Genomic changes in Fanconi anemia: implications for diagnosis, pathogenesis and prognosis

Dissertation zur Erlangung des
naturwissenschaftlichen Doktorgrades der
bayerischen Julius-Maximilians-Universität
Würzburg

vorgelegt von
Michaela Groß

geboren in Coburg

Würzburg, November 2002

Eingereicht am 14. November 2002
bei der Fakultät für Biologie
der Julius-Maximilians-Universität Würzburg

Mitglieder der Promotionskommission:
Vorsitzender: Prof. Dr. R. Hedrich
1. Gutachter: Prof. Dr. H. Höhn
2. Gutachter: Prof. apl. Dr. G. Pflugfelder

Tag des Promotionskolloquiums:
Doktorurkunde ausgehändigt am:

Contents

Chapter 1

Introduction

1.1 Fanconi Anemia

Fanconi anemia (FA) is a rare autosomal recessive disease first reported by the Swiss pediatrician Guido F. Fanconi in 1927, who descibed a family with three affected children suffering from birth defects and aplastic anemia (Fanconi, 1927). In 1967 the disorder was named "Fanconi´s anemia" (Fanconi, 1967). The worldwide prevalence is inbetween 1-5 per million newborns (Auerbach et al., 1998) with a heterozygous mutation carrier frequency of 5-10 per thousand newborns, depending on the considered population (Auerbach et al., 2001; Verlander et al., 1995; Digweed and Sperling, 1996; Auerbach et al., 1998). Populations with an unusual high prevalence due to founder effects include Ashkenazi Jews as well as the white Africaans-speaking population of South Africa. In the latter, at least 1/22000 newborns has FA and 1 person out of 77 is a heterozygous carrier. The most prevalent founder mutation there (deletion of exons 12-31) accounts for 60% of FA-patients (Tipping et al., 2001). In the Ashkenazi population the heterozygous carrier frequency is about 1/90 and about 1/150 carry the founder mutation IVS4+4A>T (Verlander et al., 1995; Whitney et al., 1994).

Fanconi anemia as well as other hereditary diseases, such as Xeroderma pigmentosum (XP), Bloom syndrome (BS) and Ataxia telangiectasia (AT), are members of the so-called "caretaker-gene family" (table 1.1), because the genes underlying these disorders normally safeguard the genomic integrity of somatic cells (Kinzler and Vogelstein, 1998). Cartaker-genes participate in important cellular functions like DNA-repair, cell cycle control and apoptosis. Affected patients show a genome-wide accelerated accumulation of mutations leading to a predisposition to various malignancies as an indirect result of the primary defect. The specific spectrum of cancers generally depends on the caretaker-gene that is defective (table 1.1). In patients suffering from XP, the defective genes are part of the nucleotide excision repair pathway. Genetic changes caused by sunlight (especially UV-light) remain unrepaired, leading to skin cancer (Bootsma et al.,

2001). In BS cells, a recQ DNA helicase is mutated, resulting in a defective DNA unwinding process and subsequently in a predisposition to all common cancers (German and Ellis, 2001). In contrast to most caretaker syndromes where the molecular functions were solved by means of highly conserved structural motifs or homologies of the gene products with other known proteins, in FA cells the molecular process remains unclear, since none of the known FA genes (until the identification of $FANCD_2$) (section 1.6.5) has any homologies to other known proteins. The consistent clinical feature of hypogonadism in FA-patients, infertility in $FancC^{-/-}$ knock out mice (Chen et al., 1996; Whitney et al., 1996) and high expression of FA proteins in testis tissue as well as the recently detected $FANCD_2$-gene provide clues to their caretaker functions. These functions obviously include DNA repair processes that may have elements in common with meiosis-related recombination. In particular, the involvement of the FA proteins in certain types of double-strand break repair (DSB) is suggested by the recent finding, that the breast cancer gene BRCA2 turns into a FA gene if both copies of the gene are mutated (Howlett et al., 2002).

1.2 Clinical phenotype

The clinical phenotype of FA is characterized by hematological problems, congenital malformations and predisposition to malignancies, with the hematological problems and the cancer predisposition generally representing the life-threatening problems.

1.2.1 Hematological problems

Bone marrow failure that is typical for FA starts during childhood at the age of 7-8 years (Fanconi, 1967; Alter, 1995; Butturini et al., 1994). Anemia is caused by progressive loss of hematopoietic stem cells and thus gradually affects all blood lineages, generally starting with thrombocytopenia and macrocytic erythrocytes, which is usually accompanied by elevated fetal hemoglobin (Young and Alter, 1994; Cheng et al., 2000).

1.2.2 Congenital malformations

There is a wide spectrum of congenital malformations; although all major organ systems can be affected, about 30% of FA-patients do not show any birth defects (Giampietro et al., 1993; Giampietro et al., 1997). Many patients show pre- and postnatal growth retardation as well as cutaneous abnormalities, such as hyper- or hypopigmentation of the skin and café-au-laît-spots. Of the skeletal malformations the absence or abnormality of thumbs and/or radii are the most

Disease	Genes identified	Molecular process	DNA damaging agent	Predominant neoplasia	Reference
Ataxia telangiectasia (AT-like disorder)	ATM (MRE11)	DNA-damage response	Ionizing radiation	Acute lymphoblastic leukemia	(Gatti, 2001)
Fanconi anemia	FANCA, FANCB, FANCC, $FANCD_1$, $FANCD_2$, FANCE, FANCF, FANCG	?	Cross-linking agents	Acute myeloblastic leukemia	(Auerbach et al., 2001)
Werner syndrome	WRN	DNA unwinding	unknown	Non-epithelial-derived carcinomas and sarcomas	(Schellenberg et al., 2001)
Xeroderma pigmentosum	XPA-G	Nucleotide excision/transcription coupled repair	UV light	Skin cancers	(Bootsma et al., 2001)
Hereditary breast/ovary cancer	BRCA1, BRCA2	DNA-damage response/repair, recombination, transcription			(Couch and Weber, 2001)
Hereditary non-polyposis colorectal cancer	MSH2, MSH6, MLH1, PMS1, PMS2		unknown	Colorectal cancer	(Boland, 2001)

Table 1.1: Caretaker-gene diseases.

common defects with about 50%, but there are also reported abnormalities of the head, the face ("elfin" facial appearance), the limbs and many organs like kidneys, urinary tract, gastrointestinal system, heart, ears, eyes (microphthalmia) and central nervous system. Furthermore FA-patients show hypogonadism and reduced fertility (Giampietro et al., 1993; Young and Alter, 1994; Auerbach et al., 1998; Joenje and Patel, 2001), and they also show elevated α-fetoprotein levels (Cassinat et al., 2000).

