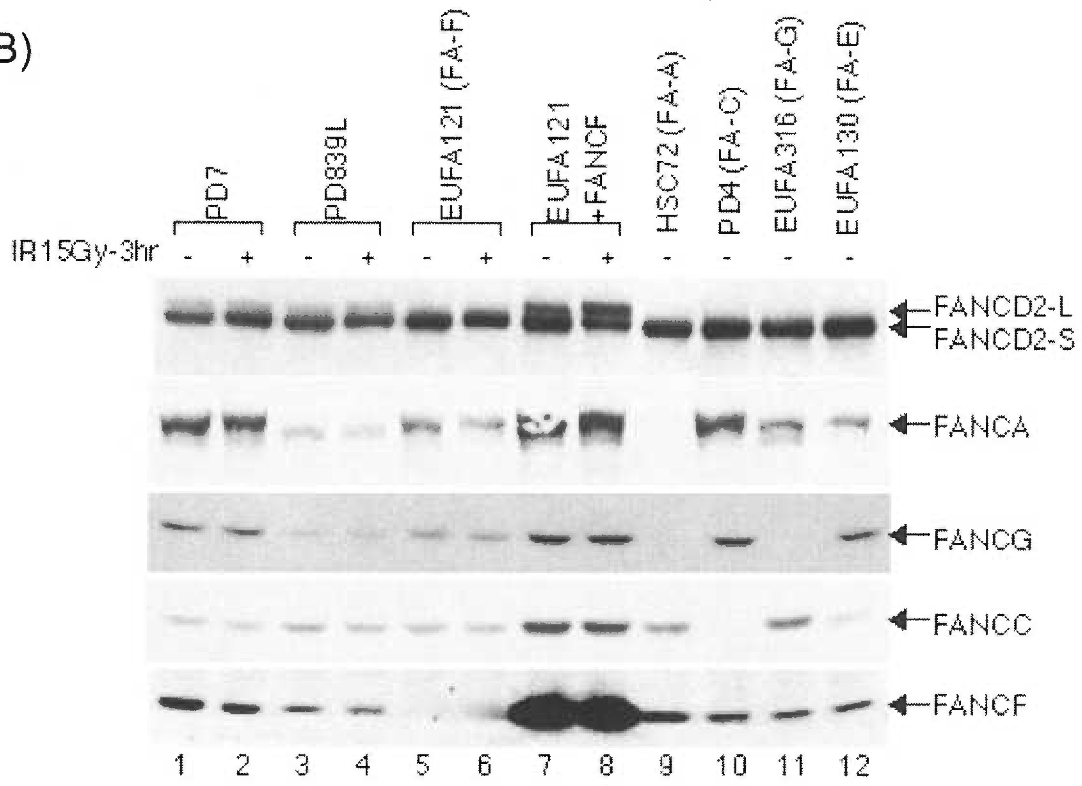
Unlike wild-type cell lines, FA cells do not exhibit monoubiquitination of FANCD2 protein[412]. Patient fibroblasts did not exhibit FANCD2 monoubiquitination until they were retrovirally infected with wild-type *FANCA* (Figure 4A, courtesy of Dr. Toshiyasu Taniguchi). Patient lymphoblasts demonstrated FANCD2 monoubiquitination without retroviral correction (Figure 4B, courtesy of Dr. Taniguchi), supporting the

hypothesis of somatic mosaicism. Infection of GM6914 cells with site-directed mutagenesis-derived retroviral constructs (pMMPpuro FANCA R880Q, E966K, and R880Q/E966K) imparted correction of the FANCD2 monoubiquitination phenotype with all constructs, but not with pMMPpuro alone (Figure 4C, courtesy of Dr. Taniguchi). Unexpectedly, PD839.L cells (Figure 4B) showed a downward mobility shift of FANCA protein, as did cells infected with pMMPpuroFANCA E966K and R880Q/E966K constructs (Figure 4C).

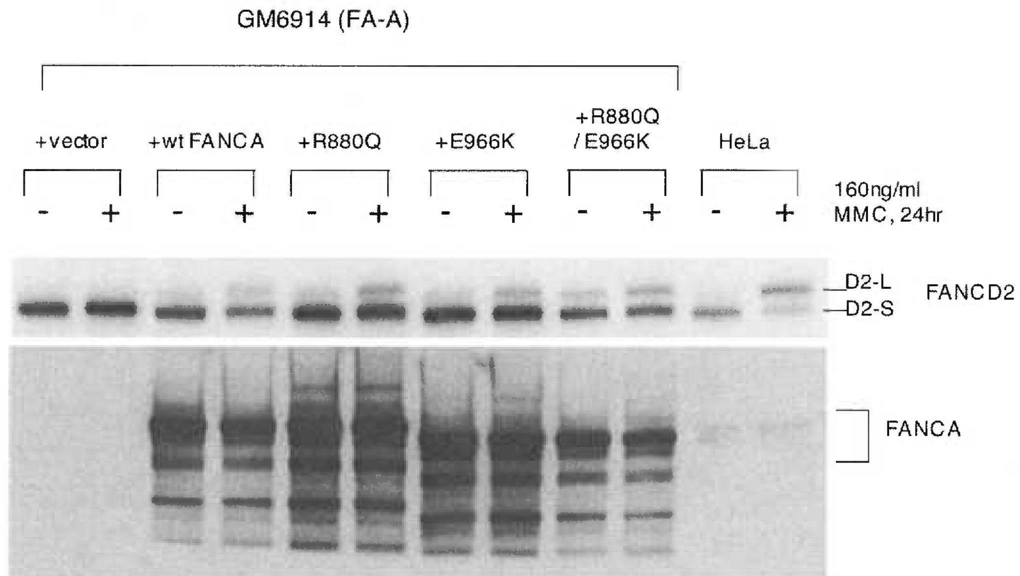
Figure 4: FANCD2 monoubiquitination immunoblots.
A) Patient fibroblasts expressing various FA genes by retroviral infection. B) Patient lymphoblasts demonstrated wild type monoubiquitination C) Constitutive expression of all site directed mutagenesis constructs corrected FANCD2 monoubiquitination.



B)



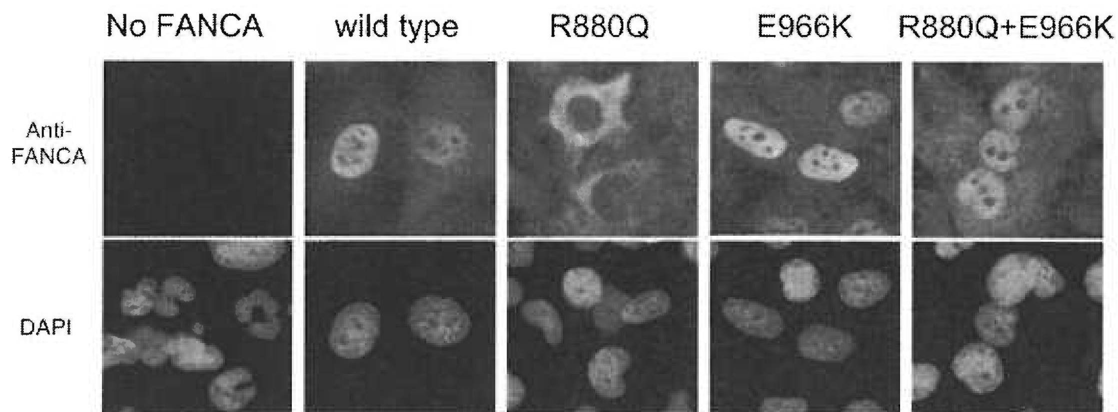
C)



Cellular localization of FANCA protein variants.

As nuclear localization of FANCA protein is known to be required for function[281,282], the cellular localization of each of the mutants was studied. GM6914 cell lines infected with site-directed mutagenesis pMMPpuroFANCA based constructs were assayed for protein localization by immunofluorescence (Figure 5, courtesy of Dr. Taniguchi). As expected, wild-type FANCA was largely nuclear, but FANCA protein with the R880Q mutation localized mostly to the cytoplasm, indicating that not much FANCA was required to enter the nucleus for functional correction to occur. FANCA protein carrying either an E966K mutation or the R880Q+E966K double mutation in combination localized mostly to the nucleus. Hence, the initial maternal mutation negatively affected sub-cellular localization of FANCA protein, and the acquired downstream mutation corrected the mislocalization.

Figure 5: Subcellular localization of FANCA by immunofluorescence.
FANCA expression was followed by immunofluorescence in GM6914 cells infected with wild type pMMPpuroFANCA retrovirus or site-directed-mutagenesis constructs. The R880Q mutation prevents nuclear localization, but the E966K mutation does not. Both mutations in cis restore nuclear localization.



Cellular localization was also determined by immunoblot, using cell lines expressing both RNA-derived and site-directed mutagenesis-derived pMMPpuroFANCA-based constructs. When subcellular localization was assayed for cells that expressed wild-type allele, maternal allele, and lymphoblast-allele, all three constructs demonstrated both cellular and nuclear localization (Figure 6). Similar results were found for all three (R880Q, E966K, and R880Q+E966K) site-directed mutagenesis

constructs (data not shown). The discrepancy between immunofluorescence and immunoblot results can be explained by the higher sensitivity of the immunoblot technique. The immunoblot results indicated that, if constitutively expressed, sufficient amounts of both maternal-allele and lymphoblast-allele-expressed FANCA entered the nucleus to complement the MMC sensitivity of GM6914 cells.

Figure 6: Subcellular localization of FANCA by immunoblot.
Uncorrected GM6914 cells express no FANCA, while corrected cells display normal localization. All constructs demonstrated normal localization under constitutive expression.

Doxycyclin regulated expression of patient-derived constructs .

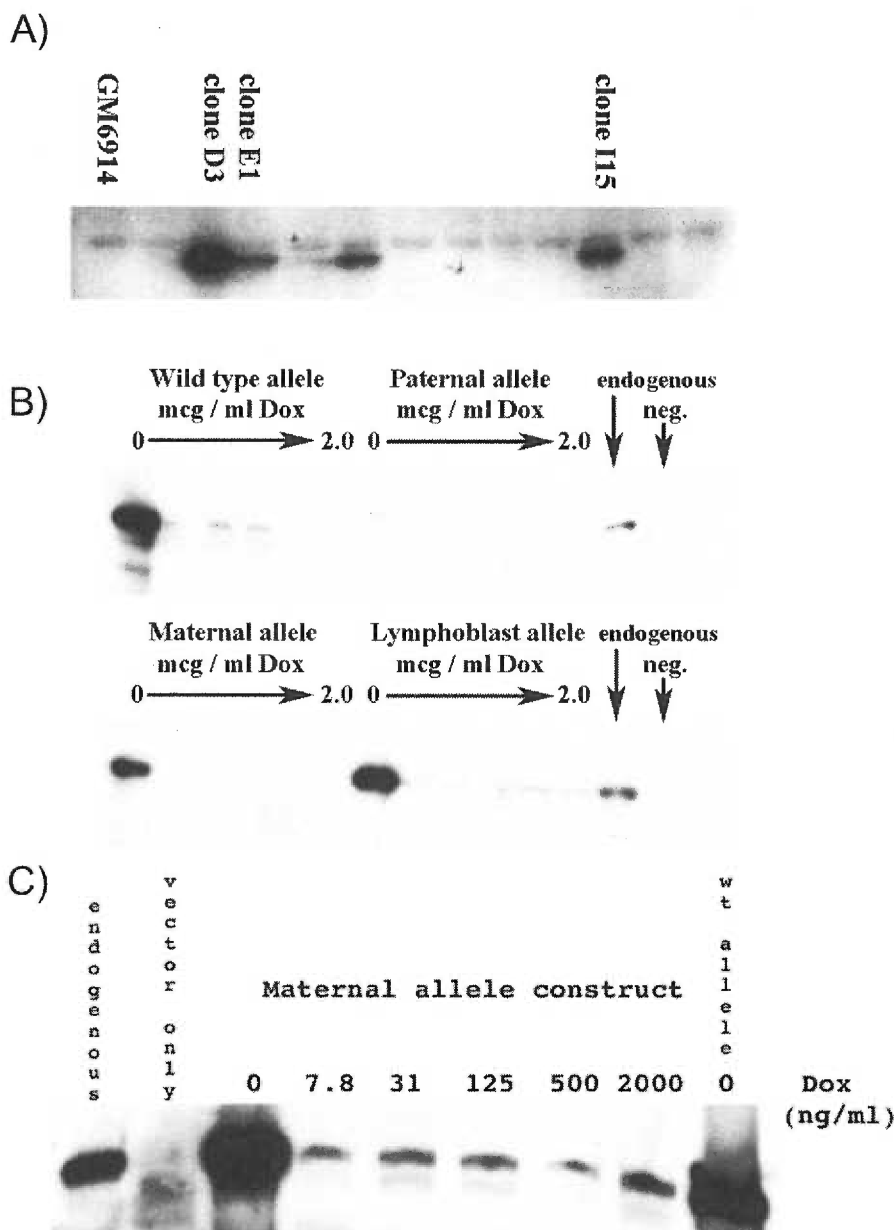
It was hypothesized that the intermediate phenotype seen in the preceding growth and CFA assays might be due to constitutive, high-level expression, common to retroviral vectors. This expression would mask mutations that reduce, but do not eliminate, function (hypomorphic mutations). This hypothesis was supported by the immunofluorescence and immunoblot results. To directly test this hypothesis, the patient-derived alleles of *FANCA* were expressed in a tetracycline-repressible fashion, using subclones of GM6914 cells transfected with pREV-Tet-Off plasmid. The three clones that expressed the highest levels of tetracycline / doxycyclin repressed transcription activator (tTA), referred to as D3, E1, and I15, (Figure 7A) were infected with retroviral

supernatants to produce, for each clone, cell lines expressing wild type, maternal, paternal, and lymphoblast FANCA alleles.

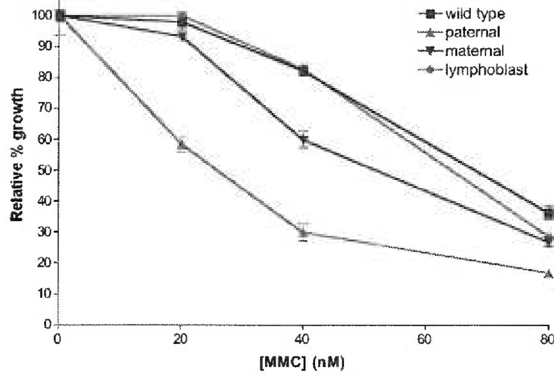
Figures 7B and 7C show the doxycyclin (Dox)-dependent effective repression of *FANCA* expression in three of the four cell lines derived from the clone D3. Since the anti-FANCA antibody used (5G9) bound to a C-terminal epitope, the expression of FANCA off of the paternal allele construct could not be detected. The expression of the maternal allele seemed to reduce more rapidly that of the wild-type or lymphoblast alleles. This was believed to be due to decreased protein stability caused by the R880Q mutation, and this reduction was apparently reversed by the acquired E966K amino acid change.

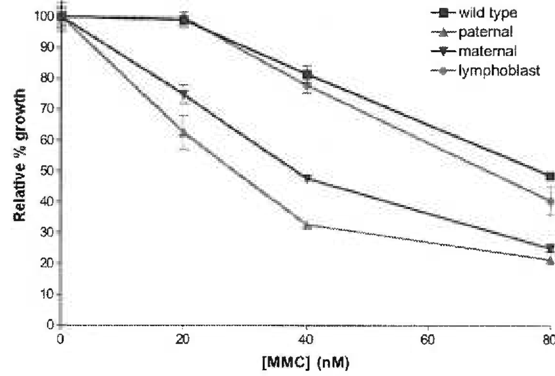
A cell growth assay, using the CyQUANT system, was employed for each set of cell lines, and cell lines within each set were compared. It was estimated, based on pulse-chase experiments reported in the literature[287], that a minimum of 12 hours was required for unregulated levels of FANCA to degrade and Dox-regulated levels to stabilize, so cells were first treated with Dox, then, about 24 hours later, with both Dox and MMC, each at various concentrations. Under conditions with no Dox, the cell lines responded similarly to pMMPpuroFANCA transfected cell lines, but as Dox levels increased, it became apparent that the protein form expressed off of the maternal allele (R880Q) had reduced function, whereas the double-mutant (R880Q + E966K) hematopoietic form demonstrated closer to wild-type function. The results were similar for all three clones. In each case, as concentration of Dox increased, the phenotype of the cell line expressing the maternal allele approached that of the cell line expressing the paternal allele, and diverged from of the cell lines that expressed wild-type or lymphoblast allele, until the Dox concentration reduced expression of wild type *FANCA* to below effective levels. These findings demonstrated that the secondary change found in hematopoietic cells represents a functional reversion of the maternal allele, and that the 2670G>A (R880Q) change is a hypomorphic mutation that can also be compensated for by overexpression. Graphs of percent cell growth, at optimal Dox concentrations for each clone, are shown in Figures 7D-F.

Figure 7: Assays of constructs expressing FANCA alleles under Dox regulation. A) Western blot demonstrating expression of tTa protein, required for activating expression of gene of interest, in four clones. B&C) Western blots demonstrating downregulation of FANCA expression to near endogenous levels in GM6914 e:pREV-Tet-Off clone D3 cell lines infected with pMMPpuroFANCA constructs containing wild type, paternal, maternal, or lymphoblast alleles of FANCA. Paternal allele expression cannot be detected by the C-terminal antibody, as expected. D-F) Growth assays, as measured by quantitation of fluorescence of DNA bound CyQUANT GR reagent, comparing sensitivity to MMC of four cell lines derived from GM6914 e:pREV-Tet-Off clones D) clone D3, no Dox (left) and 500 ng/ml Dox (right) E) clone E1, no Dox (left) and 31 ng/ml Dox (right) F) clone I15, no Dox (left) and 125 ng/ml Dox (right).



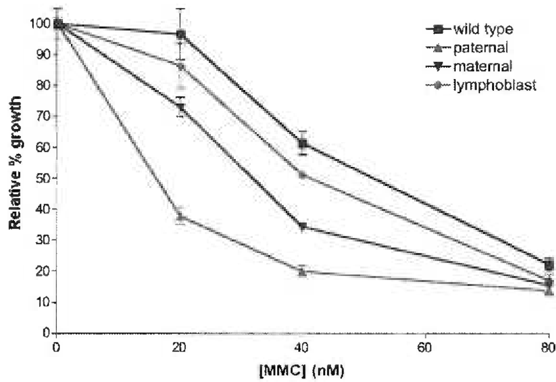
D)

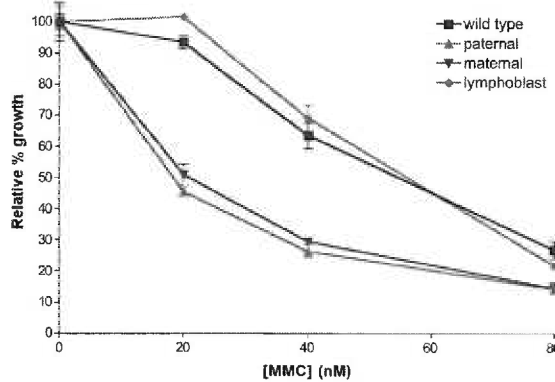




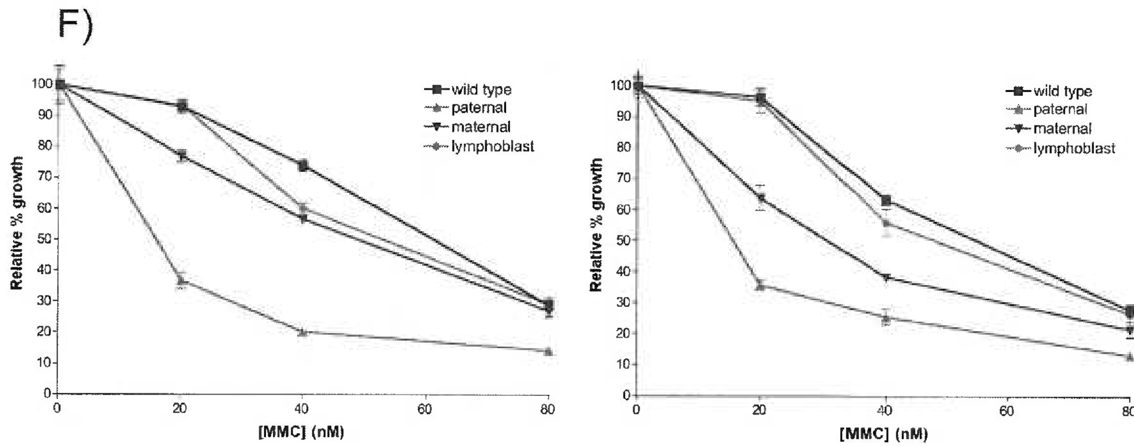
clone D3 cell lines, 500 ng/ml Dox, 40 nM MMC	
One-way analysis of variance (ANOVA)	
P value	P<0.0001
Are means signif. different? (P < 0.05)	Yes
F	155
R squared	0.9831
Tukey's Multiple Comparison Test	P value
wild type allele vs paternal allele	**P < 0.001**
wild type allele vs maternal allele	**P < 0.001**
wild type allele vs lymphoblast allele	P > 0.05
paternal allele vs maternal allele	**P < 0.01**
paternal allele vs lymphoblast allele	**P < 0.001**
maternal allele vs lymphoblast allele	**P < 0.001**

E)





clone E1 cell lines, 31 ng/ml Dox, 40 nM MMC	
One-way analysis of variance (ANOVA)	
P value	P<0.0001
Are means signif. different? (P < 0.05)	Yes
F	51.73
R squared	0.951
Tukey's Multiple Comparison Test	P value
wild type allele vs paternal allele	**P < 0.001**
wild type allele vs maternal allele	**P < 0.001**
wild type allele vs lymphoblast allele	P > 0.05
paternal allele vs maternal allele	P > 0.05
paternal allele vs lymphoblast allele	**P < 0.001**
maternal allele vs lymphoblast allele	**P < 0.001**



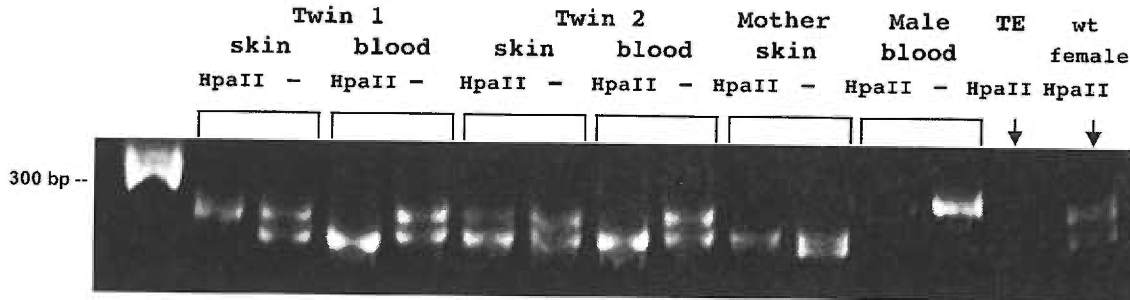
clone I15 cell lines, 125 ng/ml Dox, 40nM MMC	
One-way analysis of variance (ANOVA)	
P value	P<0.0001
Are means signif. different? (P < 0.05)	Yes
F	43.47
R squared	0.9422
Tukey's Multiple Comparison Test	P value
wild type allele vs paternal allele	**P < 0.001**
wild type allele vs maternal allele	**P < 0.001**
wild type allele vs lymphoblast allele	P > 0.05
paternal allele vs maternal allele	**P < 0.05**
paternal allele vs lymphoblast allele	**P < 0.001**
maternal allele vs lymphoblast allele	**P < 0.01**

Secondary mutation acquired in HSC, which repopulated the blood

Since the acquired mutation was found in peripheral blood from both twins, and since these twins were over two decades of age, it seemed likely that the secondary mutation occurred in a hematopoietic stem cell, leading to repopulation of the blood. This hypothesis was confirmed by testing multilineage clonal hematopoiesis using X-inactivation assays and committed progenitor assays.

First, an assay for X-inactivation skewing was undertaken using genomic DNA extracted from fibroblasts and whole blood of both twins. One twin exhibited a greater degree of skewing of X-inactivation in fibroblasts than the other twin, a phenomenon that has been previously reported in monozygotic twins[413-420]. More importantly, the assay demonstrated concordant complete skewing in the whole blood (Figure 8). Specifically, one twin showed skewing of X-inactivation in opposite directions in her blood versus her skin, and X-inactivation in her blood was skewed in the same direction as it was for her sister's blood. This finding suggests that she was repopulated by a hematopoietic precursor cell from her twin sister[414], presumably with the hematopoietic progenitor or stem cell containing the acquired mutation.

Figure 8: Skewing of X chromosome inactivation
Paired PCR products with Hpa II digested and mock digested template shown. Order of lanes: 100 bp ladder, then two lanes per DNA template: fibroblast DNA from one twin, whole blood DNA from that twin, fibroblast DNA from second twin, whole blood DNA from second twin, maternal DNA, Hpa II digested male DNA as digestion control, Hpa II "digested" TE, and Hpa II digested wild type female DNA as non-skewed control

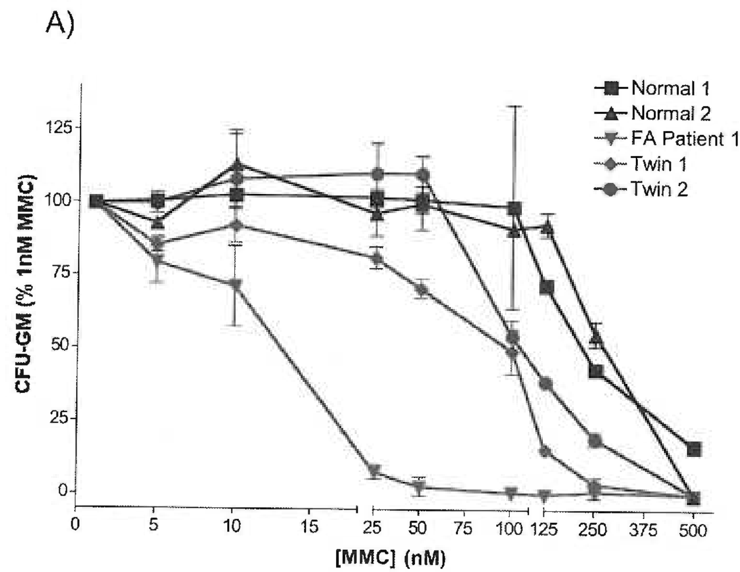


Next, bone marrow was obtained from both sisters, and granulocyte-macrophage colony forming units (CFU-GM) and erythrocyte burst forming units (BFU-E) were plucked and analyzed for presence of the acquired mutation. The acquired mutation was present in 100% of colonies evaluated (180 CFU-GM and 211 BFU-E from one twin, 99 CFU-GM and 96 BFU-E from the other twin). Along with the finding of the acquired mutation in whole blood and in cultured lymphoblasts, this result validates the notion that a multilineage bone-marrow derived hematopoietic progenitor, probably an HSC, acquired the 2927G>A compensatory mutation.

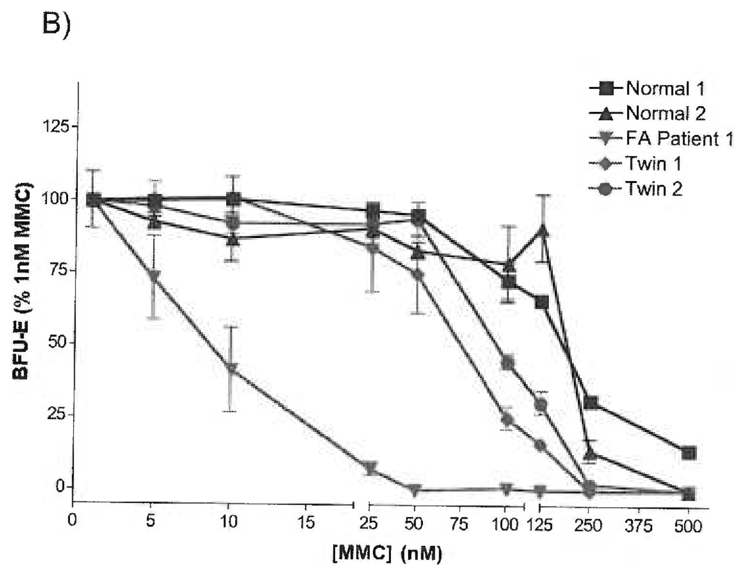
When CFU-GM and BFU-E from both twins were tested for MMC sensitivity, both cell types from both twins demonstrated dose response curves similar to those for wild type CFU-GM and BFU-E (Figure 9). This indicated normal MMC resistance, and therefore functional correction of bone marrow derived hematopoietic stem cells in both patients. In addition, the karyotype for bone marrow from both twins was normal (46 X,X) in 100% of metaphases examined (data not shown).

Figure 9: Clonal growth of hematopoietic progenitor cells
The MMC dose-survival response of bone marrow derived clonal progenitors from one Fanconi anemia patient (FA patient 1), two normal volunteers (Normal 1 and 2) and the twins (Twin 1 and 2) are shown. A) CFU-GM B) BFU-E

A)



B)

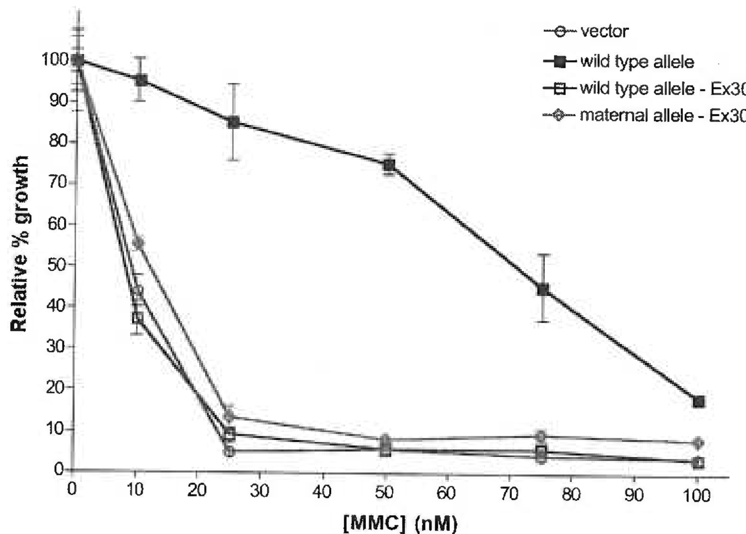


Taken together, these data support the hypothesis that a single HSC in one twin acquired the 2927G>A compensatory *FANCA* mutation, and, via self-renewal, gave rise to more hematopoietic stem cells, which repopulated the bone marrow and peripheral blood of both twins.

Alternative transcripts of FANCA lacking exon 30 do not correct MMC sensitivity.

The R880Q mutation induced partial loss of function of FANCA protein, and this function was restored by the E966K mutation in exon 30. Therefore, further study of the alternative transcripts that lacked exon 30, from both the wild-type and patient fibroblasts, gained importance. Constitutive expression of these transcripts in GM6914 cells, followed by use of the MMC sensitivity CyQUANT-based assays, revealed that neither wild-type nor mutant forms could rescue cells under MMC selection; lack of exon 30 abolished function in regards to MMC resistance (Figure 10). However, by subcellular fractionation Western blot, at least some FANCA protein from these constitutively expressed alternative transcripts could be seen in the nucleus (see Figure 6, lanes 12 & 14). Determination of subcellular localization under Dox-regulated expression or with immunofluorescence may clarify the role of exon 30 in intracellular shuttling of FANCA.

Figure 10: MMC sensitivity assay of transcripts excluding exon 30.
Neither wild type nor G2670A mutation-carrying alleles corrected MMC sensitivity.