1.2.3 Predisposition to malignancies

As one of the "caretaker-gene" syndromes (section 1.1), another consistent feature of FA is the susceptibility to malignancies, especially acute myeloid leukemia (AML) (Auerbach and Allen, 1991; Auerbach et al., 1997). According to Butturini (Butturini et al., 1994), 52% of the FA-patients develop MDS (myelodysplastic syndrome) and/or AML until they are 40 years old; in other words, the risk for developing AML for a FA-patient is about 15000 fold increased compared to healthy controls (Auerbach and Allen, 1991). To a lesser extent, FA-patients develop solid tumors, particularly squamous cell carcinomas in the oral cavity, the gastrointestinal- and the gynecologic tract (Alter and Tenner, 1994; Alter, 1996; D´Andrea, 1996; Kennedy and Hart, 1982). Adenomas and carcinomas of the liver are also reported, but they are generally attributed to the frequently used androgen-therapy in order to stimulate the hematological system after bone marrow failure (Young and Alter, 1994) (section 1.5). The risk for heterozygotes to develop any of those malignancies is unclear; Potter (Potter et al., 1983) found no evidence for an increased tumor risk, whereas recent articles postulated an increased predisposition for AML (Xie et al., 2000) or familiar T-ALL (T-cell acute lymphoblastic leukemia) (Rischewski et al., 2000).

Together, these clinical manifestations result in a markedly reduced life expectancy to an average of 23 years (Young and Alter, 1994), with death most frequently due to hematological complications or cancer. According to Butturini (Butturini et al., 1994), 81% of patients with Fanconi anemia die before the age of 40 years.

1.3 Cellular phenotype

The most consistent and the most accepted cellular phenotype is the hypersensitivity of FA cells from all complementation groups to DNA crosslinking agents, such as mitomycin C (MMC) or diepoxybutane (DEB). Exposure to these agents causes increased chromosomal instability leading to characteristic aberrations, such as radial formation or ring chromosomes (Schroeder et al., 1964). Delay in G2-phase of the cell cycle, especially after treatment with alkylating agents, is

a typical reaction of FA cells (Seyschab et al., 1995). Chromosomal breakage as well as G2-phase blockage are used as diagnostic tests for FA (Auerbach, 1993; Seyschab et al., 1995).

While the primary defect in FA cells remains unknown, a number of secondary defects have been described affecting cell growth and cell cycle regulation, detoxification, apoptosis and DNA repair (Young and Alter, 1994; Auerbach et al., 1998):

The **cell cycle** in FA cells shows spontaneous delay in the S- and G2-phase as well as a crosslinker-induced G2-arrest (Kubbies et al., 1985; Seyschab et al., 1995). Since DNA damage should be repaired before cell division, cell cycle control is linked to the DNA repair machinery through checkpoints (Paulovich et al., 1997). There are two possible causes for this arrest, a defect in DNA damage repair or a defect in cell cycle regulation. Both mechanisms have been observed in other "caretaker-syndomes". For example, a failing repair machinery has been reported in Xeroderma pigmentosum (Bootsma et al., 2001) and a disturbed cell cycle control is proposed for Ataxia telangiectasia (Gatti, 1998). After treatment with crosslinking agents FA cells do not arrest in S-phase, but perform damage resistant DNA synthesis without repairing the accumulated lesions. At the G2/M-checkpoint, those lesions are recognized leading to a G2-arrest (Centurion et al., 2000; Sala-Trepat et al., 2000). In addition, the postulated interaction of FANCC with the cyclin dependent-kinase cdc2 (Kruyt et al., 1997; Kupfer et al., 1997b) as well as the posttranscriptional, cell cycle-dependent regulation of FANCC expression (Heinrich et al., 2000) confirm the assumption that the FA proteins are involved in cell cycle control.

Increased **Oxygen sensitivity** has been proposed for primary FA skin fibroblasts after the observation that they grow better when cultured at 5% instead of 20% atmospheric oxygen and then show an alleviation of their G2-phase arrest (Schindler and Hoehn, 1988). Likewise, chromosomal damage decreases with decreasing oxygen (Joenje et al., 1981) and wildtype (WT) cells show 40-60% of the breakage level of FA cells at 20% oxygen (Joenje and Oostra, 1986). The oxygen sensitivity of FA cells decreases after overexpression of antioxidants, such as thioredoxin, at 20% atmospheric oxygen to the same extent as when cultured at 5% without any antioxidants (Ruppitsch et al., 1998). These results suggest that not the interstrand crosslinks resulting from MMC treatment are causal for oxygen sensitivity, but the generation of reactive oxygen species (ROS) (Clarke et al., 1997). There are a number of experimental observations which indicate a special role for the FANCC gene in oxygen metabolism. First, FANCC was observed to have an inhibitory effect on NADPH-cytochrome-P450 reductase, an enzyme producing ROS, suggesting an antioxidative effect (Kruyt et al., 1998b). Second, FANCC was shown to regulate GSTP1 (glutathione S-transferase P1-1) and might therefore be involved in detoxification of the cells from ROS and by-products of oxidative stress (Cumming et al., 2001). Whether the oxygen

sensitivity of FA cells has any consequences for the clinical phenotype of FA is currently unknown.

Further important functions of the FA proteins may involve a defect in **growth factor homeostasis** as well as the FANCC-mediated regulation of **apoptosis**. Abnormalities in the production of the cytokine interleukin 6 (IL-6) (Rosselli et al., 1992; Rosselli et al., 1994; Bagnara et al., 1993; De Cremoux et al., 1996) and the cellular TNF-α-levels (tumor necrosis factor alpha) (Schultz and Shahidi, 1993; Bagby et al., 1993) remain controversial. Treatment of FA-C hematopoietic precursor cells with the cytokines interferon gamma (IFN-γ) and TNF-α increases apoptosis, suggesting a connection between FANCC and the Fas-mediated death pathway, which can be blocked by FANCC overexpression (Koh et al., 1999; Rathbun et al., 1997; Wang et al., 1998). This sensitivity towards inhibitory cytokines, functioning via a Fas-mediated apoptosis pathway, has led to the assumption that this sensitivity might be the major mechanism responsible for bone marrow failure observed in FA-patients (Rathbun et al., 1997).

Like in all caretaker-gene syndromes, one of the most important functions of FA proteins is thought to be **DNA repair**. The currently accepted model of the FA pathway involves a nuclear complex containing at least FANCA, FANCC, FANCE, FANCF and FANCG. This complex activates the downstream $FANCD_2$-protein by monoubiquitylation (de Winter et al., 2000; Garcia-Higuera et al., 2000; Garcia-Higuera et al., 2001). The FA pathway, including complex formation as well as activation of $FANCD_2$, is further described in section 1.6.9. Modification of $FANCD_2$ depends on a functional nuclear complex (Garcia-Higuera et al., 2001) and results in colocalization of $FANCD_2$ with BRCA1 in nuclear foci. Because of the involvement of BRCA1 in several DNA double strand break repair systems, such as nucleotide excision repair (NER) and homologous recombinational repair (HRR), which also repairs interstrand crosslinks, a very plausible function of the FA proteins is in HRR (De silva et al., 2000; Wang et al., 2001; Dronkert and Kanaar, 2001). The recent discovery of BRCA2 as a FA gene reinforces the idea that the FA family of proteins participates in recognition and repair of specific types of DNA damage (Howlett et al., 2002; Stewart and Elledge, 2002).