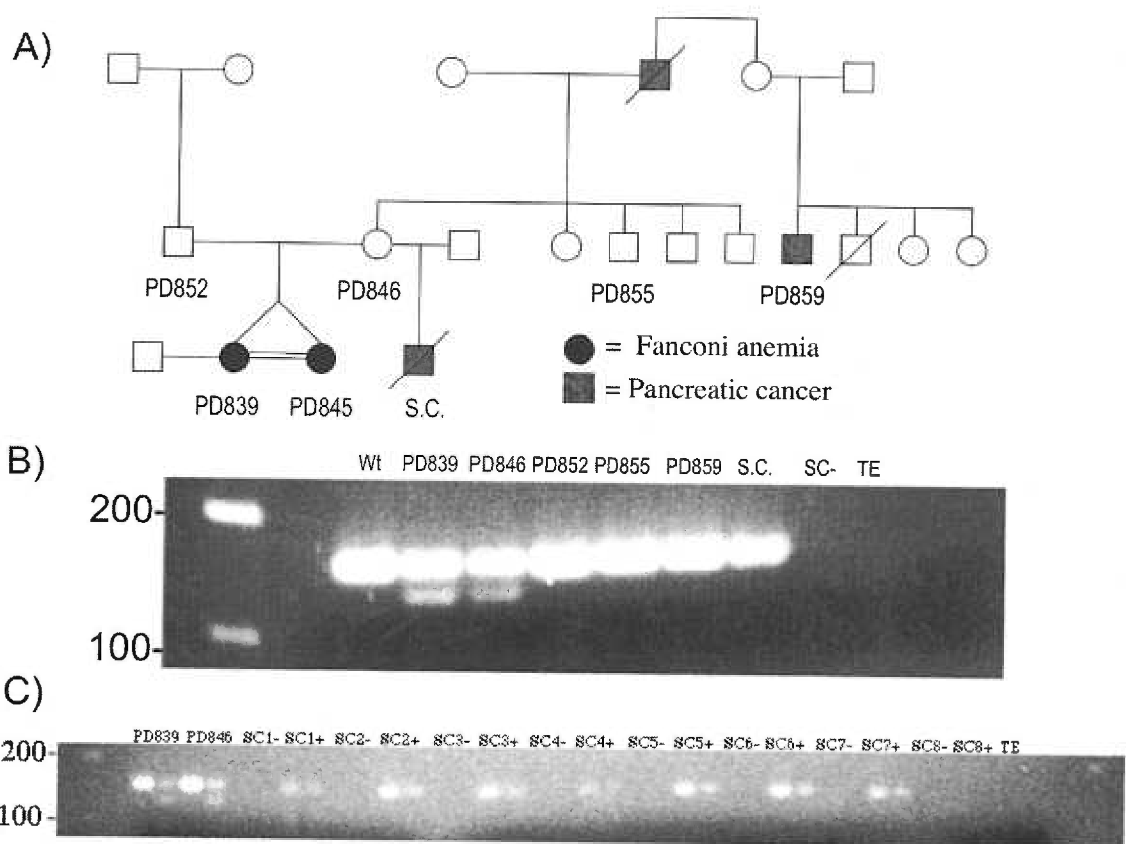


Lack of correlation between mutations of FANCA and pancreatic cancer.

Three members of the extended family, all on the maternal side, had been diagnosed with pancreatic cancer, all at a young (26-30 years) age of onset (Figure 11A).

The presence of mutations in *FANCC* and *FANCG* have been demonstrated in individuals with pancreatic cancer[27,29,362]. It was therefore important to determine whether there was a correlation between the maternal G2670A / R880Q mutation and the occurrence of this cancer. Pancreatic tumor sections, preserved in paraffin, from the deceased half-brother of the proband were obtained, and genomic DNA was extracted from these sections; genomic DNA was also obtained from a sample of whole blood from the surviving cousin of the mother. Upon PCR-digest assay examination of the genomic DNA from these sources, inheritance of the exon 28 *FANCA* mutation from a common ancestor was ruled out (Figure 11 B&C). Therefore, no correlation was found in this family between mutations in *FANCA* and pancreatic cancer.

Figure 11: Assay for correlation between G2670A mutation and pancreatic cancer. A) Complete pedigree, including patients with FA and those with pancreatic cancer. B) PCR-digest assay results for G2670A (exon 28) mutation; FA proband (PD839) and mother (PD846) used as positive controls, father of proband (PD852) for negative control, and direct uncle of proband (PD855) used as maternal relative not affected with pancreatic cancer. Mother's cousin (PD859) and son (S.C.), both diagnosed with pancreatic cancer, do not carry the exon 28 mutation. C) Same PCR-digest assay on multiple paraffin sections (1-8) from tumor tissue (+) or adjacent tissue-free paraffin (-) from individual S.C., half-brother of the twins, who died of pancreatic cancer. Undigested PCR product is paired with digested product from each sample.



Conclusions and discussion:

The case of somatic reversion reported here has important implications regarding the biology of human hematopoietic stem cells and the prospects for successful gene therapy in FA. The identical twins described herein were originally reported as examples of an FA-like syndrome because they had classic features of FA, but nonetheless were hematologically normal and did not display chromosome breakage in peripheral blood. Molecular and functional analyses demonstrate conclusively that the patients in fact have FA, complementation group A, and that they are compound heterozygotes for a previously described 2555delT mutation and a novel G2670A / R880Q mutation. The severity of their non-hematological findings and the in vitro expression studies of the mutant alleles demonstrate the expected loss of *FANCA* function, which, while partially due to mislocalization, may also result from reduced mRNA or protein stability. The fact

that hematopoiesis is normal is due to an acquired compensatory mutation (G2927A / E966K) in the blood cell lineage, in cis with the G2670A / R880Q mutation.

Importantly, both twins have the same single base change in lymphoid, myeloid, and erythroid DNA. No additional blood-specific DNA alterations were found. Repopulation by maternal cells heterozygous for FA was ruled out. This result can be explained in only two ways. First, the reversion could have occurred independently in both twins early in life followed by subsequent strong selective pressure at the stem cell level that resulted in normal hematopoiesis for over 28 years. The second and more likely explanation is that the reversion occurred prenatally in a single HSC, resulting in engraftment of both patients via the shared circulation known to occur in monozygotic twins. The presence of multiple independent reversions has rarely been seen in other cases of mosaicism in FA. Furthermore, unassisted normalization of hematopoiesis for over two decades has also not been formally reported. It therefore appears very unlikely that reversion at the stem cell level is common enough to occur multiple times, independently, in different stem cells of an individual patient. Regardless of whether the reversion occurred once in a prenatal HSC or independently in both twins postnatally, the resource for extremely long-term hematopoiesis – spanning decades of life – consisted of a very low number of HSC. To our knowledge this is the first formal report of unassisted correction of a disease phenotype by oligoclonal hematopoiesis over such a long time period in humans. These findings powerfully demonstrate the self-renewal capacity of human HSC.

Regarding the potential for gene therapy in FA, this case of "natural gene therapy" suggests that functionally corrected HSC can prevent/correct the hematological abnormalities characteristic of this disease. The normal HSC was spontaneously selected to become the dominant clone without the use of any exogenous myeloablative stress. Therefore, correction of only very few long-term repopulating HSC could be sufficient to have significant therapeutic benefit given sufficient time. The time required for successful in vivo selection of normal HSC is not known but it is noteworthy that one of the twins was reported to have some cells with abnormal chromosome breakage at 6 months of age. Spontaneous selection may therefore be a slow process taking years. However, gentle, non-myeloablative selection of corrected HSC has been reported in

murine gene therapy for Fancc[397] and could potentially be used to safely accelerate the selection process, so long as the corrected cells are introduced prior to onset of hematologic symptoms, including anemia, pancytopenia, or clonal karyotypic abnormality[392]. Based on the data presented in this thesis, once the appropriate conditions for safe selection are established, very limited numbers of HSC should be capable of repopulating the bone marrow and blood of an affected FA patient as well as any histocompatible siblings via serial stem cell transplantation.

Chapter 3: Modifications to microcell mediated chromosome transfer

Introduction:

When patients are diagnosed with a genetic disorder for which the gene is unknown, questions arise which must be answered in several successive steps, requiring the use of various techniques. There are cases where linkage mapping of genes related to human genetic disorders may be difficult, such as when there are too few patients and families are too small for linkage analysis. In such cases, the mapping may be accomplished via transfer of wild-type chromosomes into patient cell lines. Somatic-cell or whole-cell hybridization is useful for determining if two patients who are clinically diagnosed with the same cell autonomous, recessive genetic disorder share a common mutated gene. However, localizing a gene of interest, as a first step towards identifying it, is hindered by the fact that informative elimination of chromosomes from whole-cell hybrids requires extensive culturing[421].

A more direct way to map the gene responsible for a disorder is to transfer individual chromosomes, or portions of chromosomes, from a wild-type cell line, such as somatic, embryonal carcinoma, or embryonic stem cells, into a patient-derived cell line. If the transferred chromosome is marked with a cassette that confers antibiotic resistance, the resistance can be used to select for and culture hybrids. These hybrids would in turn be assayed for correction of the recessive clinical phenotype. While cells have been shown to take up chromosomes with which they are co-cultured[422] -- supposedly by phagocytosis[423] -- the chromosomes are often fragmented[424], and thus this method is feasible but inefficient.

A more efficient technique, microcell-mediated chromosome transfer (MMCT), was developed in the early 1970's[425], and it has been employed for several different purposes. Originally used to map mouse genes[426-429], MMCT was adapted for use of mapping of human DNA repair genes, including XPA[430], XPC[431], and XRCC4[432]. It was also used for physical mapping of chromosomes, such as human chromosome 17[433], for identification of tumor suppressor genes, such as p16INK4A[434], and even for studies on genomic imprinting[435].

Certain criteria must be met for MMCT to be considered for mapping a gene involved in a genetic disorder. First, the cellular defect must be corrected by introduction of a single copy of a human gene on a single wild-type chromosome. Therefore, use of this technique is generally limited to autosomal and X-linked recessive diseases. The exception to this rule is that this technique could also be applied to cases where whole-cell hybridization of patient and wild-type cells revealed a dominant negative disease phenotype. In this situation, MMCT could be used, in reverse, to transfer patient chromosomes from an immortalized cell line into wild-type cells. The gene would be localized by assaying for appearance, rather than disappearance, of the defect, following such a transfer. This version of MMCT would require some adjustments to reagent concentrations to properly process human donor cells, as well as random marking of the human cells with selectable cassettes, but it is still feasible.

There are three other criteria for use of MMCT. The second criterion is implied by the first: there must be a testable, cell-autonomous defect, so that hybrids may be assayed directly for loss (or gain) of disease phenotype. Third, both donor and recipient cells must be able to attach to tissue culture plates, although protocols for use of unattached donor cells have been described[436,437]. Finally, the cellular defect must be amenable to the technique. Namely, it should not be eliminated by immortalization of cell lines, which may be required to generate sufficient cells for repeated transfer, or abrogate cell survival under the treatment conditions described below. One must also keep in mind that the resolution of this technique is limited, and, beyond 50 cM or so, other procedures are necessary to further localize the gene of interest.

The MMCT technique has been modified several times. A high-efficiency version of MMCT, which increased the frequency of production of hybrids, has been described[438]. This high-efficiency version entailed several changes from the previous version of the procedure[439]. An MMCT protocol similar to this updated technique was used to localize the gene for Fanconi anemia group D2, *FANCD2,* to human chromosome 3p[336].

While the MMCT procedure used for this localization is a significant improvement over the original technique, certain issues remained unresolved, and, during application of the procedure, various technical difficulties arose. Solving these difficulties

required new insight into the theoretical underpinnings of the technique. One of the most long-standing unresolved issues was contamination of the microcell pool with whole cells, giving rise to unwanted whole cell hybrids after fusion of the pool to recipient cells. Technical difficulties included a failure of colcemid-treated donor cells to flatten out on the substrate to which they were supposed to attach prior to centrifugation. Also, the concentration of colcemid required to micronucleate donor cells, and the circumstances under which cells were treated, had to be adjusted. A reduction in microcell hybrid formation was noted under certain circumstances, and the filtration process had to be altered to correspond to the specifics of the enucleation process in order to correct this. Known difficulty detecting transferred human chromosomes in a human genomic background led to the use of mouse cell donors, but this required production of donor cell lines and development of assays to detect mouse chromosome transfer into human cells. Finally, complications in the use of mouse primary or embryonic stem cells as donors had to be circumvented in order to obtain donors of specific mouse chromosomes. This chapter explains how these issues were addressed and, for the most part, solved.

Materials and Methods:

Cell lines and growth conditions

Microcell donor cell lines were grown in Dubelco's Modified Eagle's Medium (DMEM) with 5% Fetal Bovine Serum (FBS), 1% Penicillin + Streptomycin (P/S), and 2mM L-Glutamine (L-Glut.); recipient cells (human fibroblasts) and microcell hybrids and were growth in Minimal Essential Medium, alpha modification (αMEM) with 15% FBS, 1% P/S, and 2 mM L-Glut. PD319 cells were immortalized patient-derived cells from an individual diagnosed with Fanconi anemia (FA); these cells were obtained from the OHSU FA cell repository. A9 cells have been previously described[440].

Recipient cell preparation

Approximately 18 to 24 hours prior to the fusion, transformed or immortalized recipient cells were plated to 6, 25 mm^2 flasks, 2 each at 1 X 10^5 cells, 2.5 X 10^5 cells, or 5 X 10^5 cells per flask. The pair of flasks, which were about 40% confluent at time of fusion--

such that cells were as spaced as densely as possible without touching--were used for fusion and control, to allow high probability of fusion with microcells while avoiding fusion between recipient cells.

Micronucleation

Donor cells were synchronized by growth to confluence in a 150 mm tissue culture plate, followed by an 8 to 12 hour growth arrest. Cells were then split to 10 150 mm plates and allowed to reenter the cell cycle for 8 to 12 hours prior to treatment with 0.05 μg/ml colcemid for 24 to 48 hours.

Enucleation

Bullet-shaped pieces of tissue culture plates[438] were sterilized by immersion in 70% ethanol and allowed to dry. Colcemid-treated, micronucleated donor cells were trypsinized and resuspended in standard donor cell medium. Cell suspension, at approximately 1 X 10^6 cells/ml, was then evenly spread over one surface of each bullet, 1ml per bullet. The donor cells were allowed to attach to the bullets for up to 10 hours at 37°C, with 6 to 8 hours being optimal for flattening out without detachment.

10 μg/ml Cytochalasin B solution in DMEM, warmed to 37°C, was aliquotted into 50 ml polycarbonate centrifuge tubes, 35 ml per tube. Next, medium was aspirated from each bullet, and the bullets were washed with PBS. One bullet was placed in each centrifuge tube, and the tube was sealed with a friction-fit closure before being placed in a 37°C tissue culture incubator. A centrifuge and an appropriate rotor (i.e. Beckman-Sorvall JA-17) were prewarmed for a half-hour at 34°C (34-37°C range) and 15,000 rpm. Alternatively, one could put the cells on bullets into enucleation medium while the rotor was being prewarmed (C. Jeff Lipps, personal communication), and this was also done when time constraints required it. Centrifuge tubes were placed in the warm rotor, with the cell coated bullet surface oriented towards the center of the rotor, and the tubes were centrifuged at 15,000 rpm for 30 minutes at 34°C.

Filtration to remove whole cells and minicells

Filters containing Whatman 8.0 μm and Whatman 5.0 μm pore size filter discs were assembled by hand in Millipore Swinnex syringe filter apparati and sterilized by autoclaving. After centrifugation, bullets were removed from the tubes with care, so as not to disturb the pellets, and the enucleation media was decanted. The pellets were each resuspended in 1ml serum-free αMEM and pooled. This microcell resuspension was transferred to a syringe with the 8 μm filter unit attached, and the suspension was passed through the filter with minimal force on the plunger. A small sample (20μl) was taken out for analysis under microscope, another (100 μl) for analysis by a Coulter Multisizer machine, and a third 0.5 ml sample was plated to 25 mm^2 flask with complete medium. The filtrate was then filtered in a similar manner through the 5 μm filter unit, and samples were taken for microscopy and Multisizer analysis. The 5 micron filtrate was then centrifuged at 2,000 rpm for 20 minutes at room temperature in a countertop centrifuge, and the pellet was resuspended in 1 ml of serum free αMEM.

Microcell fusion

Fusion was achieved essentially as previously described[336]. Specifically, one ~40% confluent 25 mm^2 flask of recipient cells was rinsed twice with serum-free αMEM, and the 1 ml microcell suspension was added; the flask was rocked back and forth to spread the microcells evenly. Next, 1 ml of a twofold concentrated solution of phytohemagglutinin P (PHA; Diffco catalog #3110-56-4) was added to the flask, to encourage attachment of microcells to the recipient cell surface, and then the flask was incubated at 37°C for 15 to 20 minutes. The PHA solution was aspirated and a 50% polyethylene glycol (PEG; Sigma) solution in serum-free αMEM was added to the bottom of the flask so that it did not immediately touch the cells. Cells were exposed to PEG for 55 seconds while the flask was rocked from side to side to allow even coverage, then the solution was rapidly aspirated out. PEG can be toxic to cells at the concentration used, and while the pretreatment with PHA reduced its toxicity[441], the exposure period was carefully controlled. The flask was gently washed thrice with serum free medium, and, after the last wash, 5 ml of complete αMEM (with 10% Fetal Bovine Serum) was added to the flask before putting the flask in the tissue culture incubator.

Selection for microcell hybrids

About 24 to 48 hours after fusion, the flask of fused cells was split into 5, 150 mm or 10, 100 mm tissue culture plates; parental recipient cells from the paired, unused flask, as well as the donor cell derived 8 micron filtrate, were moved to corresponding plates. Double selection was added to all plates, with one selective agent acting against each parental cell line. Hybrid clones were allowed to grow for 10 days to 2 weeks, or, in rare cases, up to 4 weeks, and then were picked by placing small glass rings, first dipped in sterile, non-toxic vacuum grease, around the clone. Once the grease had formed a seal with the plate, the clone was collected with trypsin and plated to a well of a multi-well plate. Clones were expanded to achieve at least one near confluent 100 mm plate, and then the cells were analyzed for loss of cellular defect, or complementation.

Retroviral infection of mouse cells to produce donor cell lines

Mouse A9 cells were infected with the pFAH-G15 based retrovirus, which contained both a neomycin resistance cassette and the complete cDNA for the human fumaryl acetoacetate hydrolase (FAH) gene[442,443]. Retroviral supernatant was applied to 50% confluent A9 cells for four hours, along with 4 μg / ml polybrene (Sigma), before changing the media and growing the cells under 500 μg / ml G418 selection.

Electroporation of mouse cells to produce donor cell lines

For each electroporation, one plate of 80% confluent A9 cells were trypsinized, washed with PBS, and resuspended in 2.4 ml Cytomix[408]; 75 μg of neomycin resistance cassette containing plasmid, previously linearized with Not I, was mixed into the cell suspension, and the mix was divided into three equal volume portions. Each portion was electroporated separately (BioRad GenePulser electroporator set at 300V and 960μF), and mock electroporations were also carried out. Electroporated cells were plated to 3 plates per electroporation, and after 24 hours, grown under G418 selection until mock electroporation control cells died off.

Production of mouse tail fibroblasts

A ~ 1 cm tail section from one mouse was finely minced with a surgical scalpel and digested at 37°C in, sequentially, 4 mg/ml collagenase / dispase solution in DMEM and 0.5 mg/ml collagenase in DMEM, for approximately 1/2 hour each. Further digestion was inhibited by addition of DMEM medium containing 5% Fetal Bovine Serum (FBS). Solid material was collected and plated to a 75 mm^2 flask, and cells were allowed to attach to the flask surface and spread out. Further culturing, in 150 mm plates, and under G418 selection, used complete DMEM medium containing 15% FBS.

Fusion of mouse knockout fibroblasts and A9 cells

Equal numbers of primary mouse tail fibroblasts and A9 cells were co-plated, at ~50% overall confluence, to a 25 mm^2 flask, in complete medium, and allowed to attach overnight. Medium was removed, and the flask was washed with serum-free medium. A 50% PEG solution was applied as described for the microcell fusion, and, after aspiration, the flask was washed thrice in serum free medium. Complete donor cell medium was added. After overnight incubation, cells were moved to 150 mm plates, and cultured under G418 selection until parental cell controls died—A9 cells dying of G418 toxicity, and primary knockout fibroblasts, of senescence.

Genomic DNA extraction

Genomic DNA was extracted as previously described[405]; briefly, cells were digested overnight, at 56°C, in lysis buffer (3ml 10 mM Tris-HCl / 400 mM NaCl / 2mM EDTA + 0.5 ml 1% SDS / 2mM EDTA + 0.2 ml 10% SDS) with 1 mg/ml proteinase K, proteins were salted out, and DNA was precipitated with ethanol before being spooled out on the end of a closed Pasteur pipette.

FAH PCR

A 296 bp product was amplified using the primers 5' TGTACTGGACGATGCTG 3' and 5' GGACGAGAGGACGGTAGTACT 3' in the following mix: 2.5 µl 10X PCR Buffer with 1.5 mM $MgCl_2$ (Roche) + 0.2mM each dNTP mix + 0.2µl Taq polymerase (Roche)

+ 100 ng each primer + 100 ng genomic DNA + PCR dH_2O to 25µl. Stratagene RoboCycler Gradient 96 thermocycler program was 1 cycle of 94°C for 2 min., 48°C for 25 sec., and 72°C for 30 sec., then 37 cycles of 94 °C for 25 sec., 48 °C for 25 sec., and 72 °C for 30 sec., followed by final extension at 72°C for 2 minutes.

FAH Southern blot

Genomic DNA samples were digested with Pst I and run on a 1% agarose gel before being transferred to Hybond N+ membrane (Amersham) using NaOH. FAH cDNA was excised with EcoR I and Pst I from pFAH-G15, radiolabled using the Random Primed DNA Labeling Kit (Roche), and used as probe. Autoradiographic film was exposed to the membrane for 2 days before developing.

B2 Repeat PCR

This PCR was similar to ones previously described[444,445]. PCR mix per reaction consisted of 2.5 µl 10X PCR buffer + 1.0 µl 200 ng/µl primer: 5' TCTTCTGGAGTGTCTGAAGA 3' + 0.2 µl 100mM dNTP Mix + 0.2 µl Taq + 50ng/µl genomic DNA + PCR H_20 to 25 µl. The Stratagene RoboCycler Gradient 96 thermocycler program was set for 94°C for 4 min., then 40 cycles of 94°C for 1 min., 62°C for 48 sec., and 68°C for 5 min., followed by final extension at 68°C for 7 minutes.

B2 Repeat PCR Southern blot

The Southern blot was performed in a manner similar to FAH Southern blot, except that labeled sheared genomic DNA from ANM2 cells was used to probe PCR products from B2 repeat PCR, which was performed on genomic DNA extracted from hybrid clones.

Use of B2 repeat PCR products for characterization of transferred material

Amplified products from B2 repeat PCR of genomic DNA from a clone were ligated into the pCR4-Topo vector (Invitrogen) according to the TA Topo Cloning Kit (Invitrogen) protocol, and sent for sequencing to the Microbiology and Molecular Biology Sequencing Core. Sequences returned were modified to exclude vector sequence, and then compared to the Celera Mouse Genomic DNA Database (courtesy of Dr. Syndi Timmers).

Cytogenetic analysis

Cytogenetic analysis of mitomycin C (MMC; Sigma-Aldrich) and diepoxybutane (DEB; Sigma-Aldrich) sensitivity by chromosomal breakage (DEB) and radial formation (MMC) assay was done as previously reported[131,267,402,403]. Mouse chromosome paint and G-banding was done as previously described[403].

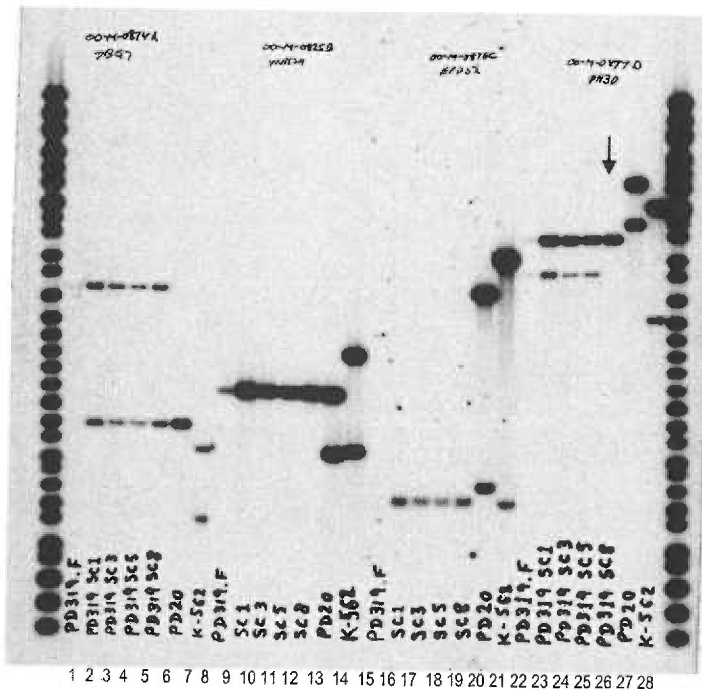
Results:

Additions to preparation of recipient cells.

Recipient cells were plated about 24 hours prior to fusion. One minor change that was made to prior protocols was to place these cells under the selective agent to which they were resistant (but to which the donor cells were sensitive), to "prime" them. Hybrids from primed cells were somewhat less stressed initially than if both selective agents were added to hybrids from unprimed recipient cells.

In addition, it was discovered that the recipient cells must be carefully tested prior to MMCT for sensitivity to the second selective agent. With one recipient cell line, PD319, it was discovered after fusion that, while the recipient cells were generally sensitive to the selective agent, either contaminating cells or individual subclones of the patient-derived cells were resistant to neomycin. These clones were indistinguishable from hybrid clones, invalidating the experiment. To overcome this issue, subclones had to be picked. Subclones (PD319 SC1) that were sensitive to both G418 and to mitomycin C — the agent used to assay for the cellular defect—were then further tested by variable number tandem repeat Southern blot to demonstrate a match to patient-derived primary fibroblasts (Figure 1). The Southern blot was performed as previously described[446].

Figure 1: Southern blot of PD319 subclones.
Note that SC8, which was the most neomycin-resistant clone, is missing one band for one VNTR (arrow). PD319.F (lanes 1, 8, 15, and 22) is the primary, patient-derived fibroblast control, SC1, 3, 5, and 8 are subclones of the immortalized PD319 cell line, PD20 (lanes 6, 13, 20, and 27) is a non-PD319 cell line control, as is K-562 (lanes 7, 14, 21, and 28).



Optimizing micronucleation of the donor cell line.

Microcell-mediated chromosome transfer is a multistep process. The first step is the micronucleation of donor cells. Various donor cells can be used, and several examples are given in the second half of the chapter. Colcemid (Sigma, catalog # D-7385 or D-6279), also known as demecolcine or N-Deacetyl-N-methylcolchicine, is the micronucleating agent. Colcemid works by preventing polymerization, or, at higher concentrations, by inducing depolymerization, of microtubule fibers; the specific microtubule fibers targeted for the micronucleation process are those of the mitotic spindle. Without proper spindle formation, cells going through mitosis cannot segregate their chromosomes appropriately, so rather than the two sets of chromosomes distributing evenly and ending up at opposite poles of the nuclear region during karyokinesis, the chromosomes end up scattered, and the cells enter the mitotic checkpoint and arrest[447]. Eventually, however, the cells surmount the checkpoint to enter an abnormal telophase. In this phase, instead of forming two new nuclear envelopes, one around each set of chromosomes, several nuclear envelopes form, each surrounding one or a few chromosomes[448].

In this project, several randomly marked mouse cell lines, produced in the laboratory, were used as chromosome donors. The concentrations of colcemid required to effect the cellular process described above depends upon the donor cell line used, although the overall effects of colcemid on these cells are generally the same. Different concentrations of colcemid were tested, for various lengths of time, on one of the donor cell lines, ANM01 cells (described below). The results are shown in Table 1. It was noted that, while the percentage of micronucleated cells to total cells increased at all concentrations over time, the amount of cell death did as well. For example, at 96 hours, 0.04 μg/ml colcemid resulted in a higher percentage of micronucleation of living cells than did 0.03 μg/ml, but the amount of cell death reduced the number of useful micronucleated cells relative to 0.03 μg/ml colcemid at that time point. In addition, colcemid concentrations at or greater than 0.07 μg/ml were disadvantageous, causing excessive cell death within 24 hours. Cells adapted to concentrations at less than 0.01 μg/ml, such that micronucleation did not occur or was quickly lost. It was also observed, as listed in Table 1, that 5 to 6 days of treatment in 0.02 μg/ml colcemid provided the highest percentage of micronucleated cells; this was only the case in early passage cells, however, and as the original population of cells was expanded through passaging, the cells became resistant to this concentration of colcemid.

Table 1 : Micronucleation curve for ANM01 cells

Colcemid concentration (μg/ml):	Hours treated:	% cells micronucleated
0.05	24-48	~50%
0.04	72	~60%
0.03	96	70-75%
0.02	120-144	80-90%

One way to increase the micronucleation efficiency would be to use synchronized cells (personal communication, Dr. Matt Thayer), since micronucleation occurs in mitosis, and, if a large percentage of cells entered mitosis simultaneously, then colcemid treatment could affect the most cells in the least amount of time. This was tested by synchronizing cells by confluence-induced growth arrest in medium containing 5% Fetal Bovine Serum (FBS), and then splitting the cells to 10 plates (in the same media) before

adding colcemid. Incidentally, use of higher than 5% FBS was found to confer some resistance to colcemid, presumably by accelerating the cell cycle. Relative to unsynchronized cells, the percent of micronucleated cells, under 0.05 μg/ml colcemid, did increase, as observed under phase-contrast microscopy, as did the degree of micronucleation -- that is, the number of cells with many, very small micronuclei, rather than fewer, larger micronuclei. The former is desired, as smaller micronuclei are indicative of fewer chromosomes in each micronucleus. The increase in degree of micronucleation had also been observed under 0.02 μg/ml colcemid for 6 days, but treating synchronized cells with 0.05 μg/ml colcemid worked even on cells that had been passaged several times. It was also observed that optimal micronucleation -- around 70% of all cells -- occurred at 36 to 40 hours post-treatment, rather than 6 days after treatment. Therefore, this methodology was adopted.

Initiating donor cell enucleation: refining the use of bullets.

Once donor cells have been micronucleated, they must be enucleated to produce microcells. This part of the MMCT technique is a multi-step procedure, and it is with these steps that the significant portion of modifications to the MMCT technique were made, beginning with the substratum on which the micronucleated cells were re-plated.

In the first step of the enucleation process, the micronucleated cells are plated to "bullets", or pieces of tissue culture plate material cut and shaped to precisely fit inside a 50ml centrifuge tube, with one semicircular end and one flat end[438]. Previous protocols[336] recommended that the bullets be coated with a chemical crosslinker, followed by concanavalin A (Con A), in order to crosslink the Con A to the bullets and allow it to attach to the surface molecules of the cells. However, it was found that, with reuse of a limited set of bullets, which are difficult to produce, repeated application of Con A apparently increased concentrations of this compound to toxic levels, and actually interfered with cell attachment to, and flattening out on, the bullet surface. Therefore, colcemid treated, micronucleated donor cells were plated directly onto untreated bullets, 1 ml of 1 X 10^6 cells/ml cell suspension per bullet. Addition of more media to the plates permitted the bullets to float and overlap, scraping cells off of each other, and is therefore not recommended.

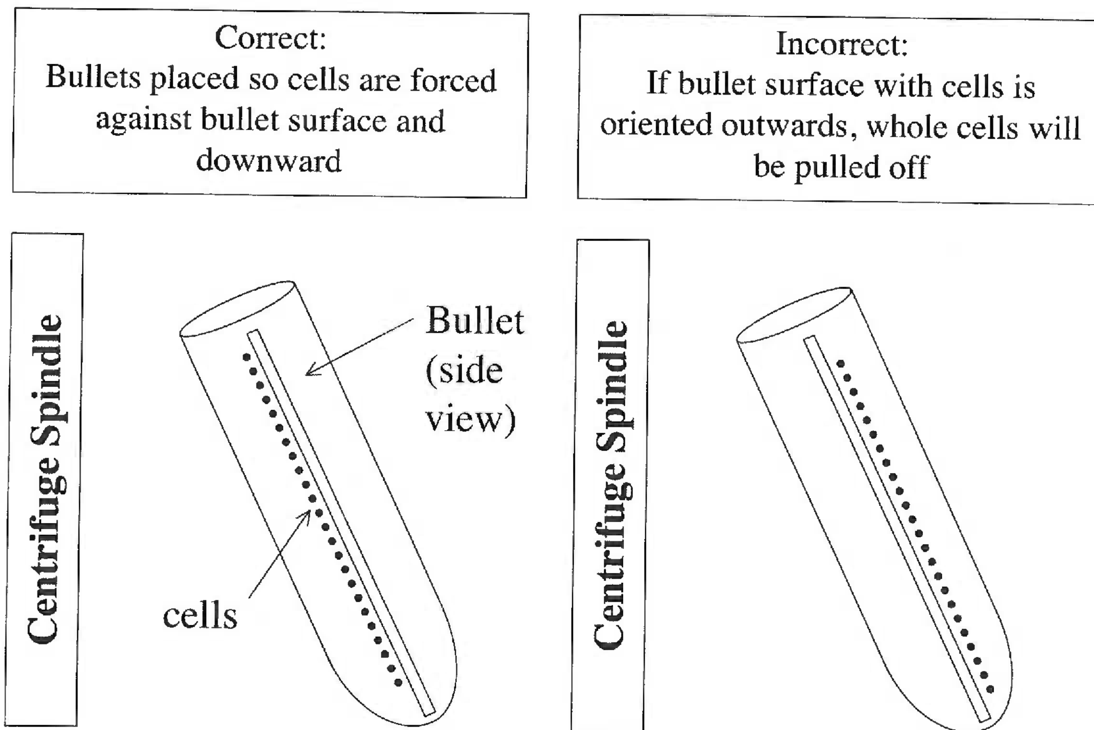
After 8 hours, on average, to allow cells to attach and flatten, cells on bullets were enucleated using a 10 μg/ml cytochalasin B solution, referred to as enucleation medium. Cytochalasin B acts to induce depolymerization of the actin cytoskeleton. Without this cytoskeleton, the micronuclei become essentially free floating particles in the cytoplasm. Centrifugation forces the micronuclei through the plasma membrane, in such a way as to form microcells, or micronuclei surrounded with portions of the cytoplasm and plasma membrane[425,449]. These microcells are very small, on the order of 2 to 5 microns in diameter. Between 60 and 90 % of the cytoplasm is lost in the enucleation process, so that the microcells by themselves can not survive long in culture[425].

Removal of whole cells and minicells.

In addition to producing microcells, however, this process can also produce minicells, with intact nuclei and a substantial portion of the cytoplasm[449]. Loosely attached whole cells, whether micronucleated or not, can also come off and collect in the centrifugation pellet. Indeed, several publications refer to contaminating whole cells in the pellet[438,450,451]. These minicells and whole cells not only clog filters, but, if they are not completely removed by filtration, they can fuse to the recipient cells, forming whole cell hybrids. When mouse fibroblast-derived cell lines are used as donors, the whole-cell hybrids take on both the morphology and rapid growth characteristic of the mouse cells, and the resulting clones can take over a culture plate to the extent of crowding out microcell fusion hybrids or making the latter harder to pick for expansion purposes. This occurrence was observed several times early on, before several changes, described below, were made to the MMCT protocol to deal with this significant nuisance.

To begin with, after aspirating media from bullets, but prior to placing them in centrifuge tubes filled with enucleation medium, bullets were washed with PBS, which removed a significant proportion of loosely attached whole cells. Furthermore, only one bullet was placed in each centrifuge tube, and the tubes were placed in the centrifuge such that each bullet surface coated with cells was oriented to face the center of rotor, as shown in Figure 2.

Figure 2: Placement of tubes with bullets in centrifuge.



The latter change was a significant modification from previous protocols and publications, in which two bullets were placed back-to-back in the tubes. In this configuration, the cell-coated side of one bullet faced the centrifuge spindle, while that of the other bullet faced the outside of the rotor. It was discovered that, after centrifugation, only one of these two bullets retained the "cell ghosts" indicative of properly enucleated cells. Apparently, cells from the other bullet came off whole, contributing to the whole cell contamination in the pellet. A subsequent experiment, with six tubes, one bullet per tube and three bullets oriented in each direction, indicated that the ghosts appeared on bullets oriented with the cell-coated surface facing the center of the rotor, such that the cells were pressed against the bullet when the force of the centrifuge was applied. Once all bullets were oriented this way, the amount of whole cells in the pellet dropped dramatically, and virtually no whole-cell hybrids were produced. Thus, this orientation was adopted as a modification of the MMCT protocol. Since the bullet can occasionally rotate slightly as the rotor reaches proper speed, it should be noted that opaque pellets larger than 5 mm in diameter were observed to contain more whole cells, while slightly

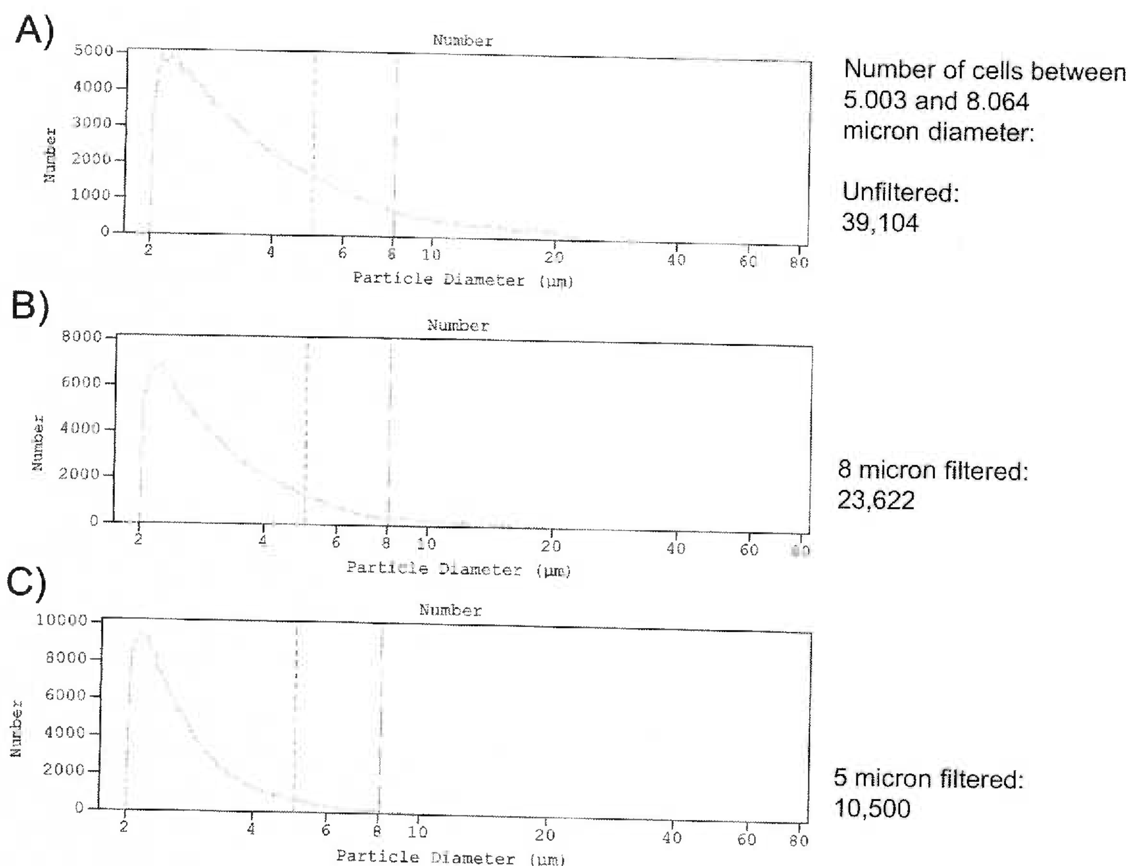
translucent, 3 to 5 mm diameter pellets had a more favorable microcell-to-whole-cell ratio.

Tailoring filtration and fusion of microcell pellets to the experimental set-up.

Despite improvements made to the procedure, filtration was still required to remove some whole cell and large minicell contamination from the microcell pellet. Filtration is recommended instead of unit gravity sedimentation to simplify the purification process, even though it was found to be less effective[438]. However, there is significant potential for loss of all microcells if filtration is done incorrectly. Therefore, it is important to understand how enucleation and filtration are interrelated and to employ the enucleation process-specific filter set.

Two stages of filtration, 8 micron followed by 5 micron filtration, were required to ensure that whole cells and minicells were removed without clogging the 5 micron filter. In the procedure described here, filtration was performed using either a combination of hand-built filters, containing Whatman 8.0 μm and Whatman 5.0 μm pore size filter discs in Millipore Swinnex syringe filter apparati and sterilized by autoclaving, or a combination of hand-built 8.0 μm filter followed by manufactured 5.0 μm syringe filter. In each case, a 100 μl sample of filtrate was analyzed, at each step, by a Coulter Multisizer machine. Examples of Multisizer profiles can be seen in Figure 3.

Figure 3: Microcell size profiles by Coulter Multisizer.
A) unfiltered microcell suspension B) 8 micron filtrate C) 5 micron filtrate. Example values of counts for microcells and minicells between ~5 and ~8 microns in size, from one MMCT run, are stated to indicate effectiveness of filtration.



The 5 micron filtrate was pelleted and resuspended in 1ml serum free medium, and then was fused to one of the two ~ 40% confluent 25 mm^2 flask of recipient cells. Between 24 and 48 hours after fusion, the cells used in fusion were split to several plates and grown under double selection. When clones grew out, usually after 10 days to 2 weeks, they were picked, expanded, and analyzed for correction of cellular defect.

When manufactured 5.0 µm syringe filters were used for filtration, a significant drop in production of microcell hybrids was observed. This was especially true when the bullets had been placed in enucleation medium while the rotor was prewarming. Consideration of this correlation revealed a possible explanation for the reduction in the number of hybrid clones produced. The amount of time required to fill the centrifuge tubes with enucleation media, place all of the bullets in the tubes and seal the tubes, allowed at most 15 minutes incubation time for the cells in the cytochalasin B containing medium if this was done while the rotor was warming; in contrast, setting the rotor to prewarm after the bullets were placed in the tubes allowed a full 30 minute incubation.

The shorter amount of time was apparently sufficient to break down the actin cytoskeleton enough to allow the microcells to be pelleted, but not enough to depolymerize all of the actin filaments. Some longer actin filaments must have remained associated with the micronuclei. As the micronuclei tore through the plasma membrane during centrifugation, the longer actin filaments ensnared more membrane and cytoplasm, resulting in slightly larger microcells.

The hand-assembled filters were autoclaved; as a result of the heating during autoclaving, the pores may have expanded slightly, from 8 and 5 micron diameter pores to 10 and 6 micron ones, respectively. The larger pore would allow the larger microcells to pass through, while still removing whole cells. This hypothesis was born out by the fact that, when the filtrate from the 8 micron filters was analyzed using a Coulter Multisizer, a portion of the particles were larger than 8 microns in size (Figure 3B). In contrast, the manufactured syringe filters were sterilized by gamma irradiation, which may not heat the filter material sufficiently to increase the pore size, so the filters retained the larger microcells.

Based on this hypothesis, and on the abovementioned drop in hybrid production efficiency, it is strongly recommended that the cells on bullets be placed in enucleation medium and that the centrifuge tubes be placed in the incubator prior to setting the rotor to prewarm, to encourage production of smaller microcells. If time allowed for incubation is constrained, hand-built autoclave filters should be favored over manufactured ones in order to ensure that larger microcells are not lost to the filter.

Successful transfer of human chromosomes.

By definition, the critical stages of microcell mediated chromosome transfer are micronucleation of and microcell formation from donor cells. Several different cell types can be used as donors, including both human and mouse fibroblasts, as well as mouse embryonic stem cells. Human fibroblasts require much higher levels of colcemid to induce micronucleation than do mouse fibroblasts[451]. Therefore, routine transfer of human chromosomes for purposes of gene mapping could best be accomplished by using various mouse cell lines, each containing a single, selectably marked, human chromosome, transferred into the mouse cells by previous MMCT, as donors. While

various groups have produced or attempted to produce such cell lines[452-455], a particular set of A9 cell lines, in which a fusion hygromycin phosphotransferase – thymidine kinase (HyTK) cassette was used to mark the human chromosome[456], was determined to be the optimal source for human genetic material, mainly because hygromycin resistance is not an endogenous mammalian phenotype and does not commonly develop in cultured cells. These cell lines were referred to as A9-N HyTK cells, where N was the number of the human chromosome present in the cell line. One application of the modified MMCT protocol involving one of these cell lines, A9-13 HyTK, has already been published[30]. The FA-D1 cell line VU423 was shown to be complemented by the *BRCA2* cDNA[30], and *BRCA2* resides on human chromosome 13[263,264]. Transfer of this chromosome from A9-13 HyTK cells into VU423 cells confirmed the cDNA finding[30], since multiple hybrid clones were complemented. Analysis of one such hybrid clone, VU423-h13 clone I3, is shown in Table 2 (data courtesy of Dr. Yassmine Akkari, Cytogenetics).

Table 2: Cytogenetic evidence for correction of VU423 cell line.
Clone I3 shows correction with human chromosome 13. Uncorrected clone J1 is also shown for comparison

VU423-h13 Clone	Clastogen	Conc ng/ml	Number Of radials	1 radial per cell	more than 1 radial per cell	Percent radials	Total number of cells
I3	MMC	40	8	7	1	16%	50
J1	MMC	40	26	16	10	52%	50

Rationale for mouse chromosome transfer.

Transfer of mouse chromosomes instead of human one was also undertaken. The transfer of mouse chromosomes into patient cell lines to map genes defective in human cells has advantages and disadvantages. While the use of A9-N HyTK cells allows for transfer of specific, predetermined human chromosomes, establishing the location of a gene involved in an autosomal recessive disorder might require up to 22 separate fusions. In contrast, randomly marked mouse cells provide better genomic coverage; even with only one chromosome marked per cell, all chromosomes should be represented in a population of such cells. Theoretically, each donor cell in a population would provide

unique chromosome content, although, in practice, certain mouse chromosomes might be transferred or retained at a higher frequency than others.

While the random nature of transfer does require a determination of which mouse chromosome is retained in any complemented clones, this is not a significant disadvantage. On the contrary, species differences greatly simplify detection of successful transfer of mouse chromosomes into a human genomic background, as well as detection of translocations and deletions in chromosomes, the latter of which can be exploited to further localize the gene of interest. Three methods for detection of successful transfer of mouse chromosomes into patient cell lines are described below.

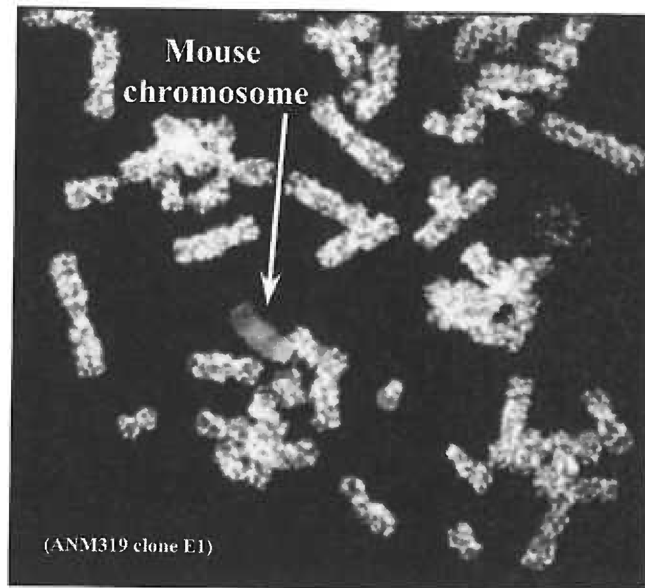
One major issue with attempted functional complementation with a mouse chromosome is the requirement for correct interaction between the mouse homolog to the defective human gene product and other members of the appropriate genetic pathway, which are all human proteins. Unfortunately, this does not always occur. However, if the mouse gene does phenotypically correct the patient cell line, then use of parallel human and mouse chromosome transfer, along with synteny mapping, can be used to narrow the region of localization of the gene of interest. Hence, randomly marked mouse cells could be effective MMCT donors.

Production of randomly marked mouse chromosome donors.

Mouse cells were randomly marked, by retroviral infection, with a cassette that conferred neomycin resistance, and then used as mouse chromosome donors. The retroviral supernatant used was previously produced[442] from a plasmid with both a neomycin resistance cassette as well as the complete cDNA for the human fumaryl acetoacetate hydrolase (FAH) gene. Two separate cell lines were produced this way; ANM01 cells were labeled by infection with 1:10 diluted supernatant, while ANM2 cells were marked by twice infecting with full concentration supernatant.

The former cell line was successfully used as a mouse chromosome donor. For instance, transfer of a single, mostly intact mouse chromosome from the ANM01 cell line into the PD319 SC1 subclone, via the modified MMCT technique described above, occurred for at least one clone (Figure 4; courtesy of Carol Reifsteck).

Figure 4: MMCT hybrid clone E1.
MMCT hybrid clone E1 has a single, presumably intact mouse chromosome in a human genomic background, indicative of proper mono-chromosome transfer.



In spite of moderate successes with the retrovirally infected ANM01 cell line, it was found that use of retrovirally marked mouse donor cells should be avoided. Infection of human cells with retroviral components – without chromosome transfer – gave rise to non-hybrid neomycin resistance clones, as described shortly. While electroporation could result in multiple integrations per cell or integration of concatomers, the chances of transferring selection resistance without also transferring mouse chromosomes would be eliminated. Therefore, mouse chromosome donors ANM3 and ANM4, were produced by electroporation with pGKneo or pcDNA3 (Invitrogen), respectively, both of which contain neomycin resistance cassettes. Resulting cell lines were also successfully used as microcell donors (Dr. Barbara Cox, personal communication).

Development of assays for transfer from randomly marked mouse cells.

Because of the presence of the human FAH cDNA in the ANM01 and ANM2 cell lines, a simple PCR was used to assay for transfer of the marked mouse chromosome after MMCT with these donors. This PCR specifically amplified a 269 bp product from the FAH cDNA in the integrated retrovirus, but could not amplify from genomic DNA (Figure 5A).

Clones that grew up after MMCT between either of these two donors and PD319 SC1 recipient cells were sent down to the Cytogenetics research facility. It was found (courtesy of Carol Reifsteck, Cytogenetics) that most of these clones did not have detectable mouse material (data not shown). However, when genomic DNA samples from donor control cells and from several clones were assayed for presence of FAH by Southern blot, the results confirmed presence of human FAH cDNA in at least some of the clones which were negative for mouse chromosomes according to cytogenetic data (Figure 5B).

Therefore, a more direct method of detecting mouse chromosome transfer had to be formulated that was not specific to any particular chromosome. Mouse B2 repeats[457] are ubiquitous, common, and bidirectional in the mouse genome, and, as such, can be used to amplify fragments of genomic DNA between repeats[444,445]. A "B2 repeat PCR" assay was developed. When the abovementioned clones were subjected to B2 repeat PCR followed by mouse genomic Southern blot of the PCR products, it was found that the clones could be sorted into three categories.

One clone, A1, which came up positive for mouse material by cytogenetic evaluation, also came up positive by B2 repeat PCR and Southern blot, and the extensive smear indicated that substantial material was transferred (Figure 5C, lane 1). This demonstrated the validity of this assay method. Other cytogenetically negative clones (e.g. C2, lane 13) showed single bands by Southern, indicating that in these clones, fragments of mouse DNA were transferred that were smaller than the cytogenetic detection limit, and implied that shearing of mouse chromosomes and integration of small fragments of mouse genome into human cells could occur during MMCT. Many other clones, however, that were positive for the FAH cDNA by PCR were negative by B2 repeat Southern; in these cases, it seemed that the retrovirus may have integrated into mouse donor chromosomes, but was subsequently reactivated by endogenous retroviral genes in the mouse genome and "hopped" into human cells during the MMCT process. The B2 repeat Southern results for these clones are shown in Figure 5C.

Figure 5: Attempts at detection of mouse chromosome transfer.
A) FAH PCR indicates transfer of FAH cDNA to all clones listed B) FAH Southern blot indicates that only some clones have integrated FAH cDNA C) B2 Repeat PCR Southern

indicates only clone A1 (lane 1) has extensive mouse chromosomal material, whereas clones C2 and C3 (lanes 13 and 14, respectively) have only small fragments of mouse chromosome.

A)

B)

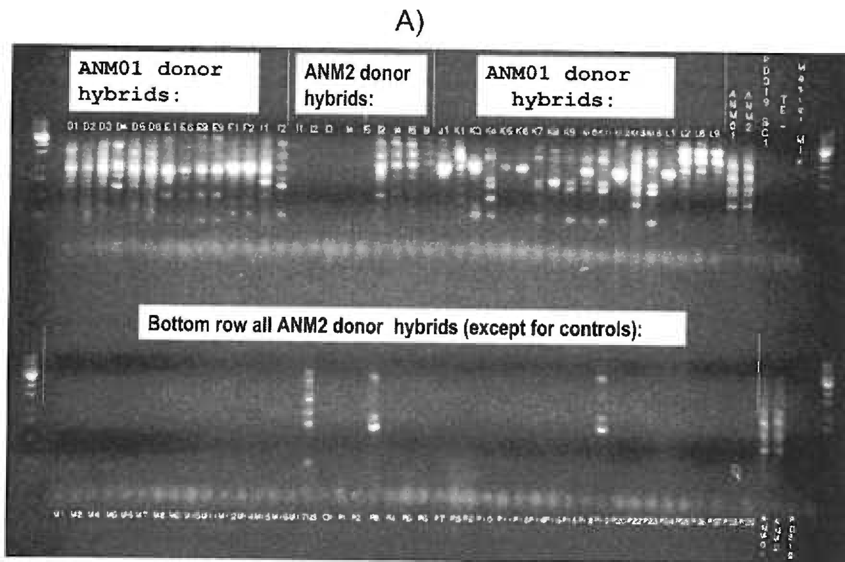
C)

When more MMCT clones were analyzed by FAH PCR and B2 repeat PCR, it was observed that almost all hybrids generated using ANM01 donor cells had mouse genomic material, while those from ANM2 donor cells generally did not (Figure 6A), even though ANM2 donor hybrid clones came up positive for the FAH cDNA (Figure 6 B). To follow up this finding, serum free medium was used to replace normal medium on plates of ANM01 and ANM2 cells, and then those media, after 0.45μm filtration, were used to infect human cells as previously described[281,407]. Plates of human cells were also treated with retroviral supernatant with known effectiveness or with serum free, virus-free medium, as controls. Clones grew in plates treated with retroviral supernatant and ANM2 supernatant, but not in plates treated with virus-free medium or ANM01 "supernatant". This indicated that infection with retrovirus to randomly mark mouse cells

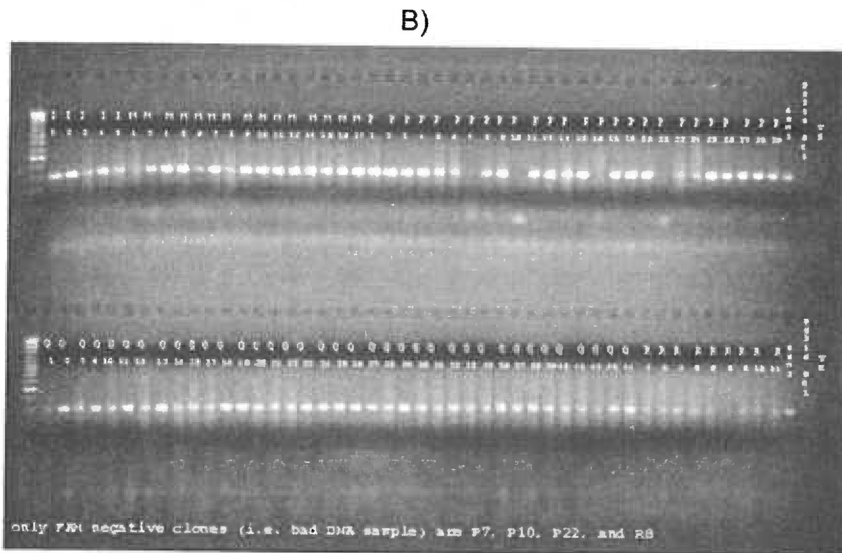
for production of microcell donors should either be avoided or performed with diluted or low titer virus. Furthermore, the B2 repeat PCR was demonstrated to be a reliable assay for presence of transferred mouse chromosomes into human cells.

Figure 6: Mouse chromosome donor cell-specific transfer.
A) ANM01 donor-derived MMCT hybrid clones have extensive B2 repeat PCR bands, indicative of mouse chromosome transfer, while ANM2 donor-derived hybrids largely do not. B) The majority of ANM2 donor-derived MMCT clones are FAH-PCR positive, implying that ANM2 cells act as retroviral producers, infecting human cells, rather than transferring mouse chromosomes with integrated FAH cDNA.

A)



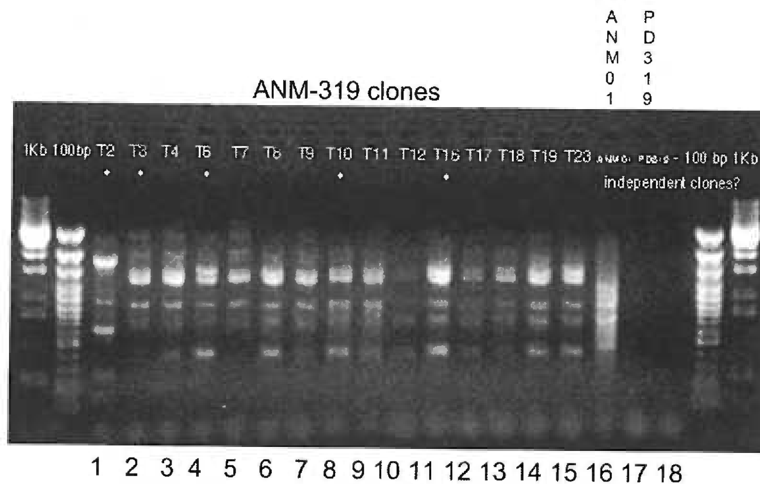
B)



Moreover, the ability of the assay to detect small fragments of transferred mouse material indicated that the B2 repeat PCR could also be employed to assay for lack of co-transfer of mouse chromosomes along with desired human chromosome transfer, such as MMCT with A9-N HyTK cells or similar donors. A third application for the B2 repeat PCR became apparent during the analysis of the abovementioned clones. The B2 repeat PCR primer bound and initiated DNA amplification off of any two adjacent oppositely oriented B2 repeats on opposite strands of DNA. As a result, the DNA fragments amplified by this PCR varied according to the distances between these B2 repeats. Thus, when the PCR products were run out on a gel, in addition to a background smear, several bands could be detected. The pattern of bands roughly correlated with each genomic subset transferred into microcell hybrids. Therefore, independent clones could be identified, and identical subclones eliminated from further consideration, by the banding pattern in the gel.

For example, in a comparison of the PCR products for a subset of hybrid clones from an MMCT between ANM01 donor cells and PD319 SC1 recipient cells, shown in Figure 7, the clone labeled T2 was obviously unique, in terms of chromosome content, when compared to all other clones shown. Clones T3, T6, T10, and T16 were either independent clones or subclones that had lost different portions of transferred mouse material. These five clones therefore represented all the clones analyzed on the gel, and only these 5 clones required further characterization.

Figure 7: Use of B2 repeat PCR to distinguish independent clones from subclones. MMCT hybrid clones T2, T3, T6, T10, and T16 represent the entire set of clones assayed.



Of interest, clone T2 was found to be complemented by cytogenetic radial formation and chromosome breakage analysis (Table 3A; courtesy of Carol Reifsteck, Cytogenetics). Unfortunately, later reanalysis showed loss of complementation from repeated passaging (Table 3B; courtesy of Carol Reifsteck).

Table 3: Cytogenetic analysis of MMCT hybrid clone T2.
A) Initial analysis of clone T2, an MMCT hybrid of an ANM01 derived microcell with a PD319 recipient cell, indicated complementation B) Reanalysis indicated loss of complementation.

A

	Clastogen	Conc ng/ml	# Radials	Total # of Cells	% Radials
319:T2	None	0	0	52	**0%**
	MMC	60	9	51	**18%**
	DEB	300	7	50	**14%**

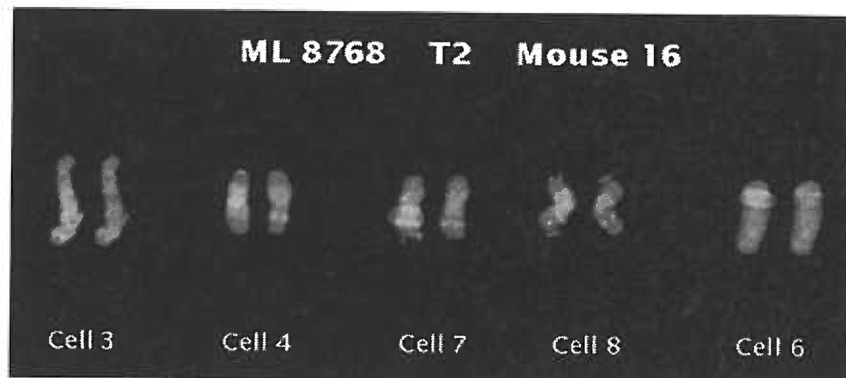
B

	Clastogen	Conc ng/ml	# Radials	Total # of Cells	% Radials
319:T2	None	0	1	50	**2%**

	MMC	60	19	52	**37%**
	DEB	300	13	50	**26%**

Prior to reanalysis, however, the B2 repeat PCR products were compared to mouse genomic sequence. The result of this search for sequence alignment indicated presence of mouse chromosome 16 in clone T2 (data not shown). This was confirmed by mouse chromosome paint for chromosome 16, although the chromosome that contained the detected material also contained different amounts of DNA from other chromosomes, indicative of ongoing translocations in the clonal cells (Figure 8; courtesy of Carol Reifsteck, Cytogenetics).

Figure 8: Mouse chromosome 16 paint of clone T2.
MMCT hybrid clone T2 has parts of mouse chromosome 16 translocated onto a chromosome containing material from at least one other source.



Continuous genomic scrambling in individual cells might explain the loss of complementation, since, if the complementing gene also slowed cell growth, it would be selected against, and repeated passages would result in enrichment of cells that had lost the gene through rearrangement. Nevertheless, the correct identification of mouse chromosome 16 material in the clone by sequencing of the B2 PCR products illustrated yet another purpose for this PCR, namely a means by which to characterize and localize transferred mouse chromosomal material.

Production and use of donors for specific mouse chromosome transfer.

The B2 repeat PCR, while extremely useful, could not be easily applied to determine which chromosome was marked in any given donor cell prior to transfer. One problem with the use of randomly marked mouse cells, whether retrovirally infected or produced by electroporation, is the lack of control over which chromosome would be transferred. While this is acceptable in cases where the purpose of the MMCT is to screen for a complementing chromosome, it would be a serious drawback if one were attempting to transfer only those mouse chromosomes which are syntenic to a complementing human chromosome, as a means by which to narrow the location of the desired gene, or to confirm complementation by a specific mouse chromosome. For such cases, embryonic stem cells or primary fibroblasts from transgenic or targeted-knockout mice could be used as donors[458]. The difficulties in employing such cells lie in obtaining sufficient numbers of primary cells, and in micronucleating ES cells, which are somewhat resistant to colcemid.

As a solution to this dilemma, fibroblasts derived from knockout mice were fused to equal numbers of an immortalized mouse cell line, A9, with 50% PEG, in a process similar to the fusion of microcells to recipient cells. This was done for three separate mouse cell lines. In the first instance, primary fibroblasts from *Atm* knockout mice[459] were obtained (courtesy of Dr. Mitch Turker) and fused to A9 cells. *Atm,* located on mouse chromosome 9[460], was disrupted with a neomycin cassette in these mice[461] . Previous MMCT hybrids, produced using randomly marked donors and a specific FA cell line as recipient, contained mouse chromosome 9 and were complemented. MMCT with the A9 X *Atm-/-* fusion hybrids was undertaken in an attempt to confirm that mouse chromosome 9 transfer was the cause of the complementation. The A9 X *Atm-/-* hybrids were used successfully as donors for MMCT, although the expected complementation of the defect in the recipient cell line by the transferred mouse chromosome 9 did not occur (Dr. Barbara Cox, personal communication). It was later determined that the gene which did complement the cell line in question did not reside on mouse chromosome 9, and that the previous MMCT hybrids had probably genetically reverted. In the other two cases of A9 fusions to mouse knockout fibroblasts, the genes which were disrupted by neomycin cassettes were dopamine receptor D2 (D2R or DRD2)[462], also on mouse chromosome

9[463], and dopamine receptor D3 (DRD3)[464], on mouse chromosome 16[463]. In each of these cases, a tail section was obtained from a knockout mouse (courtesy of Dr. Michael Hayward and Dr. Virginia Otero-Corchon, in the lab of Dr. Malcolm Low) and fibroblasts were derived from this section. The A9 X DRD3-knockout hybrids were also used successfully as MMCT donors, this time into PD319 SC1 cells, and, again, the hybrids were not complemented (data not shown), implying that, in the T2 clone mentioned above, some part other than mouse chromosome 16 of the translocation containing chromosome provided the complementing gene.

Conclusions and Discussion:

In this work, a previously described MMCT technique was modified and improved, based on observations while applying the original protocol. It was noted that use of Con-A reduced cell attachment and flattening out on the bullets. Therefore, bullets were not coated with Con-A prior to addition of cells. It was determined that orientation of bullets in the rotor affected the efficiency of enucleation of micronuclei, and the proper orientation was discovered. One bullet was placed into each centrifuge tube instead of two, and the cell-coated side of the bullet was turned toward the centrifuge spindle. In this way, as well as by washing the bullets with PBS before placing them in the tubes, the amount of whole cell contamination present in the microcell pellet was significantly reduced. The use of synchronized cells for micronucleation and the importance of proper incubation in enucleation medium prior to centrifugation of micronucleated cells were also confirmed.