1.4 Mosaicism

Mosaicism is defined as the existence of two or more cell populations with different genetic make-up in one individual. Mosaicism generally results from a genetic change in the disease locus of a person. There are two different types of mosaicism, "forward" and "reverse" mosaicism (Lo Ten Foe et al., 1997). "Forward" mosaicism can occur in heterozygous carriers, where the acquirement of a pathogenic mutation in the second allele may lead to a more or less severe phe-

notype of FA. The severity of the phenotype depends on the cell type that was hit by the second mutation and of the carrier´s age at the moment of mutation, because all cells descending from the mutated one will be affected with FA. This type of mosaicism could be responsible for sporadic cases of bone marrow failure without any congenital abnormalities. "Reverse" mosaicism represents the opposite case and creates revertants, that have regained - partially or completely - the WT phenotype by either genetic or nongenetic mechanism of reversion (Rieger et al., 1976). In those patients one (or more) affected cell(s) (homozygous or compound heterozygous) become heterozygous because of the creation of a WT allele or an allele working like a WT allele. All descendants of the reverted cell(s) will have a heterozygous status and therefore fail to show a FA-typical phenotype. Phenotypic revertants fall into two classes (Jonkman, 1999): First, so-called true reversions, where the amino acid sequence of the WT polypeptide is restored by a reverse mutation changing the mutated nucleotide to the original WT base or a third one. The underlying mechanisms are back mutation, crossing-over or gene conversion. The second type of reversions results from second site mutations, which can take place inside or outside the mutated gene without changing the original mutation. Such mutations also restore protein activity of the mutated gene, but frequently they result only in partial reversion. Mechanisms of second site mutations include base pair addition or deletion, suppressor mutation and chromosomal loss or gain. Up to now, the primary cause for mosaicism is not known, but emergence of mosaicism is clearly facilitated by chromosomal instability of FA cells. Because of a likely growth advantage of reverted cells, reverted precursor or stem cells in the bone marrow can gradually repopulate the entire bone marrow with phenotypically healthy cells leading to a gradual recovery of the patient´s hematopoiesis as described in this thesis (chapter 6). Different mechanisms for this spontaneous functional correction have been described in cultured LCLs from several FA-patients: mitotic recombination (Lo Ten Foe et al., 1997), gene conversion (Gregory et al., 2001; Lo Ten Foe et al., 1997), compensatory cis insertions and deletions which restore the reading frame (Waisfisz et al., 1999b). Back mutations and a compensatory missense mutation, where the function of a protein carrying a missense mutation is restored by a second missense mutation downstream of the first one are described in chapter 5.

About 25% of the FA-patients develop mosaicism throughout their life and own two different subpopulations of lymphocytes, one of which behaves hypersensitive to crosslinking agents and the other behaves normally in response to these agents (Lo Ten Foe et al., 1997). Generally the subpopulation of reverted cells gradually displaces the other subpopulation, so that the bulk of lymphocytes becomes more and more resistant to alkylating agents. As soon as the proportion of resistant cells is more than 20%, those cells are detectable by diagnosis based on hypersensitivity of LCLs to crosslinking agents. But as soon as the percentage of reverted cells reaches close to 100%, FA diagnosis has to be confirmed on the basis of skin

fibroblasts, because these mesenchyme cells remain sensitive to alkylating agents (Joenje and Patel, 2001). Mosaicism arises more frequently in lymphoblastoid cell lines because of many cell divisions during long-term cell culture. Development of mosaicism *in vivo* (and exclusion of a cell culture artefact) based on the LCL-status can be confirmed by the use of freshly isolated LCLs from peripheral blood samples as this was done for patient EUFA173 (chapter 5).

"Reverse" mosaicism has been reported in several other autosomal recessive diseases in the context of an attenuated phenotype (Hirschhorn et al., 1994; Kvittingen et al., 1994; Ellis et al., 1996; Stephan et al., 1996; Jonkman et al., 1997). In FA, in some - not all - patients mosaicism has been correlated with a less severe hematological outcome, although the longterm prognosis is still uncertain (chapter 5, chapter 6). In order to protect the patient from bone marrow failure, mosaicism has to extend beyond the patient´s lymphoid compartment. Differences in the clinical course between siblings or even monozygotic twins could be explained by the development or existence of somatic mosaicism.

1.5 Diagnosis and treatment

Prior to the era of chromosome breakage analysis, the diagnosis of FA was based on the presence of a typical clinical phenotype. Typical criterions for a positive diagnosis were the occurence of aplastic anemia coupled with skeletal birth defects, growth retardation and hyperpigmentation of the skin. Therefore, many patients were misdiagnosed because of a milder or less typical phenotype or they were classified to other clinical syndromes with a similar phenotype, such as VACTERL association (**V**ertebrale and vascular anomalies, **A**nale and auricular anomalies, **C**ardial anomalies, **T**racheoesophagial fistula, **E**sophagusatresia, **R**enal anomalies and **L**imb anomalies) (Sujanski and Leonhard, 1983; Cox et al., 1997). In 1964, when the spontaneous chromosome instability of cultured lymphocytes was discovered (Schroeder et al., 1964) and, about 10 years later, when the inducability of these breaks after treatment with DNA crosslinking agents, such as DEB and MMC, was reported (Sasaki and Tonomura, 1973; Auerbach and Wolman, 1976; Ishida and Buchwald, 1982), a much improved diagnosis including prenatal diagnosis became possible (Miglierina et al., 1991; Seyschab et al., 1995; Auerbach et al., 2001).