Micronucleation by short-term colcemid treatment – less than 24 hours -- followed by two hour incubation in 1 μg/ml cytochalasin B has been advocated in the literature as a means by which to increase the efficiency of microcell hybrid production[438]. The author who made this recommendation attributed the substantial increase in efficiency to the lack of extended mitotic arrest of the donor cells. However, the author observed 50% micronucleation of the donor cells after only 16 hours in 0.02 μg/ml colcemid, whereas this high a level of micronucleation was not noted at 16 hours in the donor cells used with the protocol described here, even at 0.05 μg/ml colcemid. In

addition, the donor cells described in the paper were treated with both colcemid and cytochalasin B after being plated on bullets, whereas, with the current protocol, cells were treated with colcemid, moved to bullets, and then treated for a relatively short period of time in cytochalasin B. This is because, in practice, the bullets were difficult to manufacture or obtain, and were therefore reused. Consequently, the surface upon which the cells were plated degraded over time, affecting the stability of extended attachment of cells. Under these circumstances, the optimal balance was sought between high percent micronucleation, proper attachment and flattening, and a reduced length of time in mitotic arrest. This was found to involve 36 to 40 hours in 0.05 μg/ml colcemid, followed by half an hour incubation in enucleation medium with 10 μg/ml cytochalasin B, followed by centrifugation (in enucleation medium). Nevertheless, if one wished to increase efficiency of the protocol described herein, one way to attempt this might be to treat synchronized donor cells with 0.06 μg/ml colcemid for 16 hours, move these cells to bullets, and, 6 hours later, place the PBS washed bullets in centrifuge tubes with enucleation medium for a 1 hour incubation at 37°C prior to centrifugation.

The modifications to the MMCT technique related in this chapter were applied to several cell lines. Three sets of mouse fibroblast-based cell lines, A9-N HyTK cell lines containing individual human chromosomes, randomly neo-marked A9 cells, and A9 cells fused to primary fibroblasts from targeted knockout mice, were successfully used as chromosome donors. Despite the lack of permanently complemented clones from mouse chromosome transfer, the existence of clones with stably transferred mouse chromosomes attested to the effectiveness of the modified MMCT protocol. The B2 repeat PCR was demonstrated to be a reliable means by which to detect successful or accidental mouse chromosome transfer, to differentiate independent clones from subclones, and to identify the chromosomal material transferred, via comparison of sequenced inter-repeat DNA to a mouse genomic DNA database.

The presence of mouse genomic material in MMCT hybrids, and, in at least one case, of transfer of a single mouse chromosome, as well as complementation of the VU423 cell line by human chromosome 13, indicated that the modified MMCT procedure described here worked with multiple donor cell types. As such, it could be extended to yet more types of cells. For example, for more detailed mapping,

complemented hybrids could be used repeatedly as donors into the patient-derived cell line, and the resulting clones could be analyzed for deletions in the re-transferred chromosome that lead to loss of complementation; these deletions could be used to limit the search region for the gene[336,433]. In fact, corrected clones have been used as donors in a transfer using the MMCT procedure detailed above (Dr. Barbara Cox, personal communication). Alternatively, donor cells could be irradiated to induce chromosome breaks and deletions prior to transfer of chromosomes[465]. It should also be noted that a protocol based on the modified MMCT procedure presented in this chapter has been successfully employed to map a gene that is mutated in a human disease — namely, the gene for multiple sulfatase deficiency, SUMF1[466]. Thus, the modifications to the MMCT procedure reported here should be incorporated into future applications of this technique.

Conclusions and Discussion

General findings.

Mutation analysis.

This thesis describes a novel mutation in *FANCA*, along with a secondary, acquired mutation, which results in correction of the Fanconi anemia phenotype at the cellular level. The patients described, monozygotic twins, were found to have a single nucleotide deletion on their paternal allele, 2555delT, a known mutation[301] resulting in a frameshift and premature termination codon one exon downstream of the mutation. A novel exon 28 mutation on the maternal allele, G2670A, was found to code for a R880Q missense change. This change resulted in a hypomorphic protein, as evidenced by partial correction of an FA-A cell phenotype – impeded growth under MMC treatment – with unregulated expression of the allele, but inability to correct the phenotype under doxycyclin-regulated expression. While these mutations were demonstrated to exist in patient fibroblasts, an additional, acquired mutation in exon 30, G2927A, resulting in a E966K missense change, was found in lymphoblasts and whole blood from the same patients and was determined to be in cis with the exon 28 mutation. The acquired mutation corrected the MMC sensitivity phenotype caused by the primary mutation as well as the mislocalization of FANCA protein to the cytoplasm.

Stem Cell Implications

A second topic explored in Chapter 2 was the fact that neither of the two monozygotic twin patients has anemia, and, in fact, are both hematologically normal. As the acquired mutation appears in lymphoblasts but not in fibroblasts, the twins are somatic mosaics, but the extent of the mosaicism is unknown. As these patients are 28 years old, it would seem that the secondary mutation must have occurred in a hematopoietic stem cell (HSC) in one twin, and the very early progeny of that stem cell prenatally repopulated that twin, passed through the intrauterine circulation to the other twin, and proceeded to repopulate the second twin over the course of a few years, before the age of 13. Hematopoietic development is generally polyclonal; even in cases of myelodysplastic syndromes, polyclonality of HSCs has been demonstrated[467], and

cytokine treatment of non-human primates appears to have no effect on the normally polyclonal outgrowth of hematopoietic cells[468]. However, monoclonal development has been noted in cases of leukemia in twins, including cases where intrauterine transmission of a prenatally transformed leukemic cell from one monozygotic twin to the other is suspected[469]. This is, however, the first report of phenotypic correction of monozygotic twins with Fanconi anemia in which a secondary missense mutation functionally corrects for a primary missense mutation in cis, at the level of the hematopoietic stem cell -- resulting in unassisted repopulation of the blood and averting hematopoietic symptoms for over two decades. This apparent clonal outgrowth of a single HSC demonstrates the extent of the self-renewal capacity of HSCs.

Microcell Mediated Chromosome Transfer Modifications

Chapter 3 described some modifications and improvements made to MMCT. These included treating chromosome donor cells in colcemid in medium containing 5% Fetal Bovine Serum, then placing them on plastic "bullets" without prior treatment of the bullets with concanavalin A, and, several hours later, using PBS washes to remove unattached cells. During the course of work done using the MMCT technique, the importance of a full hour incubation, including during the 1/2 hour centrifugation, in cytochalasin B, to ensure that microcells that form were small enough to pass through 5 micron filters unimpeded, was also recognized.

Additionally, it was discovered that the direction the bullets faced in the centrifuge rotor affected efficiency of enucleation of micronuclei to form microcells, as well as, conversely, the number of whole cells that were pelleted and that could cause problems with later steps of the procedure. Bullets must be placed in the rotor such that the side of the bullet that is coated with cells faces the centrifuge spindle, so that the forces generated by the centrifuge "press" the whole cells against the bullet, allowing the micronuclei to pellet without pulling the entire cell with them. Reducing the time the cells spend in colcemid and proportionally increasing the time spent in cytochalasin B may also increase efficiency of the procedure, although this was not tested directly. Thirty-six hours in 0.05 μg/ml colcemid, followed by 1 hour total in 10 μg/ml cytochalasin B was found to be effective, after which point further reductions in colcemid incubation were not attempted.

The effectiveness and low toxicity of the use of 50% PEG solution for fusion, if cells were pretreated with PHA, was also verified. The modified MMCT technique was used successfully with randomly marked mouse cells, knockout mouse tail fibroblasts pre-fused to A9 cells, human chromosome containing mouse fibroblasts, and even previous MMCT hybrid clone cells as donors. Retroviral marking of mouse cells was found to be problematic, unless low titer or diluted retrovirus was used, as some mouse cell lines retained the ability to package retrovirus introduced into them; electroporation or lipofection would be the recommended method of marking such cells. Transfer of mouse chromosomes could be detected using a B2 repeat PCR assay, and this assay was also found to be useful for detection of unwanted mouse chromosomes in human chromosome transfer experiments, for determination of status of clones as independent hybrids or subclones, and for identification and mapping of regions of the mouse chromosome transferred.

Future directions:

This thesis describes work with both scientific and clinical repercussions. Further research to answer questions raised by this work and to improve the techniques used will contribute to both science and medicine.

Comparison to R951Q and E966A mutations in EUF173

The importance of understanding the effects of the R880Q and E966K missense mutations on FANCA function is underscored by the case of a patient who was initially assigned to the now defunct FA-H complementation group and subsequently reassigned to complementation group A. This reassignment occurred once it was determined that the patient was a compound heterozygote for a large deletion and a missense mutation of arginine 951 to glutamine (R951Q) in exon 29 of *FANCA*[317]. It was later determined that the patient also exhibited somatic mosaicism due to direct reversion, in peripheral blood, of the missense mutation. Yet, functionally reverted cultured lymphoblasts from this patient had not reverted the R951Q mutation, but, instead, had acquired a mutation in exon 30 in cis. This acquisition resulted in a second, compensatory glutamate to alanine (E966A) missense mutation[394]. The position of the secondary mutation, at the protein

level, was identical to that of the patients described in this thesis. In both cases, an arginine-to-glutamine missense mutation was functionally corrected by the acquired mutation of glutamate to another amino acid.

In this context, it is instructive to re-examine publications in which the cell line derived from this patient, EUFA173, was studied, and to determine which functional defects were observed. The cell line was shown to be sensitive to MMC, as expected for an FA cell line[317]. The mutant FANCA protein from this cell line neither underwent normal phosphorylation nor interacted normally in a nuclear complex with FANCC[282]. FANCA and FANCG proteins did interact in the EUFA173 cell line, but the levels of both proteins were reduced, implying a role for R951 in protein stability, but not in FANCG binding[285,286]. In general, FANCA protein in EUFA173 did not significantly localize to the nucleus[280]. The fact that alterations to the same glutamate codon functionally corrected both the R951Q and R880Q mutations may imply a similar functional relationship, in terms of protein folding, protein stability, cellular localization, or FANCA phosphorylation, between the arginine residue in each case and the glutamate residue. This hypothesis should be explored further.

Exon 30 begins with part of the codon for R951, so that an alternate transcript lacking exon 30 expresses a protein that lacks R951. Whether this is the sole cause for the lack of ability of this transcript to complement MMC sensitivity is also a question worth answering. The fact that the E966 codon is also in exon 30, and that loss of the glutamate residue by mutation to lysine or alanine corrects loss of function, implies that the part of the protein encoded by exon 30 may have multiple regulatory or functional purposes.

Cellular localization

The R880Q mutation was shown, by immunofluorescence, to alter cellular localization, yet the allele containing this mutation was also shown to correct the MMC sensitivity phenotype when expressed in a non-regulated fashion. The interpretation that best takes these two results into account is that the maternal allele was indeed mutant, with a predominantly cytoplasmic localization, and yet, under constitutive expression, the amount of protein present in the cell was sufficient for some to enter the nucleus and functionally correct FA-A cells. This hypothesis was verified by a subcellular localization

western blot, in which detectable levels of protein appeared in the nuclear fraction. It was also supported by the fact that controlling levels of expression in the Dox-regulated MMC sensitivity experiments resulted in a mutant phenotype for the maternal allele. Constitutive expression of a mutant allele could stabilize levels of an unstable protein. Either subsequently or alternatively, constitutive expression could ensure that protein levels are simply high enough that some protein is fortuitously present in the nucleus at any given time and that this amount of nuclear protein is able to complement the MMC sensitivity defect.

Similar results to those reported here – apparent complementation by largely cytoplasmic FANCA protein – have also been reported in the literature[276,307]. In particular, the initial study of subcellular localization of C-terminal FANCA-GFP fusion proteins showed predominantly cytoplasmic localization, even though the constructs could complement an FA-A cell line[276]. A later study directly contradicts this result, demonstrating nuclear expression of a C-terminal GFP fusion construct with (full length) FANCA[278]. The major difference between these constructs, and the reason for the initial false localization, may be the way the first set of C-terminal GFP fusion constructs were produced. GFP was fused to either full length FANCA or an N-terminal 271 a.a. fragment; in either case, the penultimate amino acid in the FANCA part of the fusion was a leucine, and the fusion introduced a KLDIEFLQPGGST peptide between *FANCA* and GFP[276]. It could be hypothesized that the LXKLDIEFL peptide created by the fusion could act as a weak nuclear exclusion signal (NES)[470-473]. A weak NES could have reduced FANCA-GFP in the nucleus to below visible levels, yet allowed sufficient nuclear FANCA to correct the MMC sensitivity, explaining the initial erroneous subcellular localization results.

In much the same way, while the difference in cellular localization of the mutants reported here might be due to nuclear import, another possibility is that the R880Q protein mutant might undergo premature nuclear export, and that this is corrected by the E966K amino-acid change. Several nuclear export signals have been found in FANCA[474], and the FA complex has been shown to both enter and exit the nucleus in a regulated, cell cycle-dependent manner[371,376].

Possible molecular explanations for mislocalization

The positive charges of R880 and R951, and the negative charge at E966, may directly affect nuclear import or export, with the opposite charges having opposite effects. Another possibility is that R880 and R951 may act as binding sites for a protein or proteins, which, while bound, block access to export machinery. Once the Fanconi complex has completed its as-yet-unknown function, this protein may detach from FANCA, encouraging export of FANCA or the complex as a whole. Such regulation has been noted for p53[475]. Loss of these putative binding sites by mutation or in alternate transcripts may allow premature export of FANCA from the nucleus. There may be further binding sites in exon 30 for proteins that may affect this regulatory mechanism or for proteins required to bind for FANCA function. Some candidates for such transient binding to any one of these putative sites may include non-erythroid alpha spectrin II[294,298], BLM[364], or even FANCE[333]. Mutation of E966 may strengthen binding, or it may result in a small conformational change in the protein, which, while insufficient to affect overall function, may block binding to, or otherwise obscure, surrounding nuclear exclusion signals, such that the premature export that may occur for R880Q or R951Q mutants might not occur in double mutants.

Alignments of the human and mouse proteins[378] indicate that the glutamate at position 966 is not conserved in mice, but, instead, is exchanged with an alanine. Conversely, however, there are nearby glutamates in mouse fanca that are exchanged for alanines in the human protein; one or more of these may provide both the function and negative charge of E966 in human FANCA. To differentiate defective nuclear import from defective nuclear export, studies using the nuclear import inhibitor leptomycin B should be undertaken on cells expressing FANCA with R880Q, R951Q, E966K, and E966A mutations, and various combinations thereof, as well as the alternative transcript lacking exon 30.

Other alternative explanations for functional and localization differences between these various *FANCA* alleles may have to do with phosphorylation of FANCA, which has been shown to correlate with nuclear localization[282], or with protein or mRNA stability. Functional, phosphorylation, stability, and cellular localization assays testing the effects of combinations of all of these mutations may be highly instructive. In addition, it may

also be informative to study other mutations, including H1110P, delF1263, and R1117G, which have been shown to have similar defects[279,280,282,285,286], as well as a recently discovered arginine to tryptophan mutation at position 951 (R951W)[309], in the context of E966K/A. The study of the latter mutation would clarify whether the "loss" of the arginine or the "gain" or a glutamine is counteracted by the loss of the glutamate.

Gene therapy implications

In addition to the importance of following up on the mutation studies, based on the likelihood of hematopoietic repopulation of two individuals with functionally corrected cells as a result a single molecular event, investigation of the gene therapy implications of this work is also called for. The fact that hematopoietic repopulation of twins arose from a single HSC implies that effective intrauterine gene therapy, as seems to have occurred naturally in this case, may be possible by introduction of a vector containing the wild type or corrected gene into an HSC or into the bone marrow of the developing fetus. In addition, the findings described herein also have implications for gene therapy in infants and children, in that it may only be necessary to introduce the wild type allele into one or a few cells, or to screen for targeted homologous integration in only one of many cells, so long as the corrected cell is determined to be an HSC or early hematopoietic progenitor with proper bone marrow homing capability. This provides further impetus for ongoing studies into the nature of HSCs, the markers required to identify such cells, and the signals required for such cells to migrate from the circulation to the bone marrow for engraftment.

Potential for therapeutic application of MMCT

Studies of gene therapy for FA may benefit from the findings of Chapter 2. Unfortunately, in some non-FA disorders, such as monosomy 7 of bone marrow (OMIM # 252270)[2], treatment with a single gene may not be possible, yet introduction of the proper chromosome would not result in problems of trisomy or uniparental disomy. In such cases, using high precision MMCT to introduce single chromosomes into affected cells would be the basis for a therapeutic technique. Modifications to the accepted MMCT technique, described in Chapter 3, increased the degree of micronucleation and

reduced the number of whole cells in the microcell pool. In a therapeutic context, these modifications increase the chances of single chromosome transfer without introduction of unwanted and possibly redundant genomic material. While the changes made worked on various donor cells, further alterations would be required to increase the variety of potential recipient cells. In addition to fibroblasts and other skin cells, myocytes, hepatocytes, peripheral blood stem cells, and, especially, bone marrow stem cells, are all possible targets, and the conditions required to successfully transfer single chromosomes into such cells without changing their cell type or differentiation state should be ascertained.

Other potential future directions for MMCT

Even short of direct clinical application, MMCT has proven to be an exceptional technique for mapping of disease genes. It is possible to use chromosome transfer of syntenic mouse chromosomes to confirm complementation by human chromosome MMCT, and to narrow the region in which one must search for the complementing gene. This technique has, in fact, been used successfully[466]. As the genomes of other organisms are mapped, transfer of chromosomes from cells of these organisms may also fulfill this role. Fibroblasts from non-human primates, including Rhesus monkeys and macaques can be easily obtained, may be easier to micronucleate than human cells, and may provide better synteny mapping for certain genomic regions than mouse chromosomes. With the random marking techniques described in Chapter 2, combined with species-specific assays similar to the B2 repeat PCR, these cells may be an excellent resource as MMCT donors.

Summary

The implications of work presented in this thesis span several fields of study. Mutation analysis of twin FA-A patients revealed a novel hypomorphic mutation and the acquired mutation that could compensate for it. Understanding how these mutations interact at the molecular and cellular level would provide insight into the function of *FANCA* and the FA pathway. Given the fact that the acquired mutation seems to have occurred in an HSC, and that the twins have apparently undergone extensive repopulation

by descendents of that HSC, further examination of this phenomenon could yield benefits both for stem cell research and for gene therapy research. Finally, modifications to the MMCT technique made here and in the future could allow for therapeutic application at the chromosome level, as well as aid in identification of yet unknown genes. Pursuing opportunities suggested in this thesis and searching for answers to questions raised by this work will hopefully contribute to both science and medicine.

References

1. Poole SR, Smith AC, Hays T, McGavran L, Auerbach AD. Monozygotic twin girls with congenital malformations resembling fanconi anemia. Am J Med Genet. 1992;42:780-784.
2. OMIM. National Center for Biotechnology Information. OMIM: online Mendelian inheritance in man http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=omim.
3. Joenje H, Oostra AB, Wijker M, et al. Evidence for at least eight Fanconi anemia genes. Am J Hum Genet. 1997;61:940-944.
4. Meetei AR, de Winter JP, Medhurst AL, et al. A novel ubiquitin ligase is deficient in Fanconi anemia. Nat Genet. 2003;35:165-170.
5. Levitus M, Rooimans MA, Steltenpool J, et al. Heterogeneity in Fanconi anemia: evidence for 2 new genetic subtypes. Blood. 2004;103:2498-2503.
6. Meetei AR, Medhurst AL, Ling C, et al. A human ortholog of archaeal DNA repair protein Hef is defective in Fanconi anemia complementation group M. Nat Genet. 2005;37:958-963.
7. Fanconi G. Familial constitutional panmyelocytopathy, Fanconi's anemia (F.A.) I. Clinical aspects. Semin Hematol. 1967;4:233-240.
8. Rosendorff J, Bernstein R, Macdougall L, Jenkins T. Fanconi anemia: another disease of unusually high prevalence in the Afrikaans population of South Africa. Am J Med Genet. 1987;27:793-797.
9. Verlander PC, Kaporis A, Liu Q, Zhang Q, Seligsohn U, Auerbach AD. Carrier frequency of the IVS4 + 4 A-->T mutation of the Fanconi anemia gene FAC in the Ashkenazi Jewish population. Blood. 1995;86:4034-4038.
10. Butturini A, Gale RP, Verlander PC, Adler-Brecher-B GAP, Auerbach AD. Hematologic abnormalities in Fanconi anemia: an InternationalFanconi Anemia Registry study. Blood. 1994;84:1650-1655.
11. Joenje H, Mathew C, Gluckman E. Fanconi anaemia research: current status and prospects. Eur J Cancer. 1995;2:268-272.
12. Liu JM, Auerbach AD, Young NS. Fanconi anemia presenting unexpectedly in an adult kindred with no dysmorphic features. Am J Med. 1991;91:555-557.
13. Alter BP, Knobloch ME, Weinberg RS. Erythropoiesis in Fanconi's anemia. Blood. 1991;78:602-608.
14. Gordon-Smith EC, Rutherford TR. Fanconi anemia: constitutional aplastic anemia. Semin Hematol. 1991;28:104-112.
15. Alter BP. Hematologic abnormalities in Fanconi anemia. Blood. 1995;85:1148-1149.
16. Swift M. Fanconi's anaemia in the genetics of neoplasia. Nature. 1971;230:370-373.
17. Swift M, Zimmerman D, McDonough ER. Squamous cell carcinomas in Fanconi's anemia. Jama. 1971;216:325-326.
18. Nara N. MT, et al. Two siblings with Fanconi's Anemia developing squamous cell carcinomas. Rinsho Ketsueki. 1980;21:1944.

19. Arnold WJ, King CR, Magrina J, Masterson BJ. Squamous cell carcinoma of the vulva and Fanconi anemia. Int J Gynaecol Obstet. 1980;18:395-397.
20. Hill LS, Dennis PM, Fairham SA. Adenocarcinoma of the stomach and Fanconi's anaemia. Postgrad Med J. 1981;57:404.
21. Kozarek RA, Sanowski RA. Carcinoma of the esophagus associated with Fanconi's anemia. J Clin Gastroenterol. 1981;3:171-174.
22. Abbondanzo SL, Manz HJ, Klappenbach RS, Gootenberg JE. Hepatocellular carcinoma in an 11-year-old girl with Fanconi's anemia. Report of a case and review of the literature. Am J Pediatr Hematol Oncol. 1986;8:334-337.
23. Somers GR, Tabrizi SN, Tiedemann K, Chow CW, Garland SM, Venter DJ. Squamous cell carcinoma of the tongue in a child with Fanconi anemia: a case report and review of the literature. Pediatr Pathol Lab Med. 1995;15:597-607.
24. Alter BP. Fanconi's anemia and malignancies. Am J Hematol. 1996;53:99-110.
25. Levinson S, Vincent KA. Multifocal osteosarcoma in a patient with Fanconi anemia. J Pediatr Hematol Oncol. 1997;19:251-253.
26. Doerr TD, Shibuya TY, Marks SC. Squamous cell carcinoma of the supraglottic larynx in a patient with Fanconi's anemia. Otolaryngol Head Neck Surg. 1998;118:523-525.
27. van der Heijden MS, Yeo CJ, Hruban RH, Kern SE. Fanconi anemia gene mutations in young-onset pancreatic cancer. Cancer Res. 2003;63:2585-2588.
28. Wagner JE, Tolar J, Levran O, et al. Germline mutations in BRCA2: shared genetic susceptibility to breast cancer, early onset leukemia, and Fanconi anemia. Blood. 2004;103:3226-3229.
29. Couch FJ, Johnson MR, Rabe K, et al. Germ line Fanconi anemia complementation group C mutations and pancreatic cancer. Cancer Res. 2005;65:383-386.
30. Howlett NG, Taniguchi T, Olson S, et al. Biallelic inactivation of BRCA2 in Fanconi anemia. Science. 2002;297:606-609.
31. Garcia-Higuera I, Taniguchi T, Ganesan S, et al. Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. Mol Cell. 2001;7:249-262.
32. Folias A, Matkovic M, Bruun D, et al. BRCA1 interacts directly with the Fanconi anemia protein FANCA. Hum Mol Genet. 2002;11:2591-2597.
33. Soci'e G, Henry-Amar M, Cosset JM, Devergie A, Girinsky T, Gluckman E. Increased incidence of solid malignant tumors after bone marrow transplantation for severe aplastic anemia. Blood. 1991;78:277-279.
34. Evans DG, Rees HC, Spreadborough A, et al. Radial ray defects, renal ectopia, duodenal atresia and hydrocephalus: the extended spectrum for Fanconi anaemia. Clin Dysmorphol. 1994;3:200-206.
35. Glanz A, Fraser FC. Spectrum of anomalies in Fanconi anemia. J Med Genet. 1982;19:412-416.
36. Chaganti RS, Houldsworth J. Fanconi anemia: a pleotropic mutation with multiple cellular and developmental abnormalities. Ann Genet. 1991;34:206-211.
37. Alter BP. Fanconi's anaemia and its variability. Br J Haematol. 1993;85:9-14.
38. Aynsley-Green A, Zachmann M, Werder EA, Illig R, Prader A. Endocrine studies in Fanconi's anaemia. Report of 4 cases. Arch Dis Child. 1978;53:126-131.

39. Auerbach AD. Fanconi anemia diagnosis and the diepoxybutane (DEB) test. Exp Hematol. 1993;21:731-733.
40. Hojo ET, van-Diemen PC, Darroudi F, Natarajan AT. Spontaneous chromosomal aberrations in Fanconi anaemia, ataxia telangiectasia fibroblast and Bloom's syndrome lymphoblastoid cell lines as detected by conventional cytogenetic analysis and fluorescence in situ hybridisation (FISH) technique. Mutat Res. 1995;334:59-69.
41. Meyn MS. Ataxia-telangiectasia and cellular responses to DNA damage. Cancer Res. 1995;55:5991-6001.
42. Sasaki MS, Tonomura A. A high susceptibility of Fanconi's anemia to chromosome breakage by DNA cross-linking agents. Cancer Res. 1973;33:1829-1836.
43. Auerbach AD, Wolman SR. Susceptibility of Fanconi's anaemia fibroblasts to chromosome damage by carcinogens. Nature. 1976;261:494-496.
44. Barrett AJ, Brigden WD, Hobbs JR, et al. Successful bone marrow transplant for Fanconi's anaemia. Br Med J. 1977;1:420-422.
45. Soci'e G, Gluckman E, Raynal B, Petit T, Landman-J DA, Brison O. Bone marrow transplantation for Fanconi anemia using low-dose cyclophosphamide/thoracoabdominal irradiation as conditioning regimen: chimerism study by the polymerase chain reaction. Blood. 1993;82:2249-2256.
46. Flowers ME, Zanis J, Pasquini R, et al. Marrow transplantation for Fanconi anaemia: conditioning with reduced doses of cyclophosphamide without radiation. Br J Haematol. 1996;92:699-706.
47. Yule SM, Price L, Cole M, Pearson AD, Boddy AV. Cyclophosphamide metabolism in children with Fanconi's anaemia. Bone Marrow Transplant. 1999;24:123-128.
48. Flowers ME, Doney KC, Storb R, et al. Marrow transplantation for Fanconi anemia with or without leukemic transformation: an update of the Seattle experience. Bone Marrow Transplant. 1992;9:167-173.
49. Guardiola P, Kurre P, Vlad A, et al. Effective graft-versus-leukaemia effect after allogeneic stem cell transplantation using reduced-intensity preparative regimens in Fanconi anaemia patients with myelodysplastic syndrome or acute myeloid leukaemia. Br J Haematol. 2003;122:806-809.
50. Philpott NJ, Marsh JC, Kumaran TO, et al. Successful bone marrow transplant for Fanconi anaemia in transformation. Bone Marrow Transplant. 1994;14:151-153.
51. Ikushima S, Hibi S, Todo S, et al. Successful allogeneic bone marrow transplantation in a case with myelodysplastic syndrome which developed following Fanconi anemia. Bone Marrow Transplant. 1995;16:621-624.
52. Maschan AA, Kryzanovskii OI, Yourlova MI, et al. Intermediate-dose busulfan and cyclophosphamide as a conditioning regimen for bone marrow transplantation in a case of Fanconi anemia in myelodysplastic transformation. Bone Marrow Transplant. 1997;19:385-387.
53. Yakoub-Agha I, Damaj G, Garderet L, et al. Severe oesophagitis after allogeneic bone marrow transplantation for Fanconi's anemia. Bone Marrow Transplant. 2000;26:215-218.
54. Gluckman E. Radiosensitivity in Fanconi anemia: application to the conditioning for bone marrow transplantation. Radiother Oncol. 1990;1:88-93.

55. Di Bartolomeo P, Di Girolamo G, Olioso P, et al. Allogeneic bone marrow transplantation for Fanconi anemia. Bone Marrow Transplant. 1992;10:53-56.
56. Gluckman E. Bone marrow transplantation in Fanconi's anemia. Stem Cells. 1993;11 Suppl 2:180-183.
57. Kohli-Kumar M, Morris C, DeLaat C, et al. Bone marrow transplantation in Fanconi anemia using matched sibling donors. Blood. 1994;84:2050-2054.
58. Zanis-Neto J, Ribeiro RC, Medeiros C, et al. Bone marrow transplantation for patients with Fanconi anemia: a study of 24 cases from a single institution. Bone Marrow Transplant. 1995;15:293-298.
59. O'Donnell J, Roberts I, De la Fuente J, Daly P, New H, Dokal I. Successful second bone marrow transplant for Fanconi's anaemia following escalation of conditioning. Br J Haematol. 1997;98:772-774.
60. Mital MK, Curtis A, Spencer V, Barge D, Skinner R. Delayed engraftment and mixed chimerism after HLA-identical sibling donor BMT in Fanconi anaemia. Bone Marrow Transplant. 1999;24:201-204.
61. Medeiros C, Zanis-Neto J, Pasquini R. Bone marrow transplantation for patients with Fanconi anemia: reduced doses of cyclophosphamide without irradiation as conditioning. Bone Marrow Transplant. 1999;24:849-852.
62. Ayas M, Solh H, Mustafa MM, et al. Bone marrow transplantation from matched siblings in patients with fanconi anemia utilizing low-dose cyclophosphamide, thoracoabdominal radiation and antithymocyte globulin. Bone Marrow Transplant. 2001;27:139-143.
63. Kapelushnik J, Or R, Slavin S, Nagler A. A fludarabine-based protocol for bone marrow transplantation in Fanconi's anemia. Bone Marrow Transplant. 1997;20:1109-1110.
64. Maschan AA, Trakhtman PE, Balashov DN, et al. Fludarabine, low-dose busulfan and antithymocyte globulin as conditioning for Fanconi anemia patients receiving bone marrow transplantation from HLA-compatible related donors. Bone Marrow Transplant. 2004;34:305-307.
65. Ayas M, Al-Jefri A, Al-Mahr M, et al. Stem cell transplantation for patients with Fanconi anemia with low-dose cyclophosphamide and antithymocyte globulins without the use of radiation therapy. Bone Marrow Transplant. 2005;35:463-466.
66. Young N, Alter B. Aplastic Anemia, Aquired and Inherited. Philadelphia: Saunders Company; 1994.
67. Bacigalupo A, Hows J, Gordon-Smith EC, et al. Bone marrow transplantation for severe aplastic anemia from donors other than HLA identical siblings: a report of the BMT Working Party. Bone Marrow Transplant. 1988;3:531-535.
68. Sokal E, Michel M, Ninane J, Latinne D, de Bruyere M, Cornu G. Bone marrow transplantation from an unrelated donor for Fanconi's anaemia: two unusual complications. Bone Marrow Transplant. 1987;2:99-102.
69. Hows JM, Chapple M, Marsh JC, et al. Bone marrow transplantation for Fanconi's anaemia: the Hammersmith experience 1977-89. Bone Marrow Transplant. 1989;4:629-634.
70. Gluckman E, Auerbach AD, Horowitz MM, et al. Bone marrow transplantation for Fanconi anemia. Blood. 1995;86:2856-2862.

71. Davies SM, Khan S, Wagner JE, et al. Unrelated donor bone marrow transplantation for Fanconi anemia. Bone Marrow Transplant. 1996;17:43-47.
72. Zwaan CM, Van Weel-Sipman MH, Fibbe WE, Oudshoorn M, Vossen JM. Unrelated donor bone marrow transplantation in Fanconi anaemia: the Leiden experience. Bone Marrow Transplant. 1998;21:447-453.
73. Guardiola P, Pasquini R, Dokal I, et al. Outcome of 69 allogeneic stem cell transplantations for Fanconi anemia using HLA-matched unrelated donors: a study on behalf of the European Group for Blood and Marrow Transplantation. Blood. 2000;95:422-429.
74. Elhasid R, Ben Arush MW, Katz T, et al. Successful haploidentical bone marrow transplantation in Fanconi anemia. Bone Marrow Transplant. 2000;26:1221-1223.
75. de la Fuente J, Reiss S, McCloy M, et al. Non-TBI stem cell transplantation protocol for Fanconi anaemia using HLA-compatible sibling and unrelated donors. Bone Marrow Transplant. 2003;32:653-656.
76. Massumoto C, Moraes JR, Moraes ME, et al. Unrelated peripheral blood stem cell transplantation for Fanconi anemia. Bone Marrow Transplant. 1997;19:299-300.
77. Rossi G, Giorgiani G, Comoli P, et al. Successful T-cell-depleted, related haploidentical peripheral blood stem cell transplantation in a patient with Fanconi anaemia using a fludarabine-based preparative regimen without radiation. Bone Marrow Transplant. 2003;31:437-440.
78. Boulad F, Gillio A, Small TN, et al. Stem cell transplantation for the treatment of Fanconi anaemia using a fludarabine-based cytoreductive regimen and T-cell-depleted related HLA-mismatched peripheral blood stem cell grafts. Br J Haematol. 2000;111:1153-1157.
79. Matsuda Y, Hara J, Osugi Y, et al. Allogeneic peripheral stem cell transplantation using positively selected CD34+ cells from HLA-mismatched donors. Bone Marrow Transplant. 1998;21:355-360.
80. Broxmeyer HE, Douglas GW, Hangoc G, et al. Human umbilical cord blood as a potential source of transplantable hematopoietic stem/progenitor cells. Proc Natl Acad Sci U S A. 1989;86:3828-3832.
81. Gluckman E. European organization for cord blood banking. Blood Cells. 1994;20:601-608.
82. Gluckman E, Broxmeyer HA, Auerbach AD, et al. Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical-cord blood from an HLA-identical sibling. N Engl J Med. 1989;321:1174-1178.
83. Gluckman E, Devergie A, Bourdeau-Esperou H, et al. Transplantation of umbilical cord blood in Fanconi's anemia. Nouv Rev Fr Hematol. 1990;32:423-425.
84. Auerbach AD, Liu Q, Ghosh R, Pollack MS, Douglas GW, Broxmeyer HE. Prenatal identification of potential donors for umbilical cord blood transplantation for Fanconi anemia. Transfusion. 1990;30:682-687.
85. Broxmeyer HE, Gluckman E, Auerbach A, et al. Human umbilical cord blood: a clinically useful source of transplantable hematopoietic stem/progenitor cells. Int J Cell Cloning. 1990;1:76-89.
86. Wagner JE, Jr. Umbilical cord and placental blood transplantation: analysis of the clinical results. J Hematother. 1993;2:265-268.

87. Wagner JE, Jr. Umbilical cord blood stem cell transplantation: current status and future prospects (1992). J Hematother. 1993;2:225-228.
88. Aker M, Varadi G, Slavin S, Nagler A. Fludarabine-based protocol for human umbilical cord blood transplantation in children with Fanconi anemia. J Pediatr Hematol Oncol. 1999;21:237-239.
89. Gluckman E, Devergie A, Thierry D, et al. Clinical applications of stem cell transfusion from cord blood and rationale for cord blood banking. Bone Marrow Transplant. 1992;1:114-117.
90. Gluckman E, Wagner J, Hows J, Kernan N, Bradley B, Broxmeyer HE. Cord blood banking for hematopoietic stem cell transplantation: an international cord blood transplant registry. Bone Marrow Transplant. 1993;11:199-200.
91. Kurtzberg J, Laughlin M, Graham ML, et al. Placental blood as a source of hematopoietic stem cells for transplantation into unrelated recipients [see comments]. N Engl J Med. 1996;335:157-166.
92. Yoshimasu T, Tanaka R, Suenobu S, et al. Prompt and durable hematopoietic reconstitution by unrelated cord blood transplantation in a child with Fanconi anemia. Bone Marrow Transplant. 2001;27:767-769.
93. Kumar AR, Wagner JE, Auerbach AD, et al. Fatal hemorrhage from androgen-related hepatic adenoma after hematopoietic cell transplantation. J Pediatr Hematol Oncol. 2004;26:16-18.
94. Barker JN, Davies SM, DeFor T, Ramsay NK, Weisdorf DJ, Wagner JE. Survival after transplantation of unrelated donor umbilical cord blood is comparable to that of human leukocyte antigen-matched unrelated donor bone marrow: results of a matched-pair analysis. Blood. 2001;97:2957-2961.
95. Barker JN, Martin PL, Coad JE, et al. Low incidence of Epstein-Barr virus-associated posttransplantation lymphoproliferative disorders in 272 unrelated-donor umbilical cord blood transplant recipients. Biol Blood Marrow Transplant. 2001;7:395-399.
96. Verlinsky Y, Rechitsky S, Schoolcraft W, Strom C, Kuliev A. Preimplantation diagnosis for Fanconi anemia combined with HLA matching. Jama. 2001;285:3130-3133.
97. Grewal SS, Kahn JP, MacMillan ML, Ramsay NK, Wagner JE. Successful hematopoietic stem cell transplantation for Fanconi anemia from an unaffected HLA-genotype-identical sibling selected using preimplantation genetic diagnosis. Blood. 2004;103:1147-1151.
98. Bielorai B, Hughes MR, Auerbach AD, et al. Successful umbilical cord blood transplantation for Fanconi anemia using preimplantation genetic diagnosis for HLA-matched donor. Am J Hematol. 2004;77:397-399.
99. Kuliev A, Verlinsky Y. Preimplantation HLA typing and stem cell transplantation: report of International Meeting, Cyprus, 27-8 March, 2004. Reprod Biomed Online. 2004;9:205-209.
100. Rechitsky S, Kuliev A, Tur-Kaspa I, Morris R, Verlinsky Y. Preimplantation genetic diagnosis with HLA matching. Reprod Biomed Online. 2004;9:210-221.
101. Auerbach AD. Umbilical cord blood transplants for genetic disease: diagnostic and ethical issues in fetal studies. Blood Cells. 1994;20:303-309.
102. Liu JM. Gene transfer for the eventual treatment of Fanconi's anemia. Semin Hematol. 1998;35:168-179.

103. Walsh CE, Grompe M, Vanin E, et al. A functionally active retrovirus vector for gene therapy in Fanconi anemia group C. Blood. 1994;84:453-459.
104. Walsh CE, Nienhuis AW, Samulski RJ, et al. Phenotypic correction of Fanconi anemia in human hematopoietic cells with a recombinant adeno-associated virus vector. J Clin Invest. 1994;94:1440-1448.
105. Machl AW, Planitzer S, Kubbies M. A novel, membrane receptor-based retroviral vector for Fanconi anemia group C gene therapy. Gene Ther. 1997;4:339-345.
106. Yamada K, Olsen JC, Patel M, Rao KW, Walsh CE. Functional correction of fanconi anemia group C hematopoietic cells by the use of a novel lentiviral vector. Mol Ther. 2001;3:485-490.
107. Fu KL, Foe JR, Joenje H, Rao KW, Liu JM, Walsh CE. Functional correction of Fanconi anemia group A hematopoietic cells by retroviral gene transfer. Blood. 1997;90:3296-3303.
108. Lu L, Ge Y, Li ZH, Freie B, Clapp DW, Broxmeyer HE. CD34 stem/progenitor cells purified from cryopreserved normal cord blood can be transduced with high efficiency by a retroviral vector and expanded ex vivo with stable integration and expression of Fanconi anemia complementation C gene. Cell Transplant. 1995;4:493-503.
109. Walsh CE, Mann MM, Emmons RV, Wang S, Liu JM. Transduction of CD34-enriched human peripheral and umbilical cord blood progenitors using a retroviral vector with the Fanconi anemia group C gene. J Investig Med. 1995;43:379-385.
110. Liu JM, Young NS, Walsh CE, et al. Retroviral mediated gene transfer of the Fanconi anemia complementation group C gene to hematopoietic progenitors of group C patients. Hum Gene Ther. 1997;8:1715-1730.
111. Liu JM, Kim S, Read EJ, et al. Engraftment of hematopoietic progenitor cells transduced with the Fanconi anemia group C gene (FANCC). Hum Gene Ther. 1999;10:2337-2346.
112. Stark R, Thierry D, Richard P, Gluckman E. Long-term bone marrow culture in Fanconi's anaemia. Br J Haematol. 1993;83:554-559.
113. Croop JM, Cooper R, Fernandez C, et al. Mobilization and collection of peripheral blood CD34+ cells from patients with Fanconi anemia. Blood. 2001;98:2917-2921.
114. Williams DA, Croop J, Kelly P. Gene therapy in the treatment of Fanconi anemia, a progressive bone marrow failure syndrome. Curr Opin Mol Ther. 2005;7:461-466.
115. Croop JM. Gene therapy for fanconi anemia. Curr Hematol Rep. 2003;2:335-340.
116. Schroeder TM, Anschutz F, Knopp A. Spontaneous chromosome aberrations in familial panmyelopathy. Humangenetik. 1964;1:194-196.
117. Weksberg R, Buchwald M, Sargent P, Thompson-M-W SL. Specific cellular defects in patients with Fanconi anemia. J Cell Physiol. 1979;101:311-323.
118. Berger R, Le-Coniat M, Gendron MC. Fanconi anemia. Chromosome breakage and cell cycle studies. Cancer Genet Cytogenet. 1993;69:13-16.
119. Cervenka J, Arthur D, Yasis C. Mitomycin C test for diagnostic differentiation of idiopathic aplastic anemia and Fanconi anemia. Pediatrics. 1981;67:119-127.
120. Dutrillaux B, Aurias A, Dutrillaux AM, Buriot D, Prieur M. The cell cycle of lymphocytes in Fanconi anemia. Hum Genet. 1982;62:327-332.

121. Kubbies M, Schindler D, Hoehn H, Schinzel A, Rabinovitch PS. Endogenous blockage and delay of the chromosome cycle despite normal recruitment and growth phase explain poor proliferation and frequent edomitosis in Fanconi anemia cells. Am J Hum Genet. 1985;37:1022-1030.
122. Schindler D, Kubbies M, Hoehn H, Schinzel A, Rabinovitch PS. Presymptomatic diagnosis of Fanconi's anaemia. Lancet. 1985;1:937.
123. Seyschab H, Sun Y, Friedl R, Schindler D, Hoehn H. G2 phase cell cycle disturbance as a manifestation of genetic cell damage. Hum Genet. 1993;92:61-68.
124. Seyschab H, Friedl R, Sun Y, et al. Comparative evaluation of diepoxybutane sensitivity and cell cycle blockage in the diagnosis of Fanconi anemia. Blood. 1995;85:2233-2237.
125. Akkari YM, Bateman RL, Reifsteck CA, D'Andrea AD, Olson SB, Grompe M. The 4N cell cycle delay in Fanconi anemia reflects growth arrest in late S phase. Mol Genet Metab. 2001;74:403-412.
126. Sala-Trepat M, Rouillard D, Escarceller M, Laquerbe A, Moustacchi E, Papadopoulo D. Arrest of S-phase progression is impaired in Fanconi Anemia cells. Exp Cell Res. 2000;260:208-215.
127. Kupfer GM, D'Andrea AD. The effect of the Fanconi anemia polypeptide, FAC, upon p53 induction and G2 checkpoint regulation. Blood. 1996;88:1019-1025.
128. Kruyt FA, Dijkmans LM, van-den-Berg TK, Joenje H. Fanconi anemia genes act to suppress a cross-linker-inducible p53-independent apoptosis pathway in lymphoblastoid cell lines. Blood. 1996;87:938-948.
129. Houghtaling S, Granville L, Akkari Y, et al. Heterozygosity for p53 (Trp53+/-) accelerates epithelial tumor formation in fanconi anemia complementation group D2 (Fancd2) knockout mice. Cancer Res. 2005;65:85-91.
130. Johnstone P, Reifsteck C, Kohler S, Worland P, Olson S, Moses RE. Fanconi anemia group A and D cell lines respond normally to inhibitors of cell cycle regulation. Somat Cell Mol Genet. 1997;23:371-377.
131. Heinrich MC, Hoatlin ME, Zigler AJ, et al. DNA cross-linker-induced G2/M arrest in group C Fanconi anemia lymphoblasts reflects normal checkpoint function. Blood. 1998;91:275-287.
132. Rosselli F, Moustacchi E. Cocultivation of Fanconi anemia cells and of mouse lymphoma mutants leads to interspecies complementation of chromosomal hypersensitivity to DNA cross-linking agents. Hum Genet. 1990;84:517-521.
133. Bagnara GP, Strippoli P, Bonsi L, et al. Effect of stem cell factor on colony growth from acquired and constitutional (Fanconi) aplastic anemia. Blood. 1992;80:382-387.
134. Rosselli F, Sanceau J, Wietzerbin J, Moustacchi E. Abnormal lymphokine production: a novel feature of the genetic disease Fanconi anemia. I. Involvement of interleukin-6. Hum Genet. 1992;89:42-48.
135. Rosselli F, Sanceau J, Gluckman E, Wietzerbin J, Moustacchi E. Abnormal lymphokine production: a novel feature of the genetic disease Fanconi anemia. II. In vitro and in vivo spontaneous overproduction of tumor necrosis factor alpha. Blood. 1994;83:1216-1225.

136. Haneline LS, Broxmeyer HE, Cooper S, et al. Multiple inhibitory cytokines induce deregulated progenitor growth and apoptosis in hematopoietic cells from Fac-/- mice. Blood. 1998;91:4092-4098.
137. Dufour C, Corcione A, Svahn J, et al. TNF-alpha and IFN-gamma are overexpressed in the bone marrow of Fanconi anemia patients and TNF-alpha suppresses erythropoiesis in vitro. Blood. 2003;102:2053-2059.
138. Platzbecker U, Kurre P, Guardiola P, et al. Fanconi anemia type C-deficient hematopoietic cells are resistant to TRAIL (TNF-related apoptosis-inducing ligand)-induced cleavage of pro-caspase-8. Exp Hematol. 2004;32:815-821.
139. Whitney MA, Royle G, Low MJ, et al. Germ cell defects and hematopoietic hypersensitivity to gamma-interferon in mice with a targeted disruption of the Fanconi anemia C gene. Blood. 1996;88:49-58.
140. Rathbun RK, Faulkner GR, Ostroski MH, et al. Inactivation of the Fanconi anemia group C gene augments interferon-gamma-induced apoptotic responses in hematopoietic cells. Blood. 1997;90:974-985.
141. Li X, Yang Y, Yuan J, et al. Continuous in vivo infusion of interferon-gamma (IFN-gamma) preferentially reduces myeloid progenitor numbers and enhances engraftment of syngeneic wild-type cells in Fancc-/- mice. Blood. 2004;104:1204-1209.
142. Kurre P, Anandakumar P, Grompe M, Kiem HP. In vivo administration of interferon gamma does not cause marrow aplasia in mice with a targeted disruption of FANCC. Exp Hematol. 2002;30:1257-1262.
143. Noll M, Battaile KP, Bateman R, et al. Fanconi anemia group A and C double-mutant mice: functional evidence for a multi-protein Fanconi anemia complex. Exp Hematol. 2002;30:679-688.
144. Rathbun RK, Christianson TA, Faulkner GR, et al. Interferon-gamma-induced apoptotic responses of Fanconi anemia group C hematopoietic progenitor cells involve caspase 8-dependent activation of caspase 3 family members. Blood. 2000;96:4204-4211.
145. Pang Q, Fagerlie S, Christianson TA, et al. The Fanconi anemia protein FANCC binds to and facilitates the activation of STAT1 by gamma interferon and hematopoietic growth factors. Mol Cell Biol. 2000;20:4724-4735.
146. Pang Q, Keeble W, Christianson TA, Faulkner GR, Bagby GC. FANCC interacts with Hsp70 to protect hematopoietic cells from IFN-gamma/TNF-alpha-mediated cytotoxicity. Embo J. 2001;20:4478-4489.
147. Pang Q, Keeble W, Diaz J, et al. Role of double-stranded RNA-dependent protein kinase in mediating hypersensitivity of Fanconi anemia complementation group C cells to interferon gamma, tumor necrosis factor-alpha, and double-stranded RNA. Blood. 2001;97:1644-1652.
148. Fagerlie SR, Koretsky T, Torok-Storb B, Bagby GC. Impaired type I IFN-induced Jak/STAT signaling in FA-C cells and abnormal CD4+ Th cell subsets in Fancc-/- mice. J Immunol. 2004;173:3863-3870.
149. Fagerlie SR, Diaz J, Christianson TA, et al. Functional correction of FA-C cells with FANCC suppresses the expression of interferon gamma-inducible genes. Blood. 2001;97:3017-3024.
150. Pearl-Yafe M, Halperin D, Scheuerman O, Fabian I. The p38 pathway partially mediates caspase-3 activation induced by reactive oxygen species in Fanconi anemia C cells. Biochem Pharmacol. 2004;67:539-546.

151. Pearl-Yafe M, Halperin D, Halevy A, Kalir H, Bielorai B, Fabian I. An oxidative mechanism of interferon induced priming of the Fas pathway in Fanconi anemia cells. Biochem Pharmacol. 2003;65:833-842.
152. Hadjur S, Jirik FR. Increased sensitivity of Fancc-deficient hematopoietic cells to nitric oxide and evidence that this species mediates growth inhibition by cytokines. Blood. 2003;101:3877-3884.
153. Korkina LG, Samochatova EV, Maschan AA, Suslova TB, Cheremisina ZP, Afanas'ev IB. Release of active oxygen radicals by leukocytes of Fanconi anemia patients. J Leukoc Biol. 1992;52:357-362.
154. Degan P, Bonassi S, De Caterina M, et al. In vivo accumulation of 8-hydroxy-2'-deoxyguanosine in DNA correlates with release of reactive oxygen species in Fanconi's anaemia families. Carcinogenesis. 1995;16:735-741.
155. Takeuchi T, Morimoto K. Increased formation of 8-hydroxydeoxyguanosine, an oxidative DNA damage, in lymphoblasts from Fanconi's anemia patients due to possible catalase deficiency. Carcinogenesis. 1993;14:1115-1120.
156. Ruppitsch W, Meisslitzer C, Weirich-Schwaiger H, et al. The role of oxygen metabolism for the pathological phenotype of Fanconi anemia. Hum Genet. 1997;99:710-719.
157. Ruppitsch W, Meisslitzer C, Hirsch K-M, Schweiger M. Overexpression of thioredoxin in Fanconi anemia fibroblasts prevents the cytotoxic and DNA damaging effect of mitomycin C and diepoxybutane. Febs Letters. 1998;422:99-102.
158. Joenje H, Arwert F, Eriksson AW, de Koning H, Oostra AB. Oxygen-dependence of chromosomal aberrations in Fanconi's anaemia. Nature. 1981;290:142-143.
159. Joenje H, Oostra AB. Effect of oxygen tension on chromosomal aberrations in Fanconi anaemia. Hum Genet. 1983;65:99-101.
160. Joenje H, Oostra AB. Oxygen-induced cytogenetic instability in normal human lymphocytes. Hum Genet. 1986;74:438-440.
161. Pritsos CA, Sartorelli AC. Generation of reactive oxygen radicals through bioactivation of mitomycin antibiotics. Cancer Res. 1986;46:3528-3532.
162. Clarke AA, Philpott NJ, Gordon-Smith EC, Rutherford TR. The sensitivity of Fanconi anaemia group C cells to apoptosis induced by mitomycin C is due to oxygen radical generation, not DNA crosslinking. Br J Haematol. 1997;96:240-247.
163. Schindler D, Hoehn H. Fanconi anemia mutation causes cellular susceptibility to ambient oxygen. Am J Hum Genet. 1988;43:429-435.
164. Saito H, Hammond AT, Moses RE. Hypersensitivity to oxygen is a uniform and secondary defect in Fanconi anemia cells. Mutat Res. 1993;294:255-262.
165. Joenje H, Youssoufian H, Kruyt FA, dos-Santos CC, Wevrick R, Buchwald M. Expression of the Fanconi anemia gene FAC in human cell lines: lack of effect of oxygen tension. Blood Cells Mol Dis. 1995;21:182-191.
166. Paulin-Levasseur M, Chen G, Lariviere C. The 2G2 antibody recognizes an acidic 110-kDa human mitochondrial protein. Histochem J. 1998;30:617-625.
167. Rousset S, Nocentini S, Rouillard D, Baroche C, Moustacchi E. Mitochondrial alterations in fanconi anemia fibroblasts following ultraviolet A or psoralen photoactivation. Photochem Photobiol. 2002;75:159-166.

168. Bogliolo M, Borghini S, Abbondandolo A, Degan P. Alternative metabolic pathways for energy supply and resistance to apoptosis in Fanconi anaemia. Mutagenesis. 2002;17:25-30.
169. Rosselli F, Ridet A, Soussi T, Duchaud E, Alapetite C, Moustacchi E. p53-dependent pathway of radio-induced apoptosis is altered in Fanconi anemia. Oncogene. 1995;10:9-17.
170. Ridet A, Guillouf C, Duchaud E, et al. Deregulated apoptosis is a hallmark of the Fanconi anemia syndrome. Cancer Res. 1997;57:1722-1730.
171. Guillouf C, Vit JP, Rosselli F. Loss of the Fanconi anemia group C protein activity results in an inability to activate caspase-3 after ionizing radiation. Biochimie. 2000;82:51-58.
172. Freie B, Li X, Ciccone SL, et al. Fanconi anemia type C and p53 cooperate in apoptosis and tumorigenesis. Blood. 2003;102:4146-4152.
173. Zhang X, Li J, Sejas DP, Rathbun KR, Bagby GC, Pang Q. The Fanconi anemia proteins functionally interact with the protein kinase regulated by RNA (PKR). J Biol Chem. 2004;279:43910-43919.
174. Pang Q, Christianson TA, Koretsky T, et al. Nucleophosmin interacts with and inhibits the catalytic function of eukaryotic initiation factor 2 kinase PKR. J Biol Chem. 2003;278:41709-41717.
175. Pang Q, Christianson TA, Keeble W, Koretsky T, Bagby GC. The anti-apoptotic function of Hsp70 in the interferon-inducible double-stranded RNA-dependent protein kinase-mediated death signaling pathway requires the Fanconi anemia protein, FANCC. J Biol Chem. 2002;277:49638-49643.
176. Pang Q, Christianson TA, Keeble W, et al. The Fanconi anemia complementation group C gene product: structural evidence of multifunctionality. Blood. 2001;98:1392-1401.
177. Koh PS, Hughes GC, Faulkner GR, Keeble WW, Bagby GC. The Fanconi anemia group C gene product modulates apoptotic responses to tumor necrosis factor-alpha and Fas ligand but does not suppress expression of receptors of the tumor necrosis factor receptor superfamily. Exp Hematol. 1999;27:1-8.
178. Bagby GC, Jr. Genetic basis of Fanconi anemia. Curr Opin Hematol. 2003;10:68-76.
179. Baruque GA, Bitencourt MA, Pasquini R, Castelo-Branco MT, Llerena JC, Jr., Rumjanek VM. Apoptosis and expression of anti- and pro-apoptotic proteins in peripheral blood mononuclear cells of Fanconi anaemia patients: a study of 73 cases. Eur J Haematol. 2005;75:384-390.
180. Li X, Le Beau MM, Ciccone S, et al. Ex vivo culture of Fancc-/- stem/progenitor cells predisposes cells to undergo apoptosis, and surviving stem/progenitor cells display cytogenetic abnormalities and an increased risk of malignancy. Blood. 2005;105:3465-3471.
181. Otsuki T, Nagakura S, Wang J, Bloom M, Grompe M, Liu JM. Tumor necrosis factor-alpha and CD95 ligation suppress erythropoiesis in Fanconi anemia C gene knockout mice. J Cell Physiol. 1999;179:79-86.
182. Liu TX, Howlett NG, Deng M, et al. Knockdown of zebrafish Fancd2 causes developmental abnormalities via p53-dependent apoptosis. Dev Cell. 2003;5:903-914.

183. Rey JP, Scott R, Muller H. Apoptosis is not involved in the hypersensitivity of Fanconi anemia cells to mitomycin C. Cancer Genet Cytogenet. 1994;75:67-71.
184. Clarke AA, Gibson FM, Scott J, Myatt N, Rutherford TR. Fanconi's anemia cell lines show distinct mechanisms of cell death in response to mitomycin C or agonistic anti-Fas antibodies. Haematologica. 2004;89:11-20.
185. Ferrer M, Izeboud T, Ferreira CG, Span SW, Giaccone G, Kruyt FA. Cisplatin triggers apoptotic or nonapoptotic cell death in Fanconi anemia lymphoblasts in a concentration-dependent manner. Exp Cell Res. 2003;286:381-395.
186. Guillouf C, Wang TS, Liu J, et al. Fanconi anemia C protein acts at a switch between apoptosis and necrosis in mitomycin C-induced cell death. Exp Cell Res. 1999;246:384-394.
187. Cumming RC, Liu JM, Youssoufian H, Buchwald M. Suppression of apoptosis in hematopoietic factor-dependent progenitor cell lines by expression of the FAC gene. Blood. 1996;88:4558-4567.
188. Wang J, Otsuki T, Youssoufian H, et al. Overexpression of the fanconi anemia group C gene (FAC) protects hematopoietic progenitors from death induced by Fas-mediated apoptosis. Cancer Res. 1998;58:3538-3541.
189. Buchwald M, Moustacchi E. Is Fanconi anemia caused by a defect in the processing of DNA damage? Mutat Res. 1998;408:75-90.
190. Gluckman E, Devergie A, Dutreix J. Radiosensitivity in Fanconi anaemia: application to the conditioning regimen for bone marrow transplantation. Br J Haematol. 1983;54:431-440.
191. Arlett CF, Harcourt SA. Survey of radiosensitivity in a variety of human cell strains. Cancer Res. 1980;40:926-932.
192. Burnet NG, Wurm R, Tait DM, Peacock JH. Cellular sensitivity and low dose-rate recovery in Fanconi anaemia fibroblasts. Br J Radiol. 1994;67:579-583.
193. Knox SJ, Wilson FD, Greenberg BR, et al. Increased radiosensitivity of a subpopulation ot T-lymphocyte progenitors from patients with Fanconi's anemia. Blood. 1981;57:1043-1048.
194. Fornace AJ, Jr., Little JB, Weichselbaum RR. DNA repair in a Fanconi's anemia fibroblast cell strain. Biochim Biophys Acta. 1979;561:99-109.
195. Weichselbaum RR, Nove J, Little JB. X-ray sensitivity of fifty-three human diploid fibroblast cell strains from patients with characterized genetic disorders. Cancer Res. 1980;40:920-925.
196. Moustacchi E, Diatloff-Zito C. DNA semi-conservative synthesis in normal and Fanconi anemia fibroblasts following treatment with 8-methoxypsoralen and near ultraviolet light or with X-rays. Hum Genet. 1985;70:236-242.
197. Duckworth-Rysiecki G, Taylor AMR. Effects of ionizing radiation on cells from Fanconi's anemia pateints. Cancer Res. 1985;45:2015-2020.
198. Higurashi M, Conen PE. In vitro chromosomal radiosensitivity in "chromosomal breakage syndromes". Cancer. 1973;32:380-383.
199. Higurashi M, Conen PE. In vitro chromosomal radiosensitivity in Fanconi's anemia. Blood. 1971;38:336-342.
200. Bigelow SB, Rary JM, Bender MA. G2 chromosomal radiosensitivity in Fanconi's anemia. Mutat Res. 1979;63:189-199.

201. Parshad R, Sanford KK, Jones GM. Chromatid damage after G2 phase x-irradiation of cells from cancer- prone individuals implicates deficiency in DNA repair. Proc Natl Acad Sci U S A. 1983;80:5612-5616.
202. Djuzenova CS, Rothfuss A, Oppitz U, et al. Response to X-irradiation of Fanconi anemia homozygous and heterozygous cells assessed by the single-cell gel electrophoresis (comet) assay. Lab Invest. 2001;81:185-192.
203. Carreau M, Alon N, Bosnoyan-Collins L, Joenje H, Buchwald M. Drug sensitivity spectra in Fanconi anemia lymphoblastoid cell lines of defined complementation groups. Mutat Res. 1999;435:103-109.
204. Joenje H, Oostra AB. Clastogenicity of cyclophosphamide in Fanconi's anemia lymphocytes without exogenous metabolic activation. Cancer Genet Cytogenet. 1986;22:339-345.
205. Casado JA, Nunez MI, Segovia JC, Ruiz de Almodovar JM, Bueren JA. Non-homologous End-Joining Defect in Fanconi Anemia Hematopoietic Cells Exposed to Ionizing Radiation. Radiat Res. 2005;164:635-641.
206. Yang Y, Kuang Y, De Oca RM, et al. Targeted disruption of the murine Fanconi anemia gene, Fancg/Xrcc9. Blood. 2001;98:3435-3440.
207. Houghtaling S, Newell A, Akkari Y, Taniguchi T, Olson S, Grompe M. Fancd2 functions in a double strand break repair pathway that is distinct from non-homologous end joining. Hum Mol Genet. 2005;14:3027-3033.
208. Houghtaling S, Timmers C, Noll M, et al. Epithelial cancer in Fanconi anemia complementation group D2 (Fancd2) knockout mice. Genes Dev. 2003;17:2021-2035.
209. Hinz JM, Yamada NA, Salazar EP, Tebbs RS, Thompson LH. Influence of double-strand-break repair pathways on radiosensitivity throughout the cell cycle in CHO cells. DNA Repair (Amst). 2005;4:782-792.
210. Tebbs RS, Hinz JM, Yamada NA, et al. New insights into the Fanconi anemia pathway from an isogenic FancG hamster CHO mutant. DNA Repair (Amst). 2005;4:11-22.
211. Lamerdin JE, Yamada NA, George JW, et al. Characterization of the hamster FancG/Xrcc9 gene and mutations in CHO UV40 and NM3. Mutagenesis. 2004;19:237-244.
212. Liu N, Lamerdin JE, Tucker JD, et al. The human XRCC9 gene corrects chromosomal instability and mutagen sensitivities in CHO UV40 cells. Proc Natl Acad Sci U S A. 1997;94:9232-9237.
213. Freie BW, Ciccone SL, Li X, et al. A role for the Fanconi anemia C protein in maintaining the DNA damage-induced G2 checkpoint. J Biol Chem. 2004;279:50986-50993.
214. Papadopoulo D, Guillouf C, Porfirio B, Moustacchi E. Decreased mutagenicity in Fanconi's anemia lymphoblasts following treatment with photoactivated psoralens. Prog Clin Biol Res. 1990;340A:241-248.
215. Papadopoulo D, Porfirio B, Moustacchi E. Mutagenic response of Fanconi's anemia cells from a defined complementation group after treatment with photoactivated bifunctional psoralens. Cancer Res. 1990;50:3289-3294.
216. Papadopoulo D, Guillouf C, Mohrenweiser H, Moustacchi E. Hypomutability in Fanconi anemia cells is associated with increased deletion frequency at the HPRT locus. Proc Natl Acad Sci U S A. 1990;87:8383-8387.