Treatment of FA is essentially supportive. There are several different starting points to allay/cure the hematological problems because of their life-threatening character. Progressive bone marrow failure is treated by blood transfusions. Another supportive therapy consists of androgens or growth factors in order to stimulate hematopoiesis. In many patients hematopoiesis improves directly after the beginning of androgen-therapy, but in the long run patients often develop liver adenomas or liver tumors (Young and Alter, 1994). Up to now, the only

way to cure the hematological problems is bone marrow or umbilical cord blood transplantation (Young and Alter, 1994). For bone marrow transplantation to be successful several requirements have to be met: First, the greatest success rate (about 70%) is expected when the donor is a HLA-identical sibling (Young and Alter, 1994; Guardiola et al., 1998). Transplantations with unrelated HLA-identical donors generally result in about 20% success rate (Young and Alter, 1994). The protocols of transplantation procedure have to be adapted because of hypersensitivity of FA-patients to immunosuppressive agents used to prevent graft-versus-host-disease, such as cyclophosphamide and cisplatin (Socie et al., 1993; Young and Alter, 1994; Flowers et al., 1996). The occurence of mosaicism has to be excluded. The doses of immunosuppressive agents used for non-mosaic FA-patients usually do not fit for mosaic patients because of the resistant cells in their blood (section 1.4), which cannot be destroyed by low-dose treatment. Furthermore, the prevention of bone marrow failure after transplantation does not mean that the patient is totally cured. The predisposition to malignancies still persists. Elder patients often develop tumors and they often die as a consequence of neoplasia later in life (Joenje and Patel, 2001). For patients belonging to a complementation group with the underlying gene being cloned, who have no suitable bone marrow donor, gene therapy is being explored as a curative option (section 1.7).

1.6 FA genes and FA proteins

In the beginning, the hypothesis "one gene defect for one disease" was thought to be correct for FA too, because of all diagnosed FA-patients showing a relatively uniform phenotype, until genetical heterogeneity was proposed for FA (Schroeder et al., 1976; Joenje et al., 1997). Since then, 8 different complementation groups have been identified (FA-A, FA-B, FA-C, FA-D_1, FA-D_2, FA-E, FA-F and FA-G) and perhaps there will be some more. The genes defective in those complementation groups do not seem to share any common features and they are not clustered, but widely dispersed throughout the genome. The prevalence of the different groups depends on the population examined (Jakobs et al., 1997; Joenje, 1996; Auerbach et al., 1998), the relative prevalence of the first 100 patients registered in the EUFAR program was as follows: A(71%)>G(13%)>C(7%)>E(4%)>F(2%)>B/D_1/D_2(1% respectively) (Joenje and Patel, 2001). In the beginning the complementation group was only determined by somatic cell fusion studies done by the group of Dr. Hans Joenje at Amsterdam, Netherlands. Those studies were based on the fact that fusion hybrids of cells belonging to the same complementation group cannot complement each other´s MMC-hypersensibility, because in both cells the same gene is defective. Hybrids of cells from two different complementation groups are mutated in different genes and therefore are capable to complement each

other because of the recessive character of the disease. The cell fusion method is suitable for the detection of new complementation groups and it delivered all known reference cell lines, but there are also several disadvantages. The method is very time consuming and from the first fusion with the reference cell line A (HSC72 (Duckworth-Rysiecki et al., 1985)) to the group-defining fusion it can take months or even years, if the complementation group is one of the rare ones or a new one. Besides, because of their limited proliferative life span fibroblasts have been superseded by the use of EBV-immortalized lymphoblastoid cell lines, but the experiments need a MMC-hypersensitive phenotype of the cell line and cannot be done with reverted cells. Previously, the group of Helmut Hanenberg at Düsseldorf has developed viruses containing all identified FA genes, which can be used for complementation analysis as well as for future gene therapy trials (Hanenberg et al., 2002) and (Lobitz et al., manuscript in preparation). As material T-cells and lymphoblastoid cell lines can be used as well as fibroblasts in the case of somatic mosaicism. Since the method is virus-based, the complementation group can be determined in less than two weeks (section 1.7).

Generally the determination of the complementation group is followed by linkage and/or mutation analysis. Mutation analysis can be carried out on genomic or cDNA by several methods. Most of them are used as screening methods, such as SSCP-analysis (Single strand conformation polymorphism), PTT (Protein truncation test), Southern-Blot-analysis, HPLC-analysis (High pressure liquid chromatography) or GeneScan analysis, in order to identify striking regions in the genes. Afterwards all bandshifts have to be sequenced to identify the exact mutation type or locus (chapter 2). Many of the actually identified mutations are accessible in the Fanconi Anemia Mutation Database (http://www.rockefeller.edu/fanconi/mutate/).

There are different reasons that underline the importance of determination of one´s complementation group as well as one´s mutations. First, the detection of the complementation group and the mutations represents a confirmation of the diagnosis and is a prerequisite for prenatal diagnostics. The knowledge of one´s complementation group is an important requirement for future gene therapy trials and can perhaps deliver some information about the primary defect in Fanconi anemia. Identifying the complementation group of a large amount of patients as well as many comparable mutations there might be the chance for improved genotype/phenotype correlations and prognostic information (chapter 2, (Faivre et al., 2000)).

For cloning of the FA genes, different strategies have been used by different groups. The most successful was expression cloning, leading to the identification of FANCA, FANCC, FANCE, FANCF and FANCG. The principle of this method is that a FA cell line is transfected with a vector containing a cDNA library and is afterwards tested whether MMC-hypersensitivity still persists. Those cell lines, where no more MMC-hypersensitivity can be measured, should contain a comple-

menting cDNA and have to be further examined in order to prove the correctness of the experiment, e.g. by mutation analysis in patients of that complementation group. In order to receive good results there are several requirements that have to be fullfilled: Getting complemented colonies depends on the quality of the library used. Besides, similar to the cell fusion studies, no reverted cell lines must be used because of their own MMC-resistance and not every cell type is suitable for transfection. Last not least, the protein must not be toxic after overexpression.

An alternative approach to the identification of a new gene represents the strategy of linkage analysis followed by positional cloning, which was used for identifying FANCA and $FANCD_2$, as well as for mapping FANCE. The success of this method requires many patients and a large number of affected and unaffected siblings.

In the following subsections the different FA genes will be introduced (in alphabetical rather than chronological order):

1.6.1 FANCA

The FANCA gene, defective in patients belonging to complementation group A, is located at 16q24.3; the genomic sequence spans about 80 kb and contains 43 exons ranging from 34 to 188 bp (figure 1.1) (Lo Ten Foe et al., 1996b; Ianzano et al., 1997). The encoded 163 kDa protein consists of 1455 amino acids and has no significant homologies to any other known protein. The only hint to its nuclear function represent a nuclear localization signal (NLS) in the aminoterminus of the gene and a partial leucine zipper from amino acid 1069 to 1090 (Näf et al., 1998).

FANCA was mapped by linkage analysis (Pronk et al., 1995) and then isolated by two different groups at the same time using two different techniques, expression or complementation cloning (Lo Ten Foe et al., 1996b) and positional cloning (The Fanconi anemia/Breast cancer consortium, 1996).

FANCA is a hypermutable and highly polymorphic gene (The Fanconi anemia/Breast cancer consortium, 1996; Lo Ten Foe et al., 1996b; Levran et al., 1997; Savino et al., 1997; Levran et al., 1998; Tachibana et al., 1999; Wijker et al., 1999). Up to now, there are more than 100 different mutations on record for the FANCA gene, with large deletions representing the most important group (Wijker et al., 1999; Morgan et al., 1999). More details about the mutation spectrum as well as mutation analysis are provided in chapter 2.