217. Papadopoulo D, Laquerbe A, Guillouf C, Moustacchi E. Molecular spectrum of mutations induced at the HPRT locus by a cross-linking agent in human cell lines with different repair capacities. Mutat Res. 1993;294:167-177.
218. Guillouf C, Laquerbe A, Moustacchi E, Papadopoulo D. Mutagenic processing of psoralen monoadducts differ in normal and Fanconi anemia cells. Mutagenesis. 1993;8:355-361.
219. Laquerbe A, Sala-Trepat M, Vives C, Escarceller M, Papadopoulo D. Molecular spectra of HPRT deletion mutations in circulating T- lymphocytes in Fanconi anemia patients. Mutat Res. 1999;431:341-350.
220. Sala-Trepat M, Boyse J, Richard P, Papadopoulo D, Moustacchi E. Frequencies of HPRT- lymphocytes and glycophorin A variants erythrocytes in Fanconi anemia patients, their parents and control donors. Mutat Res. 1993;289:115-126.
221. Escarceller M, Rousset S, Moustacchi E, Papadopoulo D. The fidelity of double strand breaks processing is impaired in complementation groups B and D of Fanconi anemia, a genetic instability syndrome. Somat Cell Mol Genet. 1997;23:401-411.
222. Escarceller M, Buchwald M, Singleton BK, et al. Fanconi anemia C gene product plays a role in the fidelity of blunt DNA end-joining. Journal of Molecular Biology. 1998;279:375-385.
223. Smith J, Andrau JC, Kallenbach S, Laquerbe A, Doyen N, Papadopoulo D. Abnormal rearrangements associated with V(D)J recombination in Fanconi anemia. J Mol Biol. 1998;281:815-825.
224. Thyagarajan B, Campbell C. Elevated homologous recombination activity in fanconi anemia fibroblasts. J Biol Chem. 1997;272:23328-23333.
225. Yamamoto K, Ishiai M, Matsushita N, et al. Fanconi anemia FANCG protein in mitigating radiation- and enzyme-induced DNA double-strand breaks by homologous recombination in vertebrate cells. Mol Cell Biol. 2003;23:5421-5430.
226. Yamamoto K, Hirano S, Ishiai M, et al. Fanconi anemia protein FANCD2 promotes immunoglobulin gene conversion and DNA repair through a mechanism related to homologous recombination. Mol Cell Biol. 2005;25:34-43.
227. Niedzwiedz W, Mosedale G, Johnson M, Ong CY, Pace P, Patel KJ. The Fanconi anaemia gene FANCC promotes homologous recombination and error-prone DNA repair. Mol Cell. 2004;15:607-620.
228. Fujiwara Y, Kano Y, Yamamoto Y. DNA interstrand cross-linking, repair, and SCE mechanism in human cells in special reference to Fanconi anemia. Basic Life Sci. 1984;29 Pt B:787-800.
229. Nakanishi K, Yang YG, Pierce AJ, et al. Human Fanconi anemia monoubiquitination pathway promotes homologous DNA repair. Proc Natl Acad Sci U S A. 2005;102:1110-1115.
230. Bessho T, Mu D, Sancar A. Initiation of DNA interstrand cross-link repair in humans: the nucleotide excision repair system makes dual incisions 5' to the cross- linked base and removes a 22- to 28-nucleotide-long damage-free strand. Mol Cell Biol. 1997;17:6822-6830.
231. Lambert MW, Tsongalis GJ, Lambert WC, Parrish DD. Correction of the DNA repair defect in Fanconi anemia complementation groups A and D cells. Biochem Biophys Res Commun. 1997;230:587-591.

232. Fujiwara Y. Defective repair of mitomycin C crosslinks in Fanconi's anemia and loss in confluent normal human and xeroderma pigmentosum cells. Biochim Biophys Acta. 1982;699:217-225.
233. Kumaresan KR, Lambert MW. Fanconi anemia, complementation group A, cells are defective in ability to produce incisions at sites of psoralen interstrand cross-links. Carcinogenesis. 2000;21:741-751.
234. Rothfuss A, Grompe M. Repair kinetics of genomic interstrand DNA cross-links: evidence for DNA double-strand break-dependent activation of the Fanconi anemia/BRCA pathway. Mol Cell Biol. 2004;24:123-134.
235. Pichierri P, Averbeck D, Rosselli F. DNA cross-link-dependent RAD50/MRE11/NBS1 subnuclear assembly requires the Fanconi anemia C protein. Hum Mol Genet. 2002;11:2531-2546.
236. Thompson LH, Hinz JM, Yamada NA, Jones NJ. How Fanconi anemia proteins promote the four Rs: replication, recombination, repair, and recovery. Environ Mol Mutagen. 2005;45:128-142.
237. Ball SE, Gibson FM, Rizzo S, Tooze JA, Marsh JC, Gordon S-EC. Progressive telomere shortening in aplastic anemia. Blood. 1998;91:3582-3592.
238. Kruk PA, Bohr VA. Telomeric length in individuals and cell lines with altered p53 status. Radiat Oncol Investig. 1999;7:13-21.
239. Hanson H, Mathew CG, Docherty Z, Mackie Ogilvie C. Telomere shortening in Fanconi anaemia demonstrated by a direct FISH approach. Cytogenet Cell Genet. 2001;93:203-206.
240. Leteurtre F, Li X, Guardiola P, et al. Accelerated telomere shortening and telomerase activation in Fanconi's anaemia. Br J Haematol. 1999;105:883-893.
241. Adelfalk C, Lorenz M, Serra V, von Zglinicki T, Hirsch-Kauffmann M, Schweiger M. Accelerated telomere shortening in Fanconi anemia fibroblasts--a longitudinal study. FEBS Lett. 2001;506:22-26.
242. Polychronopoulou S, Koutroumba P. Telomere length variation and telomerase activity expression in patients with congenital and acquired aplastic anemia. Acta Haematol. 2004;111:125-131.
243. Callen E, Samper E, Ramirez MJ, et al. Breaks at telomeres and TRF2-independent end fusions in Fanconi anemia. Hum Mol Genet. 2002;11:439-444.
244. Li X, Leteurtre F, Rocha V, et al. Abnormal telomere metabolism in Fanconi's anaemia correlates with genomic instability and the probability of developing severe aplastic anaemia. Br J Haematol. 2003;120:836-845.
245. Brummendorf TH, Rufer N, Holyoake TL, et al. Telomere length dynamics in normal individuals and in patients with hematopoietic stem cell-associated disorders. Ann N Y Acad Sci. 2001;938:293-303; discussion 303-294.
246. Franco S, van de Vrugt HJ, Fernandez P, Aracil M, Arwert F, Blasco MA. Telomere dynamics in Fancg-deficient mouse and human cells. Blood. 2004;104:3927-3935.
247. Reuter TY, Medhurst AL, Waisfisz Q, et al. Yeast two-hybrid screens imply involvement of Fanconi anemia proteins in transcription regulation, cell signaling, oxidative metabolism, and cellular transport. Exp Cell Res. 2003;289:211-221.
248. Duckworth-Rysiecki G, Cornish K, Clarke CA, Buchwald M. Identification of two complementation groups in Fanconi anemia. Somat Cell Mol Genet. 1985;11:35-41.

249. Pronk JC, Gibson RA, Savoia A, et al. Localisation of the Fanconi anaemia complementation group A gene to chromosome 16q24.3. Nat Genet. 1995;11:338-340.
250. Gschwend M, Levran O, Kruglyak L, et al. A locus for Fanconi anemia on 16q determined by homozygosity mapping. Am J Hum Genet. 1996;59:377-384.
251. Lo Ten Foe JR, Rooimans MA, Bosnoyan-Collins L, et al. Expression cloning of a cDNA for the major Fanconi anaemia gene, FAA. Nat Genet. 1996;14:320-323.
252. Positional cloning of the Fanconi anaemia group A gene. The Fanconi anaemia/breast cancer consortium. Nat Genet. 1996;14:324-328.
253. Ianzano L, D'Apolito M, Centra M, et al. The genomic organization of the Fanconi anemia group A (FAA) gene. Genomics. 1997;41:309-314.
254. Centra M, Memeo E, d'Apolito M, et al. Fine exon-intron structure of the Fanconi anemia group A (FAA) gene and characterization of two genomic deletions. Genomics. 1998;51:463-467.
255. Meetei AR, Levitus M, Xue Y, et al. X-linked inheritance of Fanconi anemia complementation group B. Nat Genet. 2004;36:1219-1224.
256. NCBI. National Center for Biotechnology Information. Entrez Nucleotide Database http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide.
257. Strathdee CA, Gavish H, Shannon WR, Buchwald M. Cloning of cDNAs for Fanconi's anemia by functional complementation. Nature. 1992;356:763-767.
258. Gibson RA, Ford D, Jansen S, et al. Genetic mapping of the FACC gene and linkage analysis in Fanconi anaemia families. J Med Genet. 1994;31:868-871.
259. Youssoufian H. Localization of Fanconi anemia C protein to the cytoplasm of mammalian cells. Proc Natl Acad Sci U S A. 1994;91:7975-7979.
260. Yamashita T, Barber DL, Zhu Y, Wu N, D'Andrea AD. The Fanconi anemia polypeptide FACC is localized to the cytoplasm. Proc Natl Acad Sci U S A. 1994;91:6712-6716.
261. Kruyt FA, Youssoufian H. The Fanconi anemia proteins FAA and FAC function in different cellular compartments to protect against cross-linking agent cytotoxicity. Blood. 1998;92:2229-2236.
262. Gibson RA, Buchwald M, Roberts RG, Mathew CG. Characterization of the exon structure of the fanconi anemia group C gene by vectorette PCR. Hum Mol Genet. 1993;2:35-38.
263. Wooster R, Bignell G, Lancaster J, et al. Identification of the breast cancer susceptibility gene BRCA2. Nature. 1995;378:789-792.
264. Wooster R, Neuhausen SL, Mangion J, et al. Localization of a breast cancer susceptibility gene, BRCA2, to chromosome 13q12-13. Science. 1994;265:2088-2090.
265. Tavtigian SV, Simard J, Rommens J, et al. The complete BRCA2 gene and mutations in chromosome 13q-linked kindreds. Nat Genet. 1996;12:333-337.
266. Hejna JA, Timmers CD, Reifsteck C, et al. Localization of the Fanconi Anemia Complementation Group D Gene to a 200-kb Region on Chromosome 3p25.3. Am J Hum Genet. 2000;66:1540-1551.
267. Timmers C, Taniguchi T, Hejna J, et al. Positional cloning of a novel Fanconi anemia gene, FANCD2. Mol Cell. 2001;7:241-248.
268. de Winter JP, Leveille F, van Berkel CG, et al. Isolation of a cDNA representing the Fanconi anemia complementation group E gene. Am J Hum Genet. 2000;67:1306-1308.

269. Pace P, Johnson M, Tan WM, et al. FANCE: the link between Fanconi anaemia complex assembly and activity. Embo J. 2002;21:3414-3423.
270. de Winter JP, Rooimans MA, van Der Weel L, et al. The fanconi anaemia gene FANCF encodes a novel protein with homology to ROM. Nat Genet. 2000;24:15-16.
271. de Winter JP, Waisfisz Q, Rooimans MA, et al. The Fanconi anaemia group G gene FANCG is identical with XRCC9. Nat Genet. 1998;20:281-283.
272. Cantor SB, Bell DW, Ganesan S, et al. BACH1, a novel helicase-like protein, interacts directly with BRCA1 and contributes to its DNA repair function. Cell. 2001;105:149-160.
273. Levran O, Attwooll C, Henry RT, et al. The BRCA1-interacting helicase BRIP1 is deficient in Fanconi anemia. Nat Genet. 2005;37:931-933.
274. Levitus M, Waisfisz Q, Godthelp BC, et al. The DNA helicase BRIP1 is defective in Fanconi anemia complementation group J. Nat Genet. 2005;37:934-935.
275. CCDS:. The Concensus CDS Database: http://www.ncbi.nlm.nih.gov/CCDS/CcdsBrowse.cgi.
276. Kruyt FA, Waisfisz Q, Dijkmans LM, et al. Cytoplasmic localization of a functionally active Fanconi anemia group A-green fluorescent protein chimera in human 293 cells. Blood. 1997;90:3288-3295.
277. Walsh CE, Yountz MR, Simpson DA. Intracellular localization of the Fanconi anemia complementation group A protein. Biochem Biophys Res Commun. 1999;259:594-599.
278. Lightfoot J, Alon N, Bosnoyan-Collins L, Buchwald M. Characterization of regions functional in the nuclear localization of the Fanconi anemia group A protein. Hum Mol Genet. 1999;8:1007-1015.
279. Kupfer G, Naf D, Garcia-Higuera I, et al. A patient-derived mutant form of the Fanconi anemia protein, FANCA, is defective in nuclear accumulation. Exp Hematol. 1999;27:587-593.
280. de Winter JP, van der Weel L, de Groot J, et al. The Fanconi anemia protein FANCF forms a nuclear complex with FANCA, FANCC and FANCG. Hum Mol Genet. 2000;9:2665-2674.
281. Naf D, Kupfer GM, Suliman A, Lambert K, D'Andrea AD. Functional activity of the Fanconi anemia protein FAA requires FAC binding and nuclear localization. Mol Cell Biol. 1998;18:5952-5960.
282. Yamashita T, Kupfer GM, Naf D, et al. The fanconi anemia pathway requires FAA phosphorylation and FAA/FAC nuclear accumulation. Proc Natl Acad Sci U S A. 1998;95:13085-13090.
283. Yagasaki H, Adachi D, Oda T, et al. A cytoplasmic serine protein kinase binds and may regulate the Fanconi anemia protein FANCA. Blood. 2001;98:3650-3657.
284. Otsuki T, Nagashima T, Komatsu N, et al. Phosphorylation of Fanconi anemia protein, FANCA, is regulated by Akt kinase. Biochem Biophys Res Commun. 2002;291:628-634.
285. Garcia-Higuera I, Kuang Y, Naf D, Wasik J, D'Andrea AD. Fanconi anemia proteins FANCA, FANCC, and FANCG/XRCC9 interact in a functional nuclear complex. Mol Cell Biol. 1999;19:4866-4873.

286. Waisfisz Q, de Winter JP, Kruyt FA, et al. A physical complex of the Fanconi anemia proteins FANCG/XRCC9 and FANCA. Proc Natl Acad Sci U S A. 1999;96:10320-10325.
287. Garcia-Higuera I, Kuang Y, Denham J, D'Andrea AD. The fanconi anemia proteins FANCA and FANCG stabilize each other and promote the nuclear accumulation of the Fanconi anemia complex. Blood. 2000;96:3224-3230.
288. Reuter T, Herterich S, Bernhard O, Hoehn H, Gross HJ. Strong FANCA/FANCG but weak FANCA/FANCC interaction in the yeast 2- hybrid system. Blood. 2000;95:719-720.
289. Kuang Y, Garcia-Higuera I, Moran A, Mondoux M, Digweed M, D'Andrea AD. Carboxy terminal region of the Fanconi anemia protein, FANCG/XRCC9, is required for functional activity. Blood. 2000;96:1625-1632.
290. Kruyt FA, Abou-Zahr F, Mok H, Youssoufian H. Resistance to mitomycin C requires direct interaction between the Fanconi anemia proteins FANCA and FANCG in the nucleus through an arginine-rich domain. J Biol Chem. 1999;274:34212-34218.
291. Kupfer GM, Naf D, Suliman A, Pulsipher M, D'Andrea AD. The Fanconi anaemia proteins, FAA and FAC, interact to form a nuclear complex. Nat Genet. 1997;17:487-490.
292. Christianson TA, Bagby GC. FANCA protein binds FANCG proteins in an intracellular complex. Blood. 2000;95:725-726.
293. Otsuki T, Kajigaya S, Ozawa K, Liu JM. SNX5, a new member of the sorting nexin family, binds to the Fanconi anemia complementation group A protein. Biochem Biophys Res Commun. 1999;265:630-635.
294. McMahon LW, Walsh CE, Lambert MW. Human alpha spectrin II and the Fanconi anemia proteins FANCA and FANCC interact to form a nuclear complex. J Biol Chem. 1999;274:32904-32908.
295. McMahon LW, Sangerman J, Goodman SR, Kumaresan K, Lambert MW. Human alpha spectrin II and the FANCA, FANCC, and FANCG proteins bind to DNA containing psoralen interstrand cross-links. Biochemistry. 2001;40:7025-7034.
296. Sridharan D, Brown M, Lambert WC, McMahon LW, Lambert MW. Nonerythroid alphaII spectrin is required for recruitment of FANCA and XPF to nuclear foci induced by DNA interstrand cross-links. J Cell Sci. 2003;116:823-835.
297. Lefferts JA, Lambert MW. Fanconi anemia cell lines deficient in alphaII spectrin express normal levels of alphaII spectrin mRNA. Biochem Biophys Res Commun. 2003;307:510-515.
298. Brois DW, McMahon LW, Ramos NI, Anglin LM, Walsh CE, Lambert MW. A deficiency in a 230 kDa DNA repair protein in fanconi anemia complementation group A cells is corrected by the FANCA cDNA. Carcinogenesis. 1999;20:1845-1853.
299. Otsuki T, Furukawa Y, Ikeda K, et al. Fanconi anemia protein, FANCA, associates with BRG1, a component of the human SWI/SNF complex. Hum Mol Genet. 2001;10:2651-2660.
300. Hoatlin ME, Zhi Y, Ball H, et al. A novel BTB/POZ transcriptional repressor protein interacts with the Fanconi anemia group C protein and PLZF. Blood. 1999;94:3737-3747.
301. Levran O, Erlich T, Magdalena N, et al. Sequence variation in the Fanconi anemia gene FAA. Proc Natl Acad Sci U S A. 1997;94:13051-13056.

302. Savino M, Ianzano L, Strippoli P, et al. Mutations of the Fanconi anemia group A gene (FAA) in Italian patients. Am J Hum Genet. 1997;61:1246-1253.
303. Tachibana A, Kato T, Ejima Y, et al. The FANCA gene in Japanese Fanconi anemia: reports of eight novel mutations and analysis of sequence variability. Hum Mutat. 1999;13:237-244.
304. Nakamura A, Matsuura S, Tauchi H, et al. Four novel mutations of the Fanconi anemia group A gene (FAA) in Japanese patients. J Hum Genet. 1999;44:48-51.
305. Wijker M, Morgan NV, Herterich S, et al. Heterogeneous spectrum of mutations in the Fanconi anaemia group A gene. Eur J Hum Genet. 1999;7:52-59.
306. Tamary H, Bar-Yam R, Shalmon L, et al. Fanconi anaemia group A (FANCA) mutations in Israeli non-Ashkenazi Jewish patients. Br J Haematol. 2000;111:338-343.
307. Adachi D, Oda T, Yagasaki H, et al. Heterogeneous activation of the Fanconi anemia pathway by patient-derived FANCA mutants. Hum Mol Genet. 2002;11:3125-3134.
308. Bouchlaka C, Abdelhak S, Amouri A, et al. Fanconi anemia in Tunisia: high prevalence of group A and identification of new FANCA mutations. J Hum Genet. 2003;48:352-361.
309. Levran O, Diotti R, Pujara K, Batish SD, Hanenberg H, Auerbach AD. Spectrum of sequence variations in the FANCA gene: an International Fanconi Anemia Registry (IFAR) study. Hum Mutat. 2005;25:142-149.
310. Auerbach A, Schindler D. Fanconi Anemia Mutation Database.
311. Balta G, de Winter JP, Kayserili H, Pronk JC, Joenje H. Fanconi anemia A due to a novel frameshift mutation in hotspot motifs: lack of FANCA protein. Hum Mutat. 2000;15:578.
312. Levran O, Doggett NA, Auerbach AD. Identification of Alu-mediated deletions in the Fanconi anemia gene FAA. Hum Mutat. 1998;12:145-152.
313. Morgan NV, Tipping AJ, Joenje H, Mathew CG. High frequency of large intragenic deletions in the Fanconi anemia group A gene. Am J Hum Genet. 1999;65:1330-1341.
314. Rudiger NS, Gregersen N, Kielland-Brandt MC. One short well conserved region of Alu-sequences is involved in human gene rearrangements and has homology with prokaryotic chi. Nucleic Acids Res. 1995;23:256-260.
315. Buchwald M. Complementation groups: one or more per gene? Nat Genet. 1995;11:228-230.
316. Jakobs PM, Fiddler-Odell E, Reifsteck C, Olson S, Moses RE, Grompe M. Complementation group assignments in Fanconi anemia fibroblast cell lines from North America. Somat Cell Mol Genet. 1997;23:1-7.
317. Joenje H, Levitus M, Waisfisz Q, et al. Complementation analysis in Fanconi anemia: assignment of the reference FA-H patient to group A. Am J Hum Genet. 2000;67:759-762.
318. Youssoufian H. Cytoplasmic localization of FAC is essential for the correction of a prerepair defect in Fanconi anemia group C cells. J Clin Invest. 1996;97:2003-2010.
319. Hoatlin ME, Christianson TA, Keeble WW, et al. The Fanconi anemia group C gene product is located in both the nucleus and cytoplasm of human cells. Blood. 1998;91:1418-1425.

320. Segal GM, Magenis RE, Brown M, et al. Repression of Fanconi anemia gene (FACC) expression inhibits growth of hematopoietic progenitor cells. J Clin Invest. 1994;94:846-852.
321. Kupfer GM, Yamashita T, Naf D, Suliman A, Asano S, D'Andrea AD. The Fanconi anemia polypeptide, FAC, binds to the cyclin-dependent kinase, cdc2. Blood. 1997;90:1047-1054.
322. Liebetrau W, Budde A, Savoia A, Grummt F, Hoehn H. p53 activates Fanconi anemia group C gene expression. Hum Mol Genet. 1997;6:277-283.
323. Garcia-Higuera I, D'Andrea AD. Regulated binding of the Fanconi anemia proteins, FANCA and FANCC. Blood. 1999;93:1430-1432.
324. Busch DB, Zdzienicka MZ, Natarajan AT, et al. A CHO mutant, UV40, that is sensitive to diverse mutagens and represents a new complementation group of mitomycin C sensitivity. Mutat Res. 1996;363:209-221.
325. Qiao F, Mi J, Wilson JB, et al. Phosphorylation of fanconi anemia (FA) complementation group G protein, FANCG, at serine 7 is important for function of the FA pathway. J Biol Chem. 2004;279:46035-46045.
326. Futaki M, Watanabe S, Kajigaya S, Liu JM. Fanconi anemia protein, FANCG, is a phosphoprotein and is upregulated with FANCA after TNF-alpha treatment. Biochem Biophys Res Commun. 2001;281:347-351.
327. Leveille F, Blom E, Medhurst AL, et al. The Fanconi anemia gene product FANCF is a flexible adaptor protein. J Biol Chem. 2004;279:39421-39430.
328. Tischkowitz M, Ameziane N, Waisfisz Q, et al. Bi-allelic silencing of the Fanconi anaemia gene FANCF in acute myeloid leukaemia. Br J Haematol. 2003;123:469-471.
329. Taniguchi T, Tischkowitz M, Ameziane N, et al. Disruption of the Fanconi anemia-BRCA pathway in cisplatin-sensitive ovarian tumors. Nat Med. 2003;9:568-574.
330. Olopade OI, Wei M. FANCF methylation contributes to chemoselectivity in ovarian cancer. Cancer Cell. 2003;3:417-420.
331. Bagby GC, Olson SB. Cisplatin and the sensitive cell. Nat Med. 2003;9:513-514.
332. Narayan G, Arias-Pulido H, Nandula SV, et al. Promoter hypermethylation of FANCF: disruption of Fanconi Anemia-BRCA pathway in cervical cancer. Cancer Res. 2004;64:2994-2997.
333. Medhurst AL, Huber PA, Waisfisz Q, de Winter JP, Mathew CG. Direct interactions of the five known Fanconi anaemia proteins suggest a common functional pathway. Hum Mol Genet. 2001;10:423-429.
334. Gordon SM, Buchwald M. Fanconi anemia protein complex: mapping protein interactions in the yeast 2- and 3-hybrid systems. Blood. 2003;102:136-141.
335. Taniguchi T, D'Andrea AD. The Fanconi anemia protein, FANCE, promotes the nuclear accumulation of FANCC. Blood. 2002;100:2457-2462.
336. Whitney M, Thayer M, Reifsteck C, et al. Microcell mediated chromosome transfer maps the Fanconi anaemia group D gene to chromosome 3p. Nat Genet. 1995;11:341-343.
337. Andreassen PR, D'Andrea AD, Taniguchi T. ATR couples FANCD2 monoubiquitination to the DNA-damage response. Genes Dev. 2004;18:1958-1963.
338. Taniguchi T, Garcia-Higuera I, Andreassen PR, Gregory RC, Grompe M, D'Andrea AD. S-phase-specific interaction of the Fanconi anemia protein, FANCD2, with BRCA1 and RAD51. Blood. 2002;100:2414-2420.

339. Montes de Oca R, Andreassen PR, Margossian SP, et al. Regulated interaction of the Fanconi anemia protein, FANCD2, with chromatin. Blood. 2005;105:1003-1009.
340. Taniguchi T, Garcia-Higuera I, Xu B, et al. Convergence of the fanconi anemia and ataxia telangiectasia signaling pathways. Cell. 2002;109:459-472.
341. Nakanishi K, Taniguchi T, Ranganathan V, et al. Interaction of FANCD2 and NBS1 in the DNA damage response. Nat Cell Biol. 2002;4:913-920.
342. Spain BH, Larson CJ, Shihabuddin LS, Gage FH, Verma IM. Truncated BRCA2 is cytoplasmic: implications for cancer-linked mutations. Proc Natl Acad Sci U S A. 1999;96:13920-13925.
343. Chen J, Silver DP, Walpita D, et al. Stable interaction between the products of the BRCA1 and BRCA2 tumor suppressor genes in mitotic and meiotic cells. Mol Cell. 1998;2:317-328.
344. Chen JJ, Silver D, Cantor S, Livingston DM, Scully R. BRCA1, BRCA2, and Rad51 operate in a common DNA damage response pathway. Cancer Res. 1999;59:1752s-1756s.
345. Xia F, Taghian DG, DeFrank JS, et al. Deficiency of human BRCA2 leads to impaired homologous recombination but maintains normal nonhomologous end joining. Proc Natl Acad Sci U S A. 2001;98:8644-8649.
346. Moynahan ME, Pierce AJ, Jasin M. BRCA2 is required for homology-directed repair of chromosomal breaks. Mol Cell. 2001;7:263-272.
347. Davies AA, Masson JY, McIlwraith MJ, et al. Role of BRCA2 in control of the RAD51 recombination and DNA repair protein. Mol Cell. 2001;7:273-282.
348. Lomonosov M, Anand S, Sangrithi M, Davies R, Venkitaraman AR. Stabilization of stalled DNA replication forks by the BRCA2 breast cancer susceptibility protein. Genes Dev. 2003;17:3017-3022.
349. Venkitaraman AR. A growing network of cancer-susceptibility genes. N Engl J Med. 2003;348:1917-1919.
350. Venkitaraman AR. Tracing the network connecting BRCA and Fanconi anaemia proteins. Nat Rev Cancer. 2004;4:266-276.
351. Kinzler KW, Vogelstein B. Cancer-susceptibility genes. Gatekeepers and caretakers. Nature. 1997;386:761, 763.
352. Thorlacius S, Tryggvadottir L, Olafsdottir GH, et al. Linkage to BRCA2 region in hereditary male breast cancer. Lancet. 1995;346:544-545.
353. Easton DF, Steele L, Fields P, et al. Cancer risks in two large breast cancer families linked to BRCA2 on chromosome 13q12-13. Am J Hum Genet. 1997;61:120-128.
354. Gronberg H, Ahman AK, Emanuelsson M, Bergh A, Damber JE, Borg A. BRCA2 mutation in a family with hereditary prostate cancer. Genes Chromosomes Cancer. 2001;30:299-301.
355. Edwards SM, Kote-Jarai Z, Meitz J, et al. Two percent of men with early-onset prostate cancer harbor germline mutations in the BRCA2 gene. Am J Hum Genet. 2003;72:1-12.
356. Ozcelik H, Schmocker B, Di Nicola N, et al. Germline BRCA2 6174delT mutations in Ashkenazi Jewish pancreatic cancer patients. Nat Genet. 1997;16:17-18.
357. Offit K, Levran O, Mullaney B, et al. Shared genetic susceptibility to breast cancer, brain tumors, and Fanconi anemia. J Natl Cancer Inst. 2003;95:1548-1551.

358. Hirsch B, Shimamura A, Moreau L, et al. Association of biallelic BRCA2/FANCD1 mutations with spontaneous chromosomal instability and solid tumors of childhood. Blood. 2004;103:2554-2559.
359. Ikeda H, Matsushita M, Waisfisz Q, et al. Genetic reversion in an acute myelogenous leukemia cell line from a Fanconi anemia patient with biallelic mutations in BRCA2. Cancer Res. 2003;63:2688-2694.
360. Hussain S, Witt E, Huber PA, Medhurst AL, Ashworth A, Mathew CG. Direct interaction of the Fanconi anaemia protein FANCG with BRCA2/FANCD1. Hum Mol Genet. 2003;12:2503-2510.
361. Hussain S, Wilson JB, Medhurst AL, et al. Direct interaction of FANCD2 with BRCA2 in DNA damage response pathways. Hum Mol Genet. 2004;13:1241-1248.
362. Rogers CD, van der Heijden MS, Brune K, et al. The genetics of FANCC and FANCG in familial pancreatic cancer. Cancer Biol Ther. 2004;3:167-169.
363. van der Heijden MS, Brody JR, Gallmeier E, et al. Functional defects in the fanconi anemia pathway in pancreatic cancer cells. Am J Pathol. 2004;165:651-657.
364. Meetei AR, Sechi S, Wallisch M, et al. A multiprotein nuclear complex connects Fanconi anemia and Bloom syndrome. Mol Cell Biol. 2003;23:3417-3426.
365. Meetei AR, Yan Z, Wang W. FANCL replaces BRCA1 as the likely ubiquitin ligase responsible for FANCD2 monoubiquitination. Cell Cycle. 2004;3:179-181.
366. Grompe M. FANCL, as in ligase. Nature Genetics. 2003;35:113-114.
367. Fei P, Yin J, Wang W. New advances in the DNA damage response network of Fanconi anemia and BRCA proteins. FAAP95 replaces BRCA2 as the true FANCB protein. Cell Cycle. 2005;4:80-86.
368. Litman R, Peng M, Jin Z, et al. BACH1 is critical for homologous recombination and appears to be the Fanconi anemia gene product FANCJ. Cancer Cell. 2005;8:255-265.
369. Gupta R, Sharma S, Sommers JA, Jin Z, Cantor SB, Brosh RM, Jr. Analysis of the DNA substrate specificity of the human BACH1 helicase associated with breast cancer. J Biol Chem. 2005;280:25450-25460.
370. Thompson LH. Unraveling the Fanconi anemia-DNA repair connection. Nat Genet. 2005;37:921-922.
371. Qiao F, Moss A, Kupfer GM. Fanconi anemia proteins localize to chromatin and the nuclear matrix in a DNA damage- and cell cycle-regulated manner. J Biol Chem. 2001;276:23391-23396.
372. Muchardt C, Reyes JC, Bourachot B, Leguoy E, Yaniv M. The hbrm and BRG-1 proteins, components of the human SNF/SWI complex, are phosphorylated and excluded from the condensed chromosomes during mitosis. Embo J. 1996;15:3394-3402.
373. Muchardt C, Yaniv M. The mammalian SWI/SNF complex and the control of cell growth. Semin Cell Dev Biol. 1999;10:189-195.
374. Muchardt C, Yaniv M. ATP-dependent chromatin remodelling: SWI/SNF and Co. are on the job. J Mol Biol. 1999;293:187-198.
375. Reyes JC, Muchardt C, Yaniv M. Components of the human SWI/SNF complex are enriched in active chromatin and are associated with the nuclear matrix. J Cell Biol. 1997;137:263-274.
376. Mi J, Kupfer GM. The Fanconi anemia core complex associates with chromatin during S phase. Blood. 2005;105:759-766.

377. van de Vrugt HJ, Cheng NC, de Vries Y, et al. Cloning and characterization of murine fanconi anemia group A gene: Fanca protein is expressed in lymphoid tissues, testis, and ovary. Mamm Genome. 2000;11:326-331.
378. Wong JC, Alon N, Norga K, Kruyt FA, Youssoufian H, Buchwald M. Cloning and analysis of the mouse Fanconi anemia group A cDNA and an overlapping penta zinc finger cDNA. Genomics. 2000;67:273-283.
379. Abu-Issa R, Eichele G, Youssoufian H. Expression of the Fanconi anemia group A gene (Fanca) during mouse embryogenesis. Blood. 1999;94:818-824.
380. Chen M, Tomkins DJ, Auerbach W, et al. Inactivation of Fac in mice produces inducible chromosomal instability and reduced fertility reminiscent of Fanconi anaemia. Nat Genet. 1996;12:448-451.
381. Koomen M, Cheng NC, van de Vrugt HJ, et al. Reduced fertility and hypersensitivity to mitomycin C characterize Fancg/Xrcc9 null mice. Hum Mol Genet. 2002;11:273-281.
382. Cheng NC, van de Vrugt HJ, van der Valk MA, et al. Mice with a targeted disruption of the Fanconi anemia homolog Fanca. Hum Mol Genet. 2000;9:1805-1811.
383. Rio P, Segovia JC, Hanenberg H, et al. In vitro phenotypic correction of hematopoietic progenitors from Fanconi anemia group A knockout mice. Blood. 2002;100:2032-2039.
384. Wong JC, Alon N, McKerlie C, Huang JR, Meyn MS, Buchwald M. Targeted disruption of exons 1 to 6 of the Fanconi Anemia group A gene leads to growth retardation, strain-specific microphthalmia, meiotic defects and primordial germ cell hypoplasia. Hum Mol Genet. 2003;12:2063-2076.
385. Agoulnik AI, Lu B, Zhu Q, et al. A novel gene, Pog, is necessary for primordial germ cell proliferation in the mouse and underlies the germ cell deficient mutation, gcd. Hum Mol Genet. 2002;11:3047-3053.
386. Ludwig T, Chapman DL, Papaioannou VE, Efstratiadis A. Targeted mutations of breast cancer susceptibility gene homologs in mice: lethal phenotypes of Brca1, Brca2, Brca1/Brca2, Brca1/p53, and Brca2/p53 nullizygous embryos. Genes Dev. 1997;11:1226-1241.
387. Suzuki A, de la Pompa JL, Hakem R, et al. Brca2 is required for embryonic cellular proliferation in the mouse. Genes Dev. 1997;11:1242-1252.
388. Lee H, Trainer AH, Friedman LS, et al. Mitotic checkpoint inactivation fosters transformation in cells lacking the breast cancer susceptibility gene, Brca2. Mol Cell. 1999;4:1-10.
389. Jonkers J, Meuwissen R, van der Gulden H, Peterse H, van der Valk M, Berns A. Synergistic tumor suppressor activity of BRCA2 and p53 in a conditional mouse model for breast cancer. Nat Genet. 2001;29:418-425.
390. Lo Ten Foe JR, Kwee ML, Rooimans MA, et al. Somatic mosaicism in Fanconi anemia: molecular basis and clinical significance. Eur J Hum Genet. 1997;5:137-148.
391. Waisfisz Q, Morgan NV, Savino M, et al. Spontaneous functional correction of homozygous fanconi anaemia alleles reveals novel mechanistic basis for reverse mosaicism. Nat Genet. 1999;22:379-383.
392. Gregory JJ, Jr., Wagner JE, Verlander PC, et al. Somatic mosaicism in Fanconi anemia: evidence of genotypic reversion in lymphohematopoietic stem cells. Proc Natl Acad Sci U S A. 2001;98:2532-2537.

393. Hao QL, Thiemann FT, Petersen D, Smogorzewska EM, Crooks GM. Extended long-term culture reveals a highly quiescent and primitive human hematopoietic progenitor population. Blood. 1996;88:3306-3313.
394. Gross M, Hanenberg H, Lobitz S, et al. Reverse mosaicism in Fanconi anemia: natural gene therapy via molecular self-correction. Cytogenet Genome Res. 2002;98:126-135.
395. Soulier J, Leblanc T, Larghero J, et al. Detection of somatic mosaicism and classification of Fanconi anemia patients by analysis of the FA/BRCA pathway. Blood. 2005;105:1329-1336.
396. Battaile K, Bateman R, Mortimer D, et al. In vivo selection of wild-type hematopoietic stem cells in a murine model of Fanconi anemia. Blood. 1999;94:2151-2158.
397. Noll M, Bateman RL, D'Andrea AD, Grompe M. Preclinical protocol for in vivo selection of hematopoietic stem cells corrected by gene therapy in Fanconi anemia group C. Mol Ther. 2001;3:14-23.
398. Galimi F, Noll M, Kanazawa Y, et al. Gene therapy of Fanconi anemia: preclinical efficacy using lentiviral vectors. Blood. 2002;100:2732-2736.
399. Duckworth-Rysiecki G, Toji L, Ng J, Clarke C, Buchwald M. Characterization of a simian virus 40-transformed Fanconi anemia fibroblast cell line. Mutat Res. 1986;166:207-214.
400. Medin JA, Tudor M, Simovitch R, et al. Correction in trans for Fabry disease: expression, secretion and uptake of alpha-galactosidase A in patient-derived cells driven by a high-titer recombinant retroviral vector. Proc Natl Acad Sci U S A. 1996;93:7917-7922.
401. Miller AD, Buttimore C. Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. Mol Cell Biol. 1986;6:2895-2902.
402. Lensch MW, Tischkowitz M, Christianson TA, et al. Acquired FANCA dysfunction and cytogenetic instability in adult acute myelogenous leukemia. Blood. 2003;102:7-16.
403. Newell AE, Akkari YM, Torimaru Y, et al. Interstrand crosslink-induced radials form between non-homologous chromosomes, but are absent in sex chromosomes. DNA Repair (Amst). 2004;3:535-542.
404. Pulsipher M, Kupfer GM, Naf D, et al. Subtyping analysis of Fanconi anemia by immunoblotting and retroviral gene transfer. Mol Med. 1998;4:468-479.
405. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 1988;16:1215.
406. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem. 1987;162:156-159.
407. Ory DS, Neugeboren BA, Mulligan RC. A stable human-derived packaging cell line for production of high titer retrovirus/vesicular stomatitis virus G pseudotypes. Proc Natl Acad Sci U S A. 1996;93:11400-11406.
408. van den Hoff MJB, Moorman AFM, Lamers WH. Electroporation in 'intracellular' buffer increases cell survival. Nucl Acids Res. 1992;20:2902.

409. Tanaka M, Kyo S, Kanaya T, et al. Evidence of the monoclonal composition of human endometrial epithelial glands and mosaic pattern of clonal distribution in luminal epithelium. Am J Pathol. 2003;163:295-301.
410. Senapathy P, Shapiro MB, Harris NL. Splice junctions, branch point sites, and exons: sequence statistics, identification, and applications to genome project. Methods Enzymol. 1990;183:252-278.
411. Shapiro MB, Senapathy P. RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression. Nucleic Acids Res. 1987;15:7155-7174.
412. Shimamura A, de Oca RM, Svenson JL, et al. A novel diagnostic screen for defects in the Fanconi anemia pathway. Blood. 2002;100:4649-4654.
413. Goodship J, Carter J, Burn J. X-inactivation patterns in monozygotic and dizygotic female twins. Am J Med Genet. 1996;61:205-208.
414. Jorgensen AL, Philip J, Raskind WH, et al. Different patterns of X inactivation in MZ twins discordant for red-green color-vision deficiency. Am J Hum Genet. 1992;51:291-298.
415. Tiberio G. MZ female twins discordant for X-linked diseases: a review. Acta Genet Med Gemellol (Roma). 1994;43:207-214.
416. Vickers MA, McLeod E, Spector TD, Wilson IJ. Assessment of mechanism of acquired skewed X inactivation by analysis of twins. Blood. 2001;97:1274-1281.
417. Abbadi N, Philippe C, Chery M, et al. Additional case of female monozygotic twins discordant for the clinical manifestations of Duchenne muscular dystrophy due to opposite X-chromosome inactivation. Am J Med Genet. 1994;52:198-206.
418. Redonnet-Vernhet I, Ploos van Amstel JK, Jansen RP, Wevers RA, Salvayre R, Levade T. Uneven X inactivation in a female monozygotic twin pair with Fabry disease and discordant expression of a novel mutation in the alpha-galactosidase A gene. J Med Genet. 1996;33:682-688.
419. Richards CS, Watkins SC, Hoffman EP, et al. Skewed X inactivation in a female MZ twin results in Duchenne muscular dystrophy. Am J Hum Genet. 1990;46:672-681.
420. Watkiss E, Webb T, Rysiecki G, Girdler N, Hewett E, Bundey S. X inactivation patterns in female monozygotic twins and their families. J Med Genet. 1994;31:754-757.
421. Weiss MC, Green H. Human-mouse hybrid cell lines containing partial complements of human chromosomes and functioning human genes. Proc Natl Acad Sci U S A. 1967;58:1104-1111.
422. McBride OW, Ozer HL. Transfer of genetic information by purified metaphase chromosomes. Proc Natl Acad Sci U S A. 1973;70:1258-1262.
423. McBride OW, Athwal RS. Genetic analysis by chromosome-mediated gene transfer. In Vitro. 1976;12:777-786.
424. Wullems GJ, van der Horst J, Bootsma D. Transfer of the human genes coding for thymidine kinase and galactokinase to Chinese hamster cells and human-Chinese hamster cell hybrids. Somatic Cell Genet. 1977;3:281-293.
425. Ege T, Ringertz NR. Preparation of microcells by enucleation of micronucleate cells. Exp Cell Res. 1974;87:378-382.
426. Kozak CA, Ruddle FH. Assignment of the genes for thymidine kinase and galactokinase to Mus musculus chromosome 11 and the preferential segregation of this

chromosome in Chinese hamster/mouse somatic cell hybrids. Somatic Cell Genet. 1977;3:121-133.
427. Ruddle NH, Conta BS, Leinwand L, et al. Assignment of the receptor for ecotropic murine leukemia virus to mouse chromosome 5. J Exp Med. 1978;148:451-465.
428. Kozak CA, Fournier RE, Leinwand LA, Ruddle FH. Assignment of the gene governing cellular ouabain resistance to Mus musculus chromosome 3 using human/mouse microcell hybrids. Biochem Genet. 1979;17:23-34.
429. Fournier RE, Moran RG. Complementation mapping in microcell hybrids: localization of Fpgs and Ak-1 on Mus musculus chromosome 2. Somatic Cell Genet. 1983;9:69-84.
430. Schultz RA, Saxon PJ, Glover TW, Friedberg EC. Microcell-mediated transfer of a single human chromosome complements xeroderma pigmentosum group A fibroblasts. Proc Natl Acad Sci U S A. 1987;84:4176-4179.
431. Legerski RJ, Liu P, Li L, et al. Assignment of xeroderma pigmentosum group C (XPC) gene to chromosome 3p25. Genomics. 1994;21:266-269.
432. Athwal RS, Kaur GP. Complementation Mapping in Microcell Hybrids: Localization of XRCC4 to 5q15-q21. Methods. 1996;9:12-19.
433. Leach RJ, Thayer MJ, Schafer AJ, Fournier RE. Physical mapping of human chromosome 17 using fragment-containing microcell hybrids. Genomics. 1989;5:167-176.
434. England NL, Cuthbert AP, Trott DA, et al. Identification of human tumour suppressor genes by monochromosome transfer: rapid growth-arrest response mapped to 9p21 is mediated solely by the cyclin-D-dependent kinase inhibitor gene, CDKN2A (p16INK4A). Carcinogenesis. 1996;17:1567-1575.
435. Kugoh H, Mitsuya K, Meguro M, Shigenami K, Schulz TC, Oshimura M. Mouse A9 cells containing single human chromosomes for analysis of genomic imprinting. DNA Res. 1999;6:165-172.
436. Devore-Carter DL, Pietrzak E, Kakati S. A method to generate microcells from human lymphoblasts for use in microcell mediated chromosome transfer. In Vitro Cell Dev Biol. 1986;22:615-620.
437. Sanford JA, Stubblefield E. General protocol for microcell-mediated chromosome transfer. Somat Cell Mol Genet. 1987;13:279-284.
438. Fournier RE. A general high-efficiency procedure for production of microcell hybrids. Proc Natl Acad Sci U S A. 1981;78:6349-6353.
439. Fournier RE, Ruddle FH. Microcell-mediated transfer of murine chromosomes into mouse, Chinese hamster, and human somatic cells. Proc Natl Acad Sci U S A. 1977;74:319-323.
440. Liscum L, Luskey KL, Chin DJ, Ho YK, Goldstein JL, Brown MS. Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase and its mRNA in rat liver as studied with a monoclonal antibody and a cDNA probe. J Biol Chem. 1983;258:8450-8455.
441. Mercer WE, Schlegel RA. Phytohemagglutinin enhancement of cell fusion reduces polyethylene glycol cytotoxicity. Exp Cell Res. 1979;120:417-421.
442. Overturf K, Al-Dhalimy M, Tanguay R, et al. Hepatocytes corrected by gene therapy are selected in vivo in a murine model of hereditary tyrosinaemia type I. Nat Genet. 1996;12:266-273.

443. Markowitz D, Goff S, Bank A. Construction and use of a safe and efficient amphotropic packaging cell line. Virology. 1988;167:400-406.
444. Simmler MC, Cox RD, Avner P. Adaptation of the interspersed repetitive sequence polymerase chain reaction to the isolation of mouse DNA probes from somatic cell hybrids on a hamster background. Genomics. 1991;10:770-778.
445. Cox RD, Copeland NG, Jenkins NA, Lehrach H. Interspersed repetitive element polymerase chain reaction product mapping using a mouse interspecific backcross. Genomics. 1991;10:375-384.
446. Burman RW, Anoe KS, Popovich BW. Fragile X full mutations are more similar in siblings than in unrelated patients: further evidence for a familial factor in CGG repeat dynamics. Genet Med. 2000;2:242-248.
447. Hartwell LH, Weinert TA. Checkpoints: controls that ensure the order of cell cycle events. Science. 1989;246:629-634.
448. Sekiguchi T, Shelton K, Ringertz NR. DNA content of microcells prepared from rat kangaroo and mouse cells. Exp Cell Res. 1978;113:247-258.
449. Prescott DM, Myerson D, Wallace J. Enucleation of mammalian cells with cytochalasin B. Exp Cell Res. 1972;71:480-485.
450. Fournier RE, Ruddle FH. Stable association of the human transgenome and host murine chromosomes demonstrated with trispecific microcell hybrids. Proc Natl Acad Sci U S A. 1977;74:3937-3941.
451. McNeill CA, Brown RL. Genetic manipulation by means of microcell-mediated transfer of normal human chromosomes into recipient mouse cells. Proc Natl Acad Sci U S A. 1980;77:5394-5398.
452. Tunnacliffe A, Parkar M, Povey S, et al. Integration of Ecogpt and SV40 early region sequences into human chromosome 17: a dominant selection system in whole cell and microcell human-mouse hybrids. Embo J. 1983;2:1577-1584.
453. Athwal RS, Smarsh M, Searle BM, Deo SS. Integration of a dominant selectable marker into human chromosomes and transfer of marked chromosomes to mouse cells by microcell fusion. Somat Cell Mol Genet. 1985;11:177-187.
454. Saxon PJ, Srivatsan ES, Leipzig GV, Sameshima JH, Stanbridge EJ. Selective transfer of individual human chromosomes to recipient cells. Mol Cell Biol. 1985;5:140-146.
455. Lugo TG, Handelin B, Killary AM, Housman DE, Fournier REK. Isolation of microcell hybrid clones containing retroviral vector insertions into specific human chromosomes. Mol Cell Biol. 1987;7:2814-2820.
456. Cuthbert AP, Trott DA, Ekong RM, et al. Construction and characterization of a highly stable human: rodent monochromosomal hybrid panel for genetic complementation and genome mapping studies. Cytogenetics and Cell Genetics. 1995;71:68-76.
457. Kramerov DA, Grigoryan AA, Ryskov AP, Georgiev GP. Long double-stranded sequences (dsRNA-B) of nuclear pre-mRNA consist of a few highly abundant classes of sequences: evidence from DNA cloning experiments. Nucleic Acids Res. 1979;6:697-713.
458. Jakobs PM, Smith L, Thayer M, Grompe M. Embryonic stem cells can be used to construct hybrid cell lines containing a single, selectable murine chromosome. Mamm Genome. 1999;10:381-384.

459. Turker MS, Gage BM, Rose JA, et al. Solid tissues removed from ATM homozygous deficient mice do not exhibit a mutator phenotype for second-step autosomal mutations. Cancer Res. 1999;59:4781-4783.
460. Pecker I, Avraham KB, Gilbert DJ, et al. Identification and chromosomal localization of Atm, the mouse homolog of the ataxia-telangiectasia gene. Genomics. 1996;35:39-45.
461. Barlow C, Hirotsune S, Paylor R, et al. Atm-deficient mice: a paradigm of ataxia telangiectasia. Cell. 1996;86:159-171.
462. Kelly MA, Rubinstein M, Phillips TJ, et al. Locomotor activity in D2 dopamine receptor-deficient mice is determined by gene dosage, genetic background, and developmental adaptations. J Neurosci. 1998;18:3470-3479.
463. MGI. Mouse Genome Informatics http://www.informatics.jax.org/.
464. Tomiyama K, McNamara FN, Clifford JJ, et al. Comparative phenotypic resolution of spontaneous, D2-like and D1-like agonist-induced orofacial movement topographies in congenic mutants with dopamine D2 vs. D3 receptor "knockout". Synapse. 2004;51:71-81.
465. Dowdy SF, Scanlon DJ, Fasching CL, Casey G, Stanbridge EJ. Irradiation microcell-mediated chromosome transfer (XMMCT): the generation of specific chromosomal arm deletions. Genes Chromosomes Cancer. 1990;2:318-327.
466. Cosma MP, Pepe S, Annunziata I, et al. The multiple sulfatase deficiency gene encodes an essential and limiting factor for the activity of sulfatases. Cell. 2003;113:445-456.
467. Guidetti F, Grazioli S, Capelli F, et al. Primitive hematopoietic stem cells shows a polyclonal pattern in myelodysplastic syndromes. Haematologica. 2004;89:21-28.
468. Kuramoto K, Follmann DA, Hematti P, et al. Effect of chronic cytokine therapy on clonal dynamics in nonhuman primates. Blood. 2004;103:4070-4077.
469. Greaves MF, Maia AT, Wiemels JL, Ford AM. Leukemia in twins: lessons in natural history. Blood. 2003;102:2321-2333.
470. Ossareh-Nazari B, Bachelerie F, Dargemont C. Evidence for a role of CRM1 in signal-mediated nuclear protein export. Science. 1997;278:141-144.
471. Watanabe M, Fukuda M, Yoshida M, Yanagida M, Nishida E. Involvement of CRM1, a nuclear export receptor, in mRNA export in mammalian cells and fission yeast. Genes Cells. 1999;4:291-297.
472. la Cour T, Gupta R, Rapacki K, Skriver K, Poulsen FM, Brunak S. NESbase version 1.0: a database of nuclear export signals. Nucleic Acids Res. 2003;31:393-396.
473. la Cour T, Kiemer L, Molgaard A, Gupta R, Skriver K, Brunak S. Analysis and prediction of leucine-rich nuclear export signals. Protein Eng Des Sel. 2004;17:527-536.
474. Ferrer M, Rodriguez JA, Spierings EA, de Winter JP, Giaccone G, Kruyt FA. Identification of multiple nuclear export sequences in Fanconi anemia group A protein that contribute to CRM1-dependent nuclear export. Hum Mol Genet. 2005;14:1271-1281.
475. Stommel JM, Marchenko ND, Jimenez GS, Moll UM, Hope TJ, Wahl GM. A leucine-rich nuclear export signal in the p53 tetramerization domain: regulation of subcellular localization and p53 activity by NES masking. Embo J. 1999;18:1660-1672.

Microcell Mediated Mouse Chromosome Transfer (MMMCT) Protocol

Written by Anuj Mankad (6/12/01), based on protocols by Jeff Lipps and Petra Jakobs, Ph.D., formulatedwith assistance from Maria Pia Cosma, Ph.D.

Special Reagents:

Bullets = Plastic pieces cut out of flask or tissue culture plate material and shaped to fit the inside diameter of a 50ml centrifuge tube, with one semicircular end and one flat end

Colcemid / Demecolcine: (Sigma D-7385 / D-6279, powder, store desiccated at 4°C) blocks mitosis by causing depolymerization of microtubules; used to induce micronucleation in donor cells. Also known as N-Deacetyl-N-methylcolchicine Concentrated stock solution of 1 mg/ml or 100μg/ml in 0.9% NaCl, store at -20°C, protected from light, stable for 6 months. Diluted working solution of 10 μg/ml in 0.9% NaCl, store at -20°C, protected from light, stable for 4 weeks.

Phytohemagglutinin P (PHA) (Diffco 3110-56-4, powder, store at 4°C) is normally used to cause leukocytes to adhere to plastic of flask, in this case assists in attachment of microcells to recipient cell surface
Add 5ml serum free αMEM to bottle containing 2 mg powder , then filter sterilize

Cytochalasin B (Sigma C-6762, powder, store at -20°C) blocks formation of contractile microfilaments by pushing equilibrium towards actin depolymerization 5 mg powder, dissolve in DMSO, then filter sterilize

Enucleation Media: DMEM, 2mM L-Glutamine, 1% Penicillin / Streptomycin,10 μg/ml Cytochalasin B. Make 500 ml to 1L at a time ; can be reused once following filter sterilization.

Polyethylene Glycol (PEG) Mol. Wt. 1,450 (Sigma P-5402, powder, store at room temp.) Allows dissociation of membrane lipids, inducing fusion of cell membranes. Melt PEG in stock container in 65°C water bath and aliquot 1-2 ml into 15 ml conical tubes. Store aliquots at room temp. Just before use, melt single aliquot (65°C), add equal volume of serum free αMEM, filter sterilize, & keep in 37°C waterbath until needed.

Micronucleation of Donor Cell Line:

Split donor cells from one 100+% confluent 150 mm TC dish into 10 such dishes using 20 ml DMEM media with 5% Fetal Bovine Serum (FBS) and appropriate selection.

After cells have attached and flattened (8 hours to overnight), add 100 μl 10 μg/ml colcemid solution (for a final concentration of 0.05 μg/ml) to 8 of these plates

Allow 24-48 hours for micronucleation to occur, which should be visible under phase-contrast microscopy.

Enucleation of Donor Cells:

Plating cells on bullets:

Previous protocols called for crosslinking the bullets with Water Soluble Crosslinker and Concanavalin A solution, but Pia and I have determined that this is not necessary, and that repeated application of Con A can reduce attachment and flattening of cells on bullets.

Sterilize 14 bullets by soaking in 70% ethanol for 2 hours to overnight

Place bullets in sterile 150 mm microbiological plates, 3 or 4 per plate, spaced 1/8 inch apart, and allow them to dry in the TC hood, with a pre-marked side up on all bullets.

Trypsinize colcemid-treated donor cells off of plates, spin down cells, and resuspend in slightly more than 14 ml of complete DMEM media with 5% Fetal Bovine Serum.

Overlay each bullet with 1ml of medium, working with 3 to 4 bullets at a time. Using a P1000 pipette tip and holding the pipette like a marker, use a "wide stroke" to spread the media to the edges of the bullet. Use gentle linear strokes at first, then, once the media covers the bullet surface, use a swirling motion to disperse the cells.

When all bullets on each plate are covered with cells, move that plate to the 37°C tissue culture incubator and proceed to the next plate of 3-4 bullets

Allow cells to attach to bullets for 8 hours to overnight

Further addition of media to plates containing bullets in also not necessary, and may cause bullets to float up when plate is moved, overlap upon settling, and scrape cells off each other.

Spinning down microcells:

Pour 35 ml of <u>prewarmed</u> Enucleation Media into each of 14 polycarbonate centrifuge tubes.

Aspirate media from bullets and <u>wash with PBS</u>. Then place <u>one</u> bullet in each tube.

Sterilely cap tubes so that the tab of the cap is facing <u>away</u> from the side of the bullet with cells attached to it.

Check the seal of the cap by looking at the tubes from the side to make sure cap is firmly seated into tube without excess space showing.

Prewarm Beckman J2 Centrifuge and <u>clean</u> Sorvall SS-34 rotor by running for 1/2 hour at 34°C (34-37°C range) and 15,000 rpm.

Place tubes with bullets inside into 37°C incubator.

Incubation of these tubes at 37°C for 1/2 hour is not absolutely necessary, and the 4 steps prior to the prewarming of the rotor can be done while the rotor is warming up, but treatment of the cells in Cytochalasin B for 1/2 hour before spinning down is <u>strongly</u> recommended to obtain smaller microcells (less cytoplasm and cell membrane surrounding micronuclei), which can pass more easily through the 5 micron filter. Incubation greatly in excess of 1 hour is not recommended, as whole cells may come off the bullets during spin down.

Place in the prewarmed rotor so that tabs are facing outside of rotor <u>and bullet surface with cells is facing center of rotor</u>; <u>cells should be pressed against bullet when outward force of centrifuge is applied</u>. See diagram accompanying protocol.

Spin at 15,000 rpm for 30 minutes at 34°C .

Remove bullets from tubes, being careful not to dislodge pellets (& using only still intact sealed tubes) and check bullets for cell ghosts / remnants indicative of enucleation

Place used bullets in distilled water and then remove enucleation media (transfer to bottle through 0.2 μm filter for one-time reuse, or aspirate. Be careful not to dislodge pellet.

Filtering microcell pellets:

To make filters:

Place Teflon o-ring onto bottom half of syringe filter apparatus.
Place appropriate filter on top of o-ring, making sure filter is centered.
Place second o-ring on top of filter, and gently screw top of apparatus on tightly.
Unscrew apparatus carefully.
Make sure filter is sandwiched between o-rings and not wrinkled.
Screw apparatus back together again, and tighten.
Seal filter into autoclave pouch or bag and autoclave the day of use.

Filtration and preparation of microcell pellet suspension:

Resuspend each microcell pellet in 1ml of serum free αMEM and pool all pellets.

Filter suspension through Whatman 8.0 μm filter and then through Whatman 5.0 μm filter to remove whole cells and large cell debris.

At each step (including unfiltered) pipette out 100 μl into Coulter Multisizer cuvettes for size profile and 20μl onto a glass slide for microscopic observation. Determine filtration efficiency.

Plate out 0.5 ml of 8 or 5 micron filtrate to a T25 flask as a parental control.

Spin microcells at 2,000 rpm for 20 minutes at room temperature, and resuspend combined microcell pellet in 1 ml of serum free αMEM.

Microcell Fusion:

Serially dilute recipient cells into T25 flasks 24 hours prior to fusion (1, 2.5, and 5 times 10^5 cells per flask, 2 flasks at each concentration)

On day of fusion, choose one flask to use for fusion; second flask at same density will be used for parental cell control. Cells must be as dense as possible without touching, since cells in contact with each other will fuse. (Usually ~ 40% confluent is optimal.)

Rinse flask twice with serum free αMEM.

Add microcell suspension to T25 flask and rock flask to spread microcells evenly.

Add 1 ml of freshly made and filter sterilized PHA solution and rock flask to spread evenly.

Place flask in incubator at 37°C for 15 minutes

During incubation period, prepare 1:1 PEG / serum free αMEM and leave at 37°C

Aspirate PHA solution from flask.

Add 1 ml of PEG / s.f. αMEM to bottom of flask, so that it does not touch cells.

Rotate flask so PEG flows evenly over cells, and rock back and forth for 55 seconds.

At 55 seconds, rotate flask so PEG flows to bottom of flask.

At 60 seconds, aspirate PEG and wash flask three times with serum free αMEM as quickly as possible

After aspirating third wash, add 5 ml of complete αMEM (with 10% Fetal Bovine Serum) and place flask in incubator

Plating out cells for clonal growth:

24 to 48 hours after fusion, split T25 flask of fused cells into 5 150 mm tissue culture plates

Transfer parental recipient cell control and 8 or 5 micron filtrate control to 150 mm plates.

For all plates, use complete αMEM media containing 15% Fetal Bovine Serum

To each plate, add appropriate double selection (e.g. . 400 μg/ml G418 and 1X HAT medium)

Allow clones to grow for 10 days to 2 weeks, watching plates and changing media (while keeping selection constant).

Pick clones (using medium and large cloning rings and blue tips) and transfer to 24 well format; later transfer to 6 well format and then to 100 mm plates.

If using HAT medium, switch selection on clones to antibiotic used in selection (e.g. 400 μg/ml G418) and 1X HT medium for 3 days, then wwitch selection to antibiotic (e.g. 400 μg/ml G418) alone.

Culture clones, freeze down two vials, and do genomic DNA extraction; test by B2 Repeat PCR (protocol below)for whole cell or microcell hybrid genotype.

Send T25 of microcell hybrid clones to Cytogenetics to test for clastogen sensitivity and complementation studies.

B2 (or B1) repeat PCR:

In order to test clones for mouse chromosome content, one can do a PCR for rodent B2 (or B1) SINE repeats. The product of the PCR is a background smear combined with a distinct pattern of bands for every subset of mouse chromosomes. It is recommended that the PCR include, along with the genomic DNA of the clones to be analyzed, the donor and recipient cell genomic DNA and TE as controls and for comparative purposes. Comparison of clone gDNA PCR product to donor gDNA PCR product can indicate whether the clone is a whole cell hybrid or not. The conditions are as follows:

Per Reaction:
2.5 μl 10X PCR buffer
1.0 μl 200ng/μl oligo of choice
0.2 μl 100mM dNTP Mix
0.2 μl Taq
1.0 μl 50ng/μl gDNA of choice
20.1 μl PCR H_2O

PCR thermocycler conditions:
For MJ Research thermocycler and all oligos:

94°C 4 min
40 cycles:
94°C, 1 min.; 60°C ,48 sec.; 68°C, 5 min.
68°C 7 min

The B2 oligo (oligo 261) also works well on the Robocycler and the following conditions:

94°C 4 min
40 cycles:
94°C, 1 min.; 62°C ,48 sec.; 68°C, 5 min.
68°C 7 min

Taq and buffer both from Roche Diagnostics GmbH
Roche Molecular Biochemicals; Sandhofer Strasse 116; D-68305 Mannheim, Germany

Oligo sequences:

B2 , oligo #261:	TCTTCTGGAGTGTCTGAAGA
B2B.1, oligo #1107:	GACTGCTCTTCCGAAGGTCC
B1.2 , oligo #1108:	CTGGAACTCACTCTGAAGAC
B1.1, oligo # 1109:	AGTGAGTTCCAGGACACCAG
B1.4, oligo #1110:	GAACTCAGAAATCCGCCTGC
B2R.1, oligo #1111:	AACTCTGGACCTTCGGAAG
B2R.2 , oligo#1112:	CCATGTGGTTGCTGGGATTTG
B2R.3, oligo#1113:	TCTTCAGACACTCCAGAAGA

Oligos 1107 & 1108 can be used separately or together,
as can oligos 261 & 1111 and 261 &1112
Oligos 1109 & 1110 should be used together,
as they give false positives if used alone.
Oligos 1107 through 1110 and thermocycler conditions
are based on sequences and conditions in
Simmler et al., 1991 and Cox et al, 1991
Oligos 1111 & 1112 were designed by Dr. Markus Grompe
Oligo 1113 is the reverse complement of oligo 261

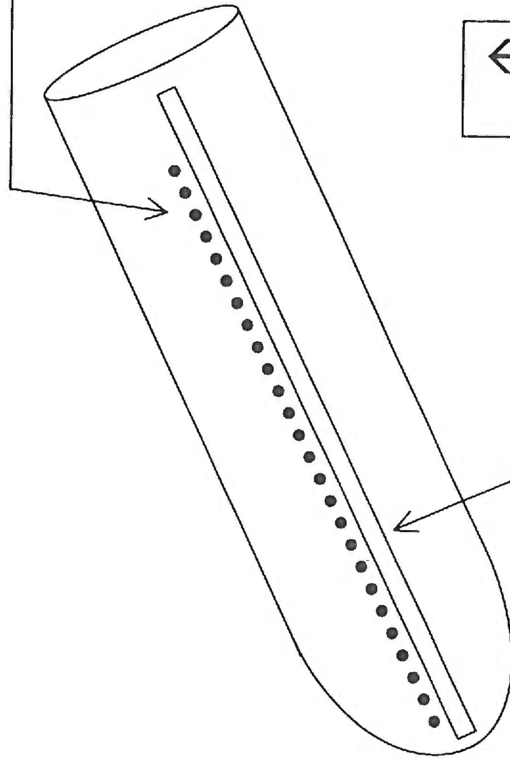
References:

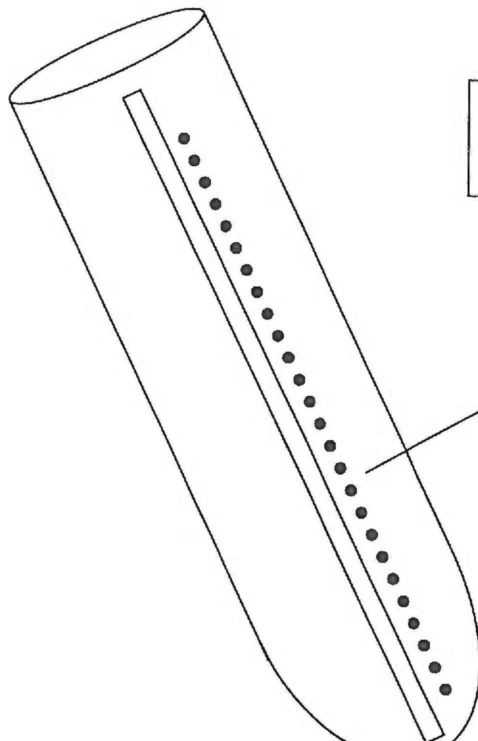
Cox, R., Copeland, N.G., Jenkins, N., and Lehrach, H. Interspersed Repetitive Element Polymerase Chain Reaction Product Mapping Using a Mouse Interspecific Backcross, Genomics **10**: 375-384 (1991).

Markowitz, D., Goff, S., Bank, A., Construction and Use of a Safe and Efficient Amphotropic Packaging Cell Line. Virology **167**: 400-406 (1988).