A $FancA^{-/-}$ knock out mouse model has been generated (Cheng et al., 2000). Those mice are reported to be viable and not to have any detectable developmental abnormalities or growth retardation. Consistent with the clinical phenotype of FA-patients, both male and female mice show reduced fertility and hypogonadism. Corresponding to the phenotype the FancA protein levels in testis, ovary and lymphoid tissues (spleen, thymus, lymph nodes) are higher than in other tissues (Van de Vrugt et al., 2000). Cultured embryonic fibroblasts of the knock-

out mouse exhibit chromosomal instability and hypersensitivity to crosslinking agents, but no predisposition to cancer suggesting further factors being involved, such as genetic background or environmental factors. In contrast to the human phenotype, fetal hemoglobin expression in adult FancA$^{-/-}$ mice was not elevated and hematopoiesis seems to be unaffected. Bone marrow failure cannot be induced by low doses of MMC, as has been reported for the FancC$^{-/-}$ knock out mice (Carreau et al., 1998).

1.6.2 FANCB

The gene defective in complementation group B (FANCB) is not yet identified, but there is postulated a possible cytoplasmic role in the phosphorylation of FANCA (de Winter et al., 2000).

1.6.3 FANCC

The FANCC gene was the first FA gene to be identified in 1992 (Strathdee et al., 1992) by the method of expression cloning. It is located on chromosome 9q22.3, spans approximately 80 kb and contains 14 exons ranging from 54 bp to 203 bp (figure 1.1). The 63 kDa hydrophobic protein consists of 558 amino acids and does - like FANCA - not have any homologies to known sequences as well as no functional motifs (Gibson et al., 1993a; Savoia et al., 1995). A confusing fact in the examination of the structure of the gene was the detection of transcripts of different length in Northern blot analysis, the function of which are not clear yet. Analysis of the primary sequence of FANCC revealed two different 5´-exons , called exon 1a and exon 1b, (Savoia et al., 1995) as well as different 3´-Untranslated regions (UTR) (Strathdee et al., 1992), causing different transcripts. Some exons can be skipped without any obvious influence on protein function (Gibson et al., 1993b).

Mutation analysis revealed to date more than 10 different mutations and almost the same number of polymorphisms (Strathdee et al., 1992; Gibson et al., 1993b; Whitney et al., 1993; Verlander et al., 1994; Gibson et al., 1996; Lo Ten Foe et al., 1996c; Lo Ten Foe et al., 1996a; Lo Ten Foe et al., 1998). Most of the mutations are truncating, and there are only two missense mutations described (Strathdee et al., 1992; Gavish et al., 1993; Gibson et al., 1996). One of those missense mutations (L554P) is reported to block nuclear translocation of the protein (Hoatlin et al., 1998; Savoia et al., 1999) and furthermore to have a dominant negative effect on non-FA cells (Youssoufian et al., 1996). More detailed information about the mutation spectrum is provided in chapter 2. Eight new mutations, including two missense mutations, are described elsewhere in this thesis (chapter 3).

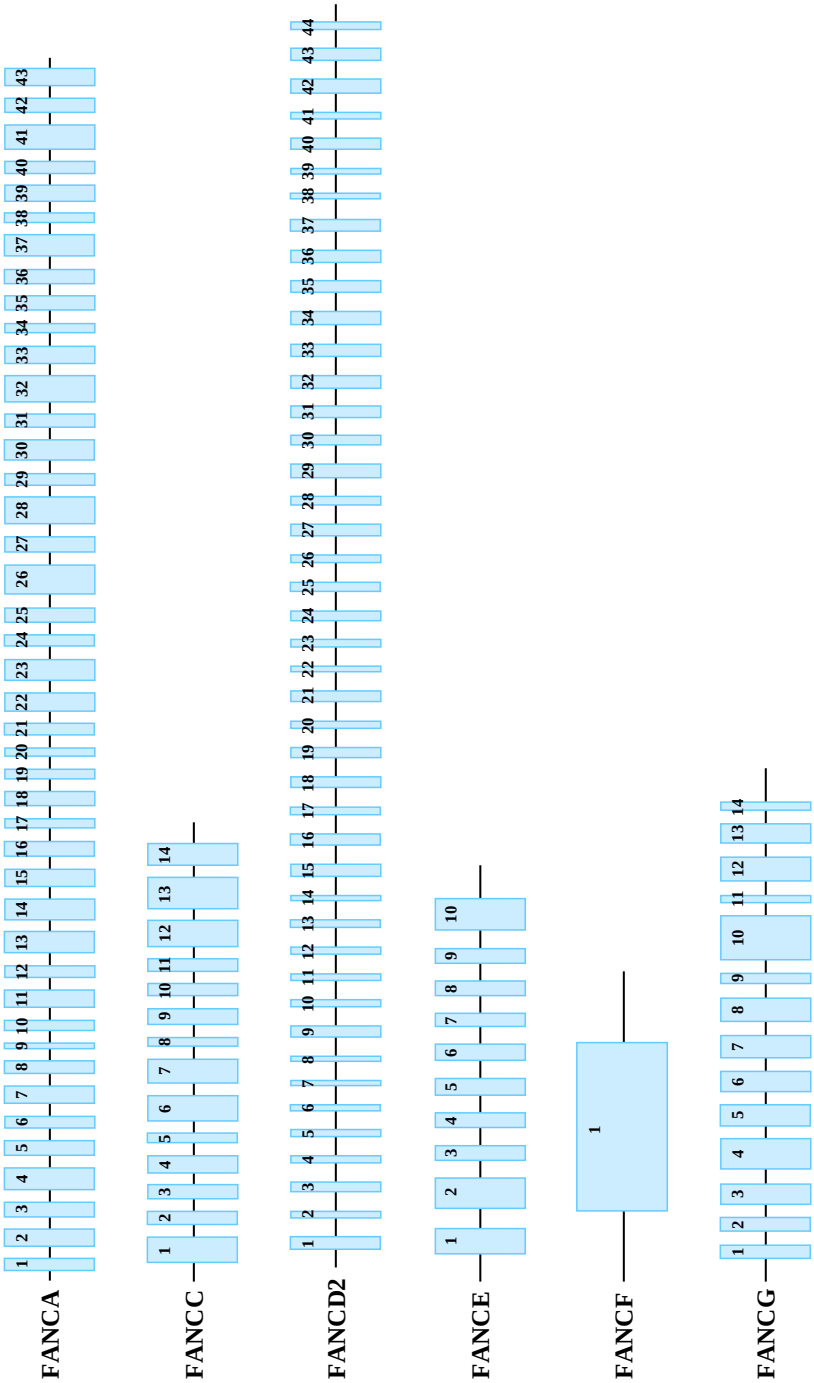


Figure 1.1: Exon-intron structure of the cloned FA genes FANCA, FANCC, $FANCD_2$, FANCE, FANCF and FANCG.