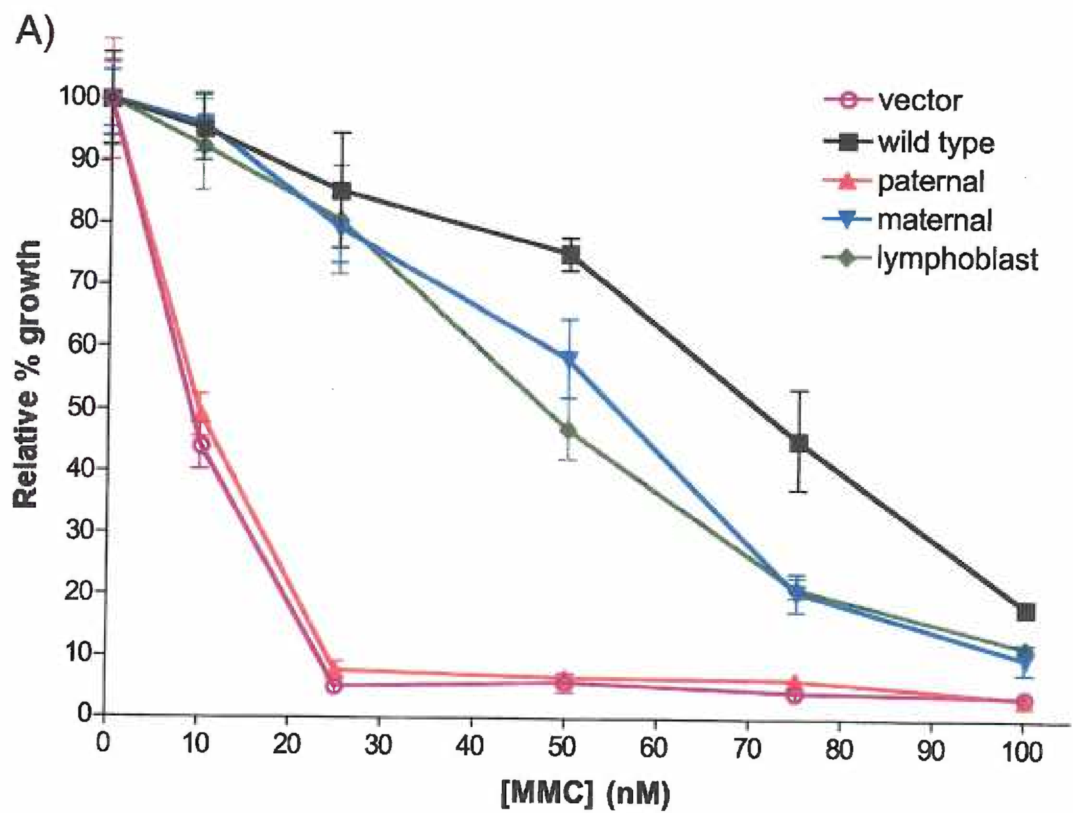
Overturf, K., Al-Dhalimy, M., Tanguay, R., Brantly, M., Ou, C.-N., Finegold, M., and Grompe, M. Hepatocytes corrected by gene therapy are selected *in vivo* in a murine model of hereditary tyrosinaemia type I, Nature Genetics **12**: 266-273 (1996).

Simmler, M.-C., Cox, R., and Avner, P. Adaptation of the Interspersed Repetitive Sequence Polymerase Chain Reaction to the Isolation of Mouse DNA Probes from Somatic Cell Hybrids on a Hamster Background, Genomics **10**: 770-778 (1991).

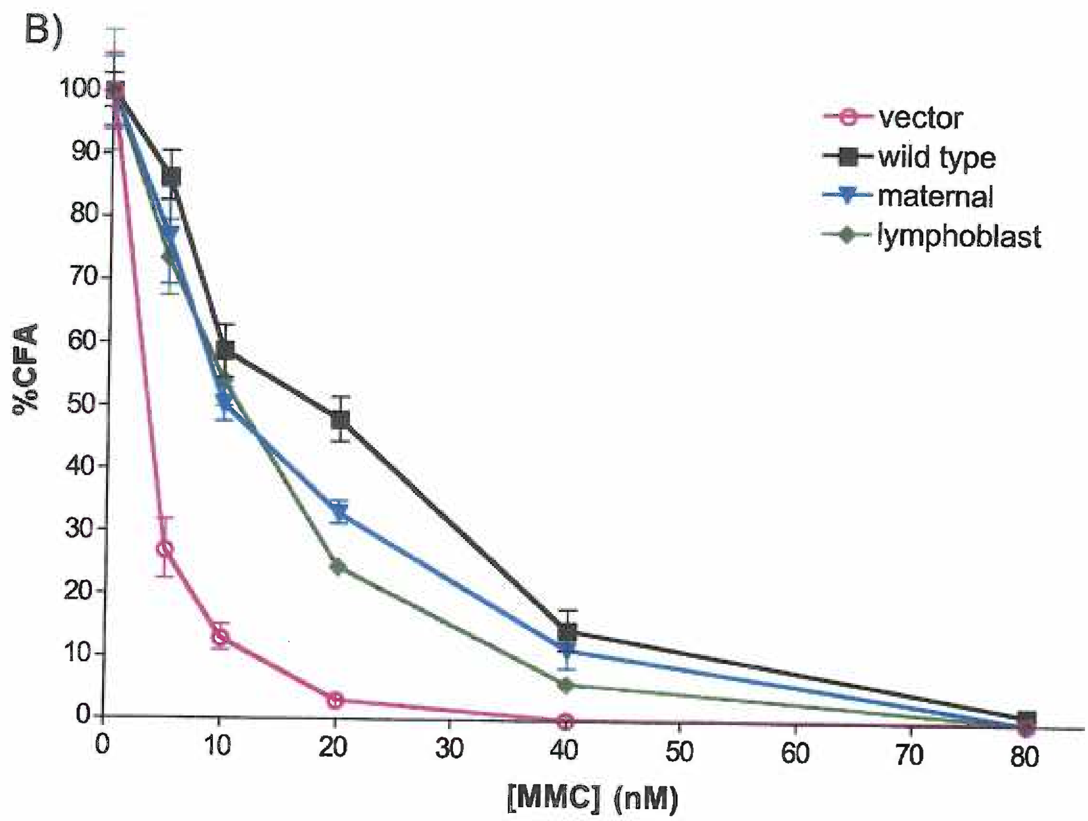

Center of rotor on this side of diagram
Bullets placed so cells are forced against the bullet surface and downwards
← Correct
Bullet (side view)


← Incorrect
Bullet surface with cells should NOT be facing towards outside, or whole cells will be pulled off

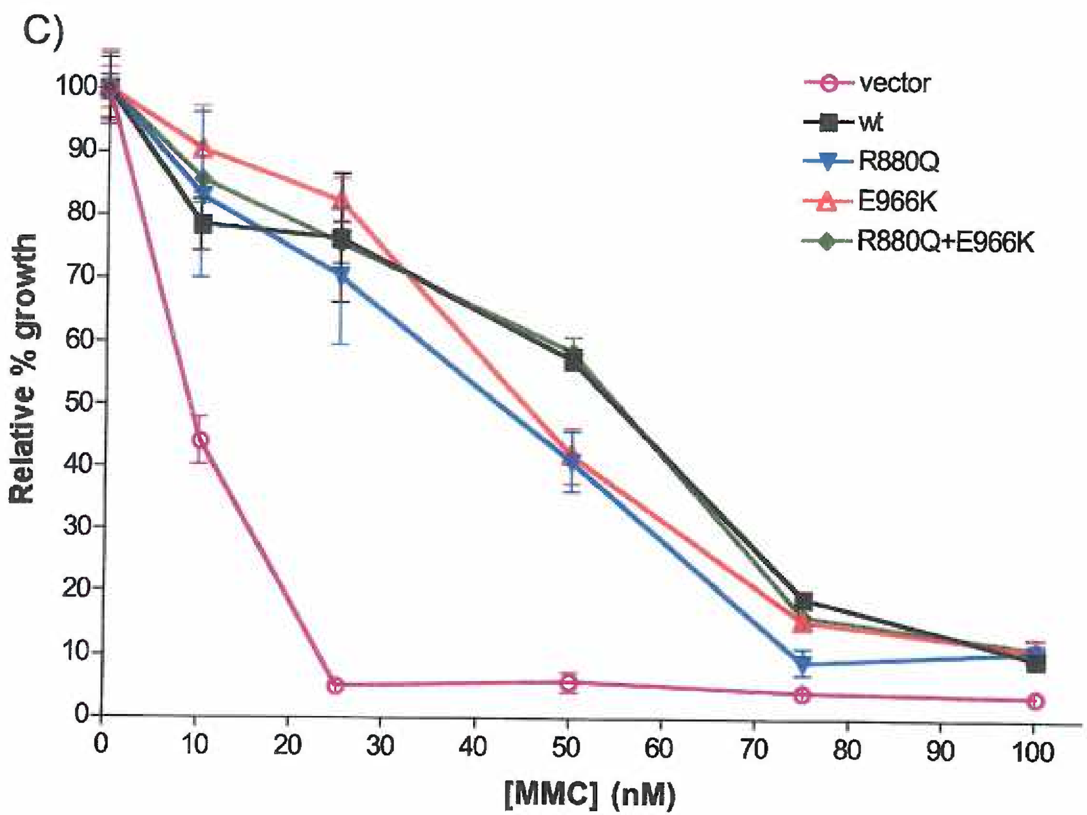
Chapter 2, Figure 3A



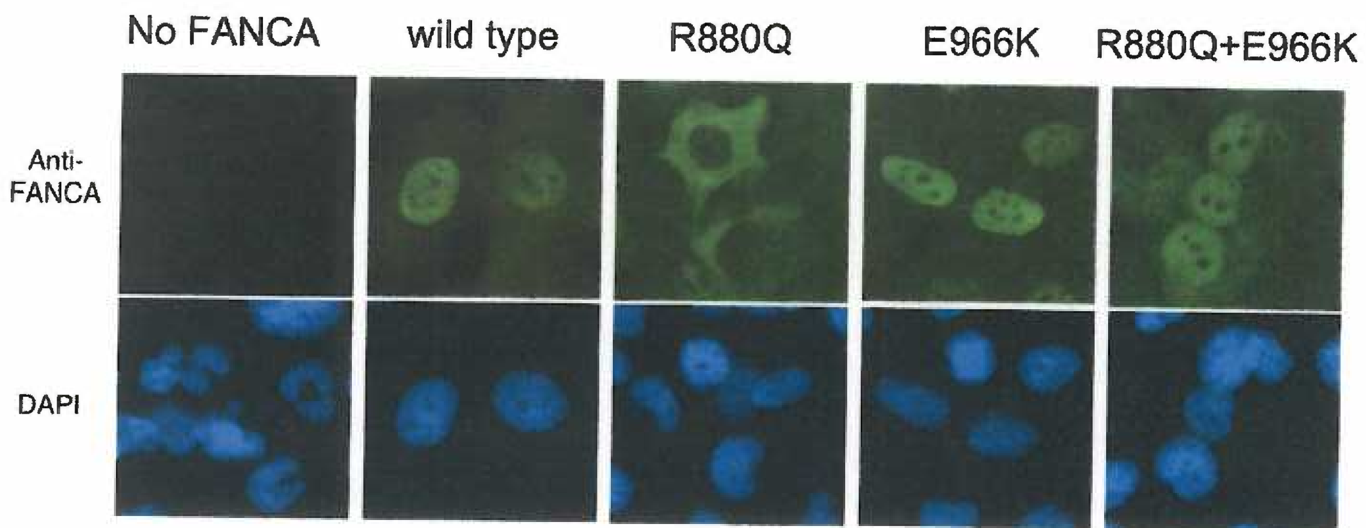
Chapter 2, Figure 3B



Chapter 2, Figure 3C

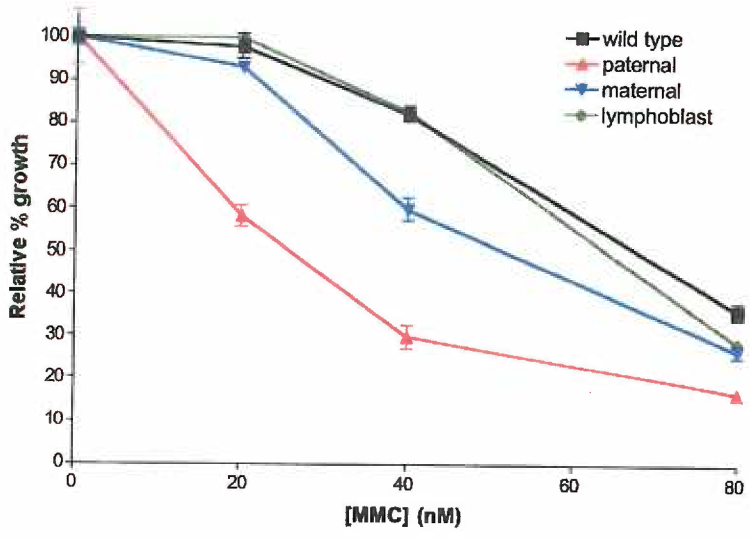


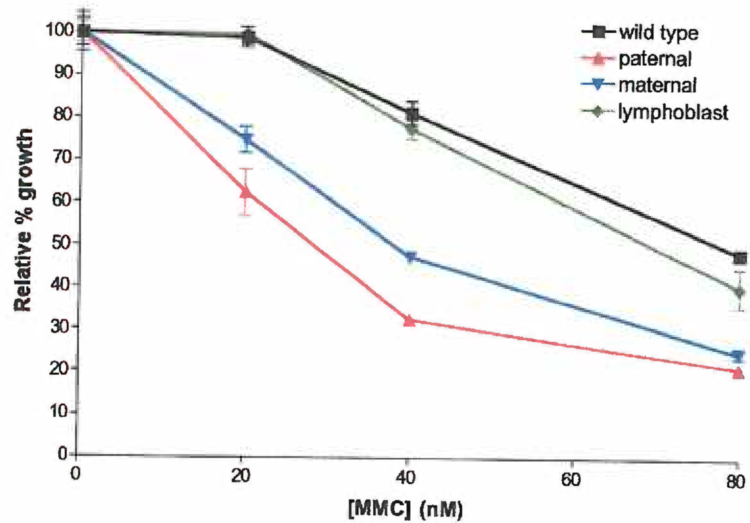
Chapter 2, Figure 5



Chapter 2, Figure 7D

D)

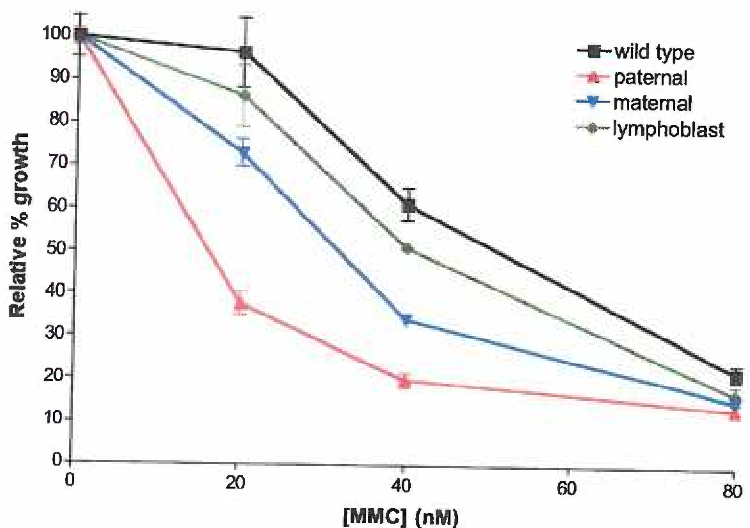


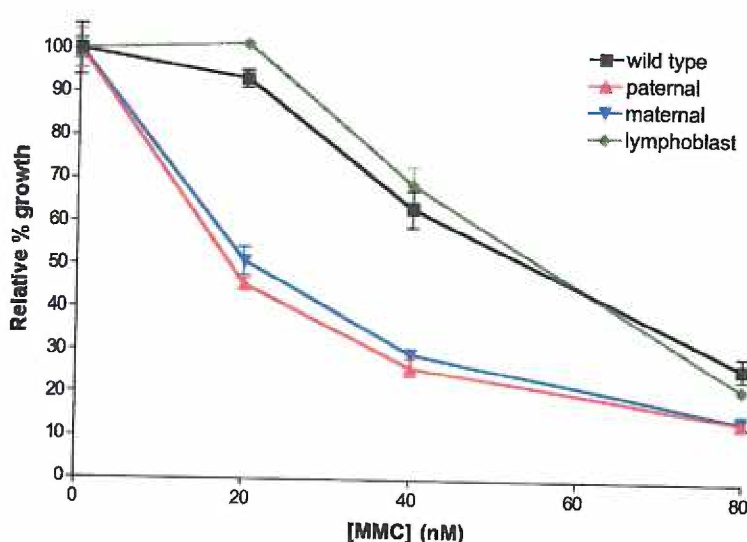


clone D3 cell lines, 500 ng/ml Dox, 40 nM MMC	
One-way analysis of variance (ANOVA)	
P value	P<0.0001
Are means signif. different? (P < 0.05)	Yes
F	155
R squared	0.9831
Tukey's Multiple Comparison Test	P value
wild type allele vs paternal allele	**P < 0.001**
wild type allele vs maternal allele	**P < 0.001**
wild type allele vs lymphoblast allele	P > 0.05
paternal allele vs maternal allele	**P < 0.01**
paternal allele vs lymphoblast allele	**P < 0.001**
maternal allele vs lymphoblast allele	**P < 0.001**

Chapter 2, Figure 7E

E)

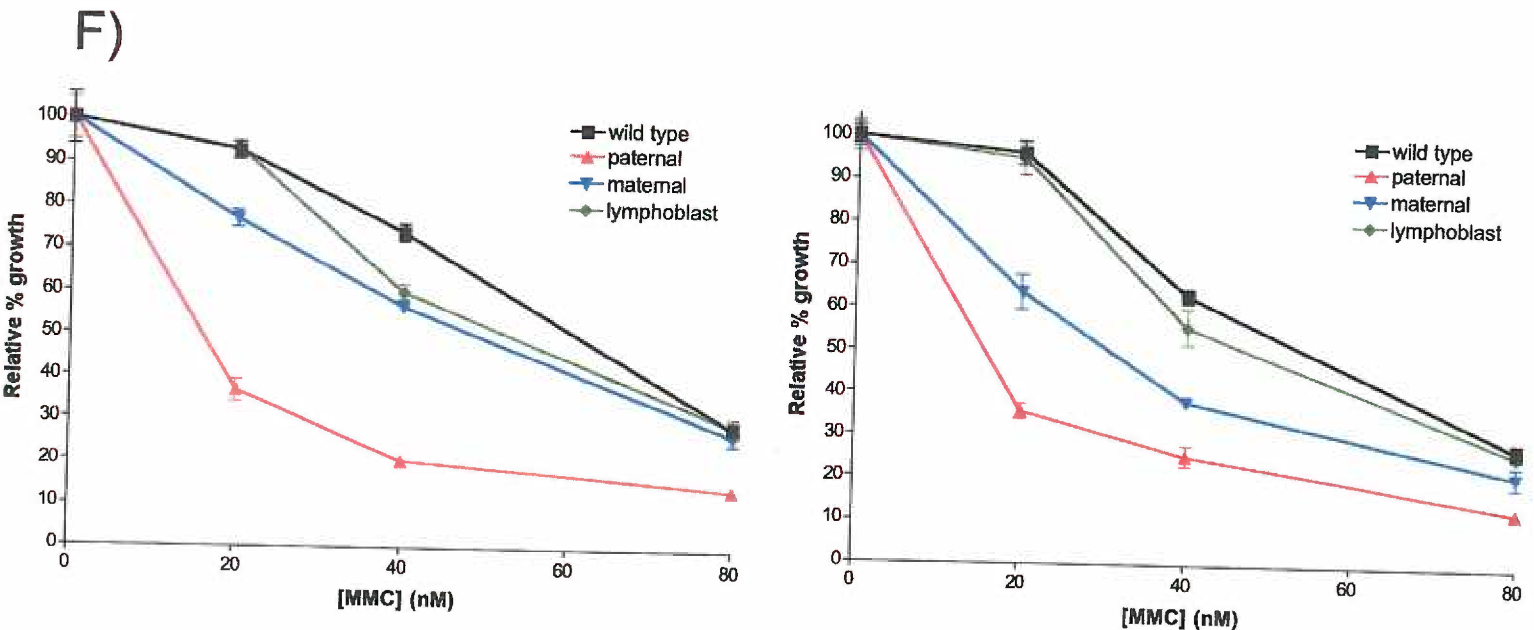




clone E1 cell lines, 31 ng/ml Dox, 40 nM MMC	
One-way analysis of variance (ANOVA)	
P value	P<0.0001
Are means signif. different? (P < 0.05)	Yes
F	51.73
R squared	0.951
Tukey's Multiple Comparison Test	P value
wild type allele vs paternal allele	**P < 0.001**
wild type allele vs maternal allele	**P < 0.001**
wild type allele vs lymphoblast allele	P > 0.05
paternal allele vs maternal allele	P > 0.05
paternal allele vs lymphoblast allele	**P < 0.001**
maternal allele vs lymphoblast allele	**P < 0.001**

Chapter 2, Figure 7F

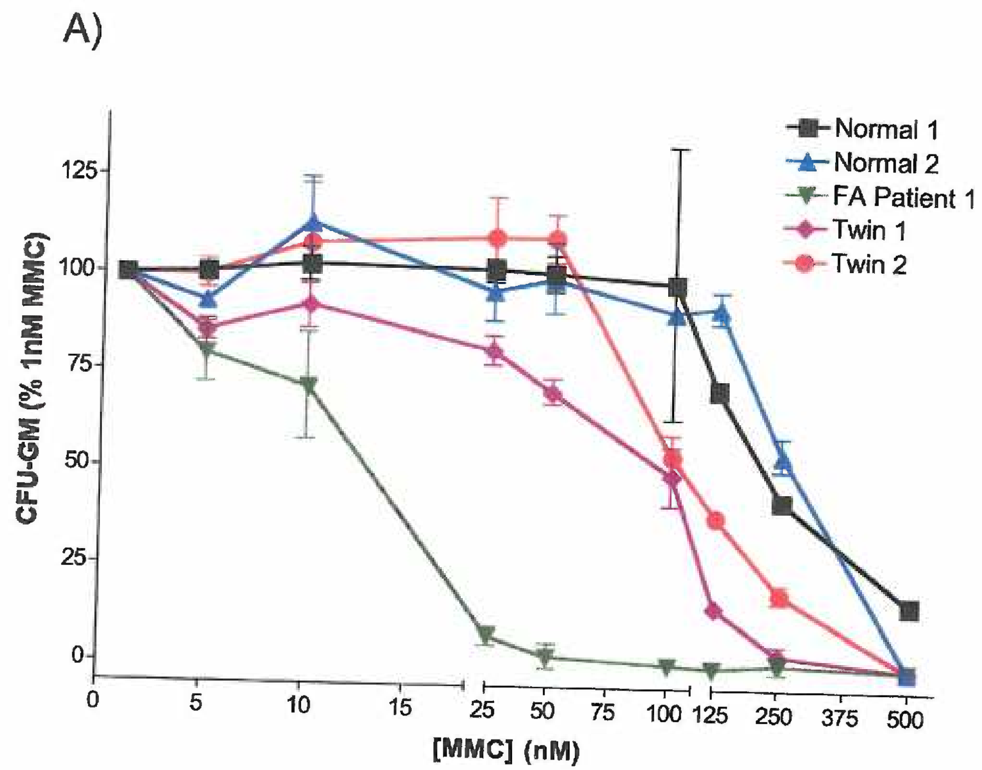
F)



clone I15 cell lines, 125 ng/ml Dox, 40nM MMC	
One-way analysis of variance (ANOVA)	
P value	P<0.0001
Are means signif. different? (P < 0.05)	Yes
F	43.47
R squared	0.9422
Tukey's Multiple Comparison Test	P value
wild type allele vs paternal allele	**P < 0.001**
wild type allele vs maternal allele	**P < 0.001**
wild type allele vs lymphoblast allele	P > 0.05
paternal allele vs maternal allele	**P < 0.05**
paternal allele vs lymphoblast allele	**P < 0.001**
maternal allele vs lymphoblast allele	**P < 0.01**

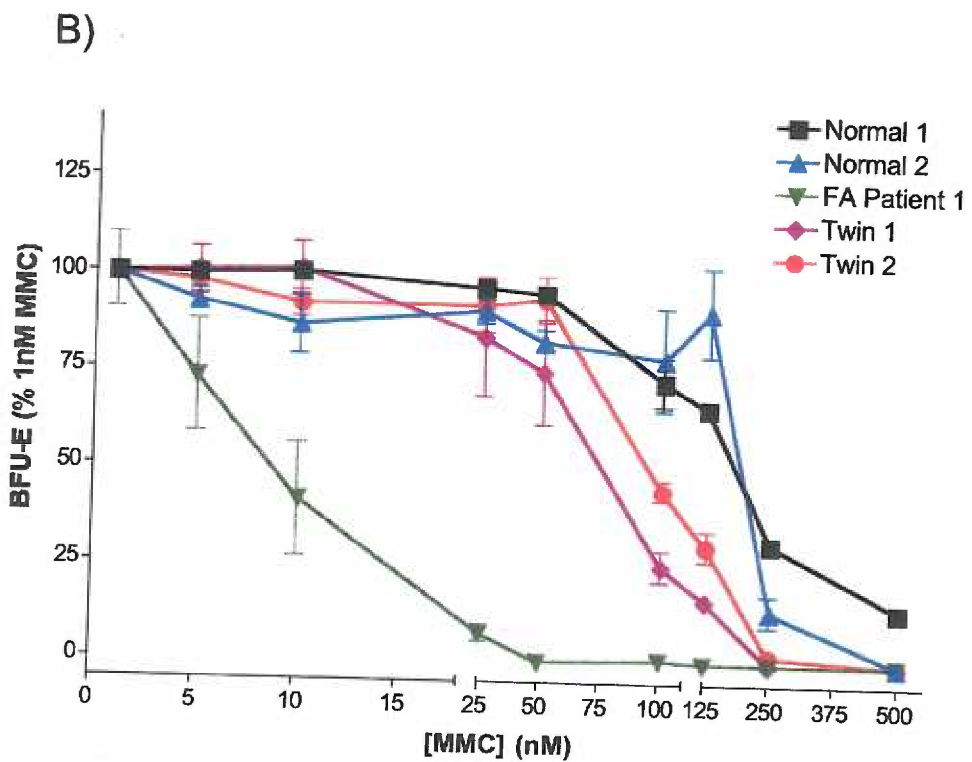
Chapter 2, Figure 9A

A)

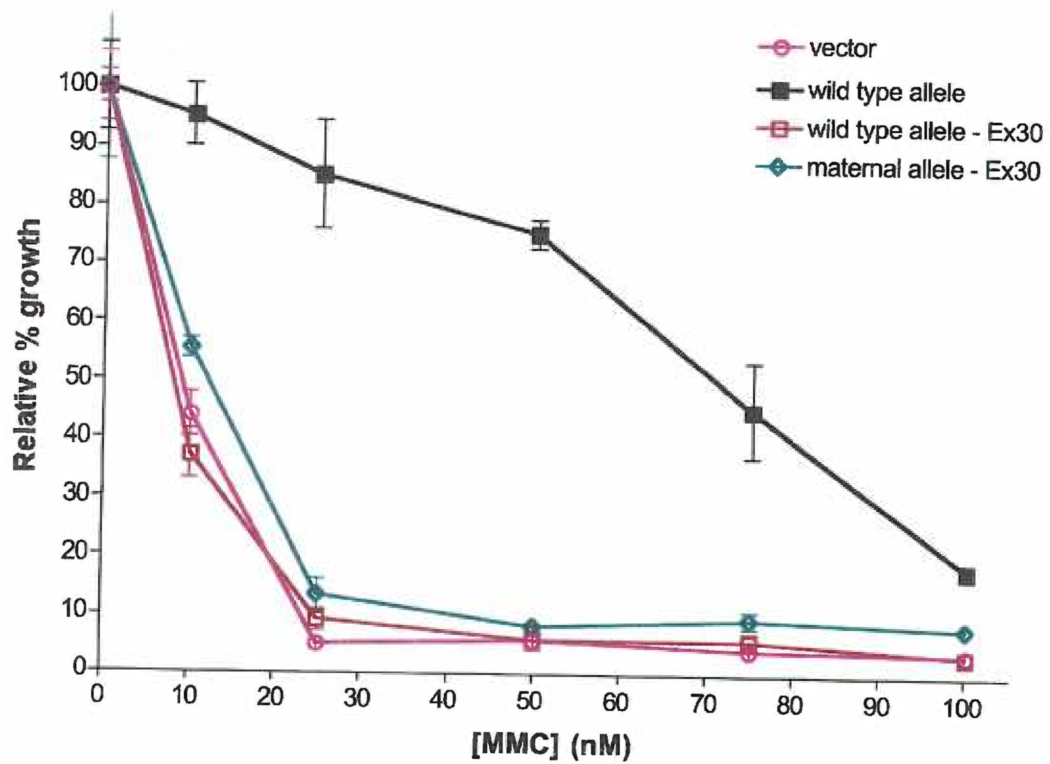


Chapter 2, Figure 9B

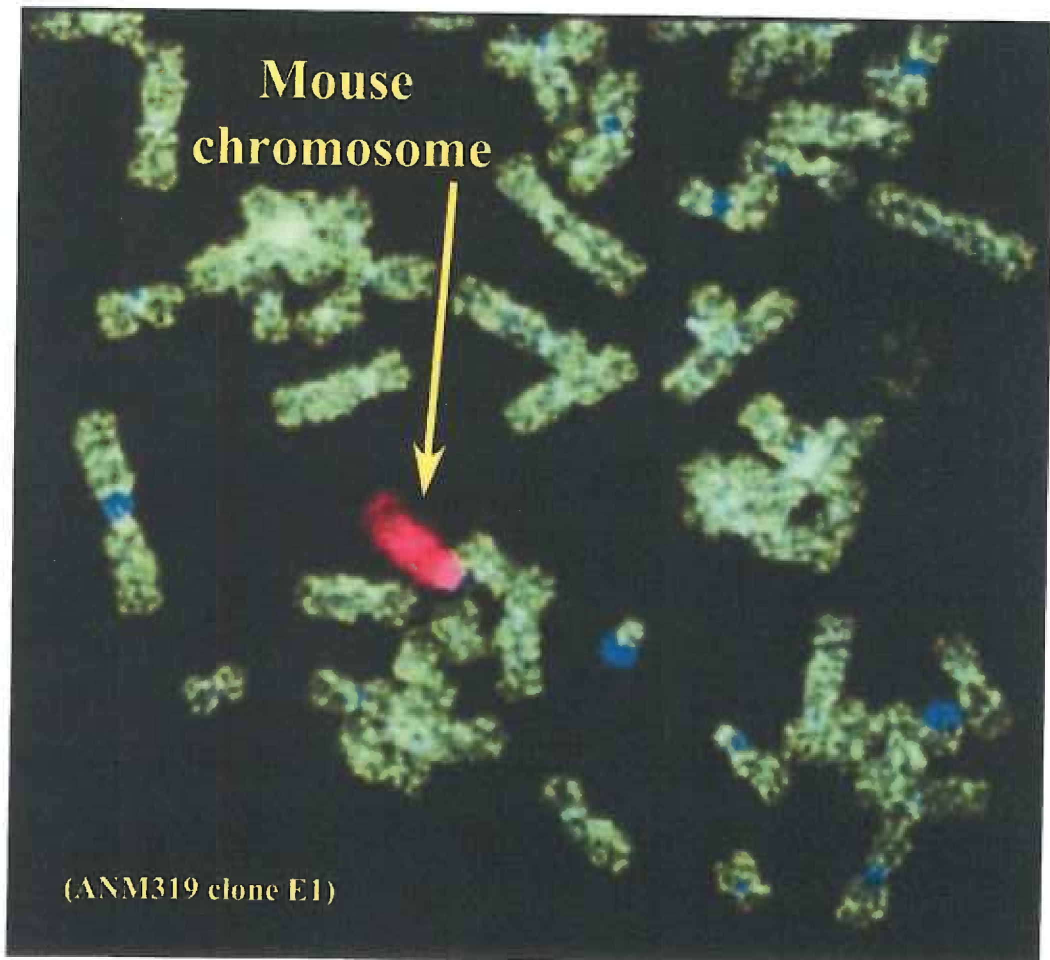
B)



Chapter 2, Figure 10



Chapter 3, Figure 4



Chapter 3, Figure 8