The human FANCC protein and the murine FancC protein are ubiquitously expressed (Wevrick et al., 1993). Expression levels are relatively high in bone tissue and in progenitor cell populations, fitting to the clinical phenotype with skeletal malformations and bone marrow failure (Wevrick et al., 1993; Krasnoshtein and Buchwald, 1996). Considering the RNA- and protein levels, FANCC mRNA levels are constant during cell cycle (Joenje et al., 1995b; Tower et al., 1998) independently from environmental factors like crosslinking agents, oxidative stress, radiation or heat. FANCC protein levels in overexpression, however, fluctuate during cell cycle, showing high levels at G2/M boundary, but degradation in G1 phase, suggesting posttranscriptional regulation of the protein (Heinrich et al., 2000). Following the suggestion of cytoplasmic location (Youssoufian, 1994; Yamashita et al., 1994; Youssoufian, 1996a), FANCC was more recently localized also in the nuclear compartment (Kupfer et al., 1997a; Hoatlin et al., 1998; Näf et al., 1998; Yamashita et al., 1998) where it appears to function as a part of the FA multiprotein complex. The fact that more FANCC protein is localized in the cytoplasm than in the nucleus suggests that the protein must have additional function(s). *In vitro* binding studies revealed that three unidentified cytoplasmatic proteins (30, 50 and 70 kDa) co-precipitate with FANCC (Youssoufian et al., 1995). Other laboratories found interactions between FANCC and NADPH cytochrome P450 reductase, suggesting a possible detoxification role (Kruyt et al., 1998b), or interactions with GRP94, a molecular chaperone, that could be responsible for the regulation of the intracellular expression of FANCC protein levels (Hoshino et al., 1998). An interaction with cdc2 was reported in the context of a possible role of FANCC protein in cell cycle regulation (Kupfer et al., 1997b), but this could not be confirmed by others (Kruyt et al., 1998b; Kruyt and Youssoufian, 1998).

Two FancC$^{-/-}$ knock out mouse models have been generated (Chen et al., 1996; Whitney et al., 1996). In spite of a relative low level of amino acid conservation, human FANCC is able to complement murine FancC cells and vice versa (Gush et al., 1999). The clinical phenotype of the mice is characterized by reduced fertility and hypogonadism, but they show no obvious birth defects of the skeletal or urinary systems. Cells derived from these animals exhibit the classic hypersensitivity to bifunctional DNA crosslinking agents; nonetheless, pancytopenia did not develop during the first year of life and no leukemia and cancer susceptibility was observed. Bone marrow failure could be induced by acute exposure to MMC, leading to death of the mice within a few days, as well as by treatment with sequential, nonlethal doses of MMC causing a progressive pancytopenia, remarkably similar to that seen in FA-patients (Carreau et al., 1998). The ability of stem cell repopulation after serial transplantations seems to be reduced in FancC$^{-/-}$ mice (Haneline et al., 1999).

1.6.4 $FANCD_1$

The gene defective in complementation group D_1 ($FANCD_1$) has recently been found to be identical to the human breast cancer gene BRCA2 located on human chromosome 13q12 (Howlett et al., 2002; Stewart and Elledge, 2002), which plays a known role in the human double strand break (DSB) repair pathway. This discovery suggests an important role of the FA gene family in the maintenance of genomic stability.

1.6.5 $FANCD_2$

The $FANCD_2$ gene, defective in patients belonging to complementation group D2, is located at chromosome 3p25.3 and contains 44 exons (figure 1.1). The gene was identified by positional cloning (Timmers et al., 2001), six years after the first mapping by microcell-mediated chromosome transfer (Whitney et al., 1995). The encoded protein consists of 1451 amino acids and is found in the two isoforms $FANCD_2$-L ("long") and $FANCD_2$-S ("short") with 162 and 155 kDa because of the monoubiquitylation at lysine 561 (Timmers et al., 2001; Garcia-Higuera et al., 2001). In contrast to FANCA, FANCC, FANCE, FANCF and FANCG which all represent members of the nuclear FA complex and have no homologues in nonvertebrates, $FANCD_2$ is highly conserved in plants (Arabidopsis thaliana), insects (Drosophila melanogaster) and nematodes (Caenorhabditis elegans), although to date the functions of the homologues in those organisms are still unknown. The observation of the high conservation lead to the assumption that $FANCD_2$ might act in a conserved pathway upon which the action of the other FA genes may have been superimposed later in evolution (Joenje and Patel, 2001). Current evidence suggests that $FANCD_2$ is a target of the function of the nuclear FA protein complex and acts downstream of the FA pathway, where it becomes monoubiquitylated in response to DNA damage (Garcia-Higuera et al., 2001). Thus, the FA protein complex might represent a novel ubiquitin-ligase or regulate the activity of such an enzyme. DNA damage and monoubiquitylation can be induced by interstrand DNA crosslinking agents as well as ionizing radiation (Garcia-Higuera et al., 2001). In cells of the complementation group A, B, C, E, F, and G (all of them participating in the nuclear complex), $FANCD_2$ is only present in the non-ubiquitylated short form, suggesting that $FANCD_2$ is not required for its assembly or stability (Grompe and D´Andrea, 2001). Expression of the complementing FA protein in these cell lines results in a reconstitution of the nuclear complex and in monoubiquitylation of $FANCD_2$ after DNA damage. The localization of $FANCD_2$ is exclusively nuclear, where it can be found within nuclear foci together with BRCA1. In WT cells DNA damage provokes the formation of nuclear foci, whereas in FA-A, FA-B, FA-C, FA-E, FA-F, and FA-G cells no speckels are detected and the $FANCD_2$ protein is found to be diffuse (Garcia-Higuera et al., 2001).

Five different mutations have been described in $FANCD_2$ to date, two out of them being missense mutations. None of the patients belonging to complementation group D2 has been found to carry biallelic null mutations, suggesting that null/null mutants might be lethal (Joenje and Patel, 2001). A knock out mouse model of $FANCD_2$ has not yet been generated.

1.6.6 FANCE

FANCE, the corresponding gene to complementation group FA-E, has been mapped to chromosome 6p21-22 through linkage analysis (Waisfisz et al., 1999c) and one year later identified on chromosome 6p21.3 through expression cloning (De Winter et al., 2000a). The gene has 10 exons and spans about 15 kb (figure 1.1). The encoded 59 kDa protein consists of 536 amino acids with two potential nuclear localization signals, but, like the other FA proteins with the exception of $FANCD_2$, lacks any significant homology to other proteins (De Winter et al., 2000a). To date, three different mutations are known, all of them resulting in a premature stop-codon and therefore in a truncated protein. Unfortunately, FANCE is localized in the same region as the HLA class I genes of the major histocompatibility complex, so that these patients are very unlikely to have an HLA-matched unaffected sibling donor for bone marrow transplantation (De Winter et al., 2000a). A knock out mouse model of FANCE is not yet published.

1.6.7 FANCF

The gene defective in patients belonging to complementation group F, FANCF, is the first FA gene containing only a single exon (figure 1.1). The gene is located on chromosome 11p15. Like most of the FA genes, FANCF was identified by expression cloning (De Winter et al., 2000b). The resulting 42 kDa protein contains 374 amino acids and was the first FA gene being reported to have a homology to any known protein. Protein analysis revealed a region of homology with the prokaryotic RNA binding protein ROM, which is known to regulate the ColE1 plasmid copy number through binding and stabilizing hairpin pairs of two complementary RNA sequences, suggesting that part of the function of FANCF could be based on the capacity to bind (deoxy)ribonucleic acid (De Winter et al., 2000b). Recently, this homology was found to be nonsignificant, because mutations in the FANCF region homologous to ROM did not affect the function of FANCF (De Winter et al., 2000a). FANCF is predominantly localized in the nucleus (de Winter et al., 2000) and the protein level does not vary during cell cycle progression (Siddique et al., 2001). To date, mutation analysis revealed six different mutations, all of them leading to a premature stop-codon. As with FANCE, a knock out mouse model of FANCF is not yet published.

1.6.8 FANCG

The gene defective in Fanconi anemia complementation group G was the third FA gene identified after FANCC and FANCA (De Winter et al., 1998). It has been localized on chromosome 9p13 where it covers about 6 kb and was identified through expression cloning like most of the other FA genes (De Winter et al., 1998). The FANCG gene consists of 14 exons ranging from 47 to 290 bp each (figure 1.1). The encoded protein contains 622 amino acids and has a calculated molecular weight of 68 kDa (Demuth et al., 2000) (chapter 4). Like all other FA proteins except of $FANCD_2$, this protein has no homology to any known protein including the other FA proteins (Liu et al., 1997), but there are two putative leucine zippers in the gene (Demuth et al., 2000) (chapter 4).

FANCG was found to be identical with human XRCC9 (De Winter et al., 1998), a gene that cross-complements the MMC-sensitive Chinese hamster mutant UV40 and was therefore suggested to be involved in DNA postreplication repair or cell cycle checkpoint control (Liu et al., 1997; Busch et al., 1996). Human XRCC9 corrects hypersensitivity of CHO UV40 cells to MMC, cisplatin, ethyl methane sulphonate (EMS) as well as UV. FA cells from complementation groups A, B and D are not hypersensitive to monofunctional alkylating agents or UV (Liu et al., 1997; Busch et al., 1996). Likewise, cells from complementation group G are not hypersensitive towards UV, ethyl and methyl methane sulphonate (De Winter et al., 1998).

To date, mutation anlysis in FANCG has revealed 18 different mutations, most of them small deletions and insertions or splice-site mutations, all leading to a premature stop-codon. The mutation spectrum and mutation analysis are further discussed in chapter 2 and chapter 4. A knock out mouse model of FANCG ($FancG^{-/-}$) has recently been generated (Yang et al., 2001) which is similar to that previously described for FANCC and FANCA. The mice have normal viability, no gross developmental abnormalities, decreased fertility and abnormal gonadal histology. Primary splenic lymphocytes, bone marrow progenitor cells and murine embryo fibroblasts demonstrate spontaneous chromosomal breakage as well as increased sensitivity to MMC and, to a lesser extent, ionizing radiation.

1.6.9 Current model of the FA pathway

All FA genes identified to date, except of $FANCD_2$, encode orphan proteins with uncertain functions. Altogether they share a common pathway which contributes to genomic stability, either as components of a multiprotein nuclear complex or of a signal transduction cascade. FANCA, FANCC and FANCG are found in the cytoplasm as well as in the nucleus, whereas FANCE and FANCF are localized almost exclusively in the nucleus (de Winter et al., 2000; Hoatlin et al., 1998; Garcia-Higuera et al., 1999; Youssoufian, 1994). Direct interactions have been

observed between several proteins: FANCG binds directly to the amino terminal nuclear localization sequence (NLS) of FANCA, and together they might stabilize the nuclear FA protein complex (Garcia-Higuera et al., 2000; Waisfisz et al., 1999a). Further direct interactions have been proposed for FANCC and FANCE as well as for FANCF interacting with FANCA and FANCC (Medhurst et al., 2001; de Winter et al., 2000). A direct interaction between FANCA and FANCC remains questionable (Kupfer et al., 1997a; Näf et al., 1998; Yamashita et al., 1998; Garcia-Higuera et al., 1999; Kruyt and Youssoufian, 1998; Reuter et al., 2000). The current view concerning the structure of the FA multiprotein complex is shown in figure 1.2. After stable binding of FANCA and FANCG, FANCB seems to play an important role in the nuclear transport as well as in FANCA-phosphorylation (Yamashita et al., 1998). After binding of FANCC, the complex accumulates in the nucleus, where FANCE and FANCF join (de Winter et al., 2000). Since cell lines from complementation group D all seem to have an intact nuclear complex, $FANCD_1$ as well as $FANCD_2$ are thought to act downstream (Timmers et al., 2001; de Winter et al., 2000; Garcia-Higuera et al., 2001). The monoubiquitylation of $FANCD_2$ by the complex is a crucial step in the reaction to DNA damage. FANCF may be important for monoubiquitylation (Siddique et al., 2001), although there exists no evidence for a physical interaction between $FANCD_2$ and the other FA proteins.

The monoubiquitylated $FANCD_2$ is targeted to discrete nuclear foci, where it colocalizes and copurifies with BRCA1 (breast cancer associated protein 1) (Garcia-Higuera et al., 2001), indicating that $FANCD_2$ may function in the same DNA damage response pathway as BRCA1. These nuclear foci are detected in cells after DNA damage as well as during DNA replication. To date, it is not clear, which other proteins are present in those BRCA1/$FANCD_2$-foci, but important DNA-repair proteins, such as RAD50, NBS1 (Nijmegen breakage syndrome 1) and MRE11 (Meiotic recombination 11), are also found to be present in BRCA1-containing foci (Scully et al., 1997; Paull et al., 2000). Recently, the ATM (Ataxia telangiectasia mutated) gene was shown to phosphorylate $FANCD_2$, building a link between the FA and the ATM damage repair pathways (Taniguchi et al., 2002). To date, there is no definitive evidence how the FA proteins might be involved in DNA repair, but the nuclear complex might act as a sensor for DNA damage. Following damage recognition it might monoubiquitylate $FANCD_2$, which then might be the reacting effector molecule. The fact that all complementation groups show a similar phenotype and that knock out mouse double mutants behave like single mutants, supports the notion of a common functional pathway of the respective FA proteins.

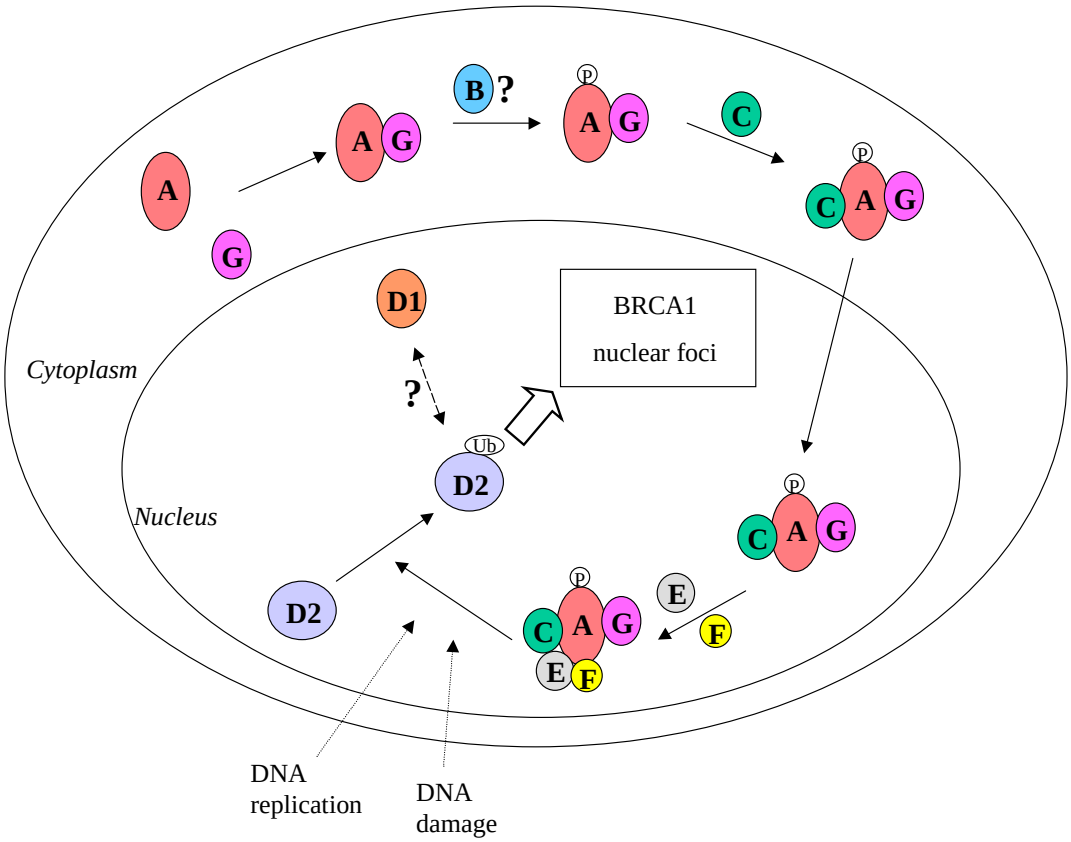


Figure 1.2: A model of the interaction of the FA proteins in a common pathway.

1.7 Retroviral systems and gene therapy

There are many ways to import genetic information, like DNA or RNA, into cells, but most of them are inefficient or instable. An optimal vehicle to transport DNA into cells are viruses, because they only exist for transporting their own genetic material from cell to cell. Since retroviruses stably integrate their genetic information into their host cell, the material is stably transmitted to the following cell generations, rendering them a very attractive medium for gene transfer. The most frequently used virus is Molony murine leukemia virus (MoMuLV). More recently, improved vectors based on a murine stem cell virus (MSCV) have been developped.

For transporting new information into cells, retroviruses have to be manipulated. On the one hand, the target DNA has to be inserted into the viral genome. And on the other hand, viral DNA or RNA has to be removed from the viral genome in order to stay below the maximum transport capacity of the retrovirus and in order to render the virus invirulent. Proteins that are necessary for infecting cells, are provided by a packaging cell line which produces "empty" retroviruses

without retroviral RNA and fills them with the target DNA surrounded by 5´- and 3´-LTRs.

A schematic presentation of packaging recombinant viruses with the target DNA is shown in figure 1.3. Construction of the vector can be done *in vitro*, packaging however has to be done *in vivo*, in so-called packaging cells. Those packaging cells carry replication-deficient proviruses as helper viruses in their genome (Cone and Mulligan, 1984; Mann et al., 1983; Miller and Rosman, 1989). These deliver all necessary viral proteins, such as group specific antigens (*gag*), reverse transcriptase and integrase (*pol*) and envelope proteins (*env*).

The *in vitro* constructed vector, generally a plasmid containing a 5´- and a 3´-LTR, the packaging signal, the targeted DNA (here: a WT FA gene) and eventually a selection marker, is transfected into an ecotropic murine packaging cell line, such as Phoenix cells, where the vector DNA is packed, within 48 hours, in helper virus encoded envelope proteins. Viruses are harvested from the supernatant. Because of the *env*-protein gp70, which defines host-specifity, those viruses can only infect murine and rat cells. The resulting viruses represent a mixture of viruses carrying different numbers of plasmids due to transfection. To get viruses with only one copy of the vector, a second, amphotropic packaging cell line, such as Gibbon ape leukemia virus (GALV) (Miller et al., 1991), is infected. Because of the retroviral infection mode, only one virus infects each cell and the DNA is stably integrated into the host genome. The *env*-protein gp70 of that amphotropic packaging cells increases the host spectrum and enables the resulting virions to infect human cells. In order to increase virus titers, eco- and amphotropic cells are infected alternately ("ping-pong-amplification") resulting in amplification of the recombinant retrovirus.

The subsequent infection of a FA cell line of unknown complementation group is shown in figure 1.4, where a cell line is tested whether it belongs to complementation group A. The recombinant retrovirus containing FANCA binds to host specific receptors and penetrates into the host cell of unknown complementation group. In the cytoplasma, the RNA of the virion is transcribed into DNA and transported into the nucleus, where it is stably integrated elsewhere in the host´s genome by the viral reverse transcriptase and integrase. The virus-mediated FANCA now is transcribed and translated like any other FA gene in the cell. In order to identify the complementation group, the cells are selected by MMC for survival. If the host cells are resistant to MMC, FANCA is the gene defective in that patient and the complementation group is FA-A. Those cell lines that remain MMC-hypersensitive do not belong to complementation group A, because the introduction of WT FANCA cannot compensate the lack of a FA gene. In that case the cell line is successively infected with other retroviruses containing the FA genes FANCC, $FANCD_2$, FANCE, FANCF as well as FANCG. If the tested cell line remains hypersensitive to MMC after all given retroviruses, the gene defective in that patient has to be FANCB or $FANCD_1$ or a new FA gene.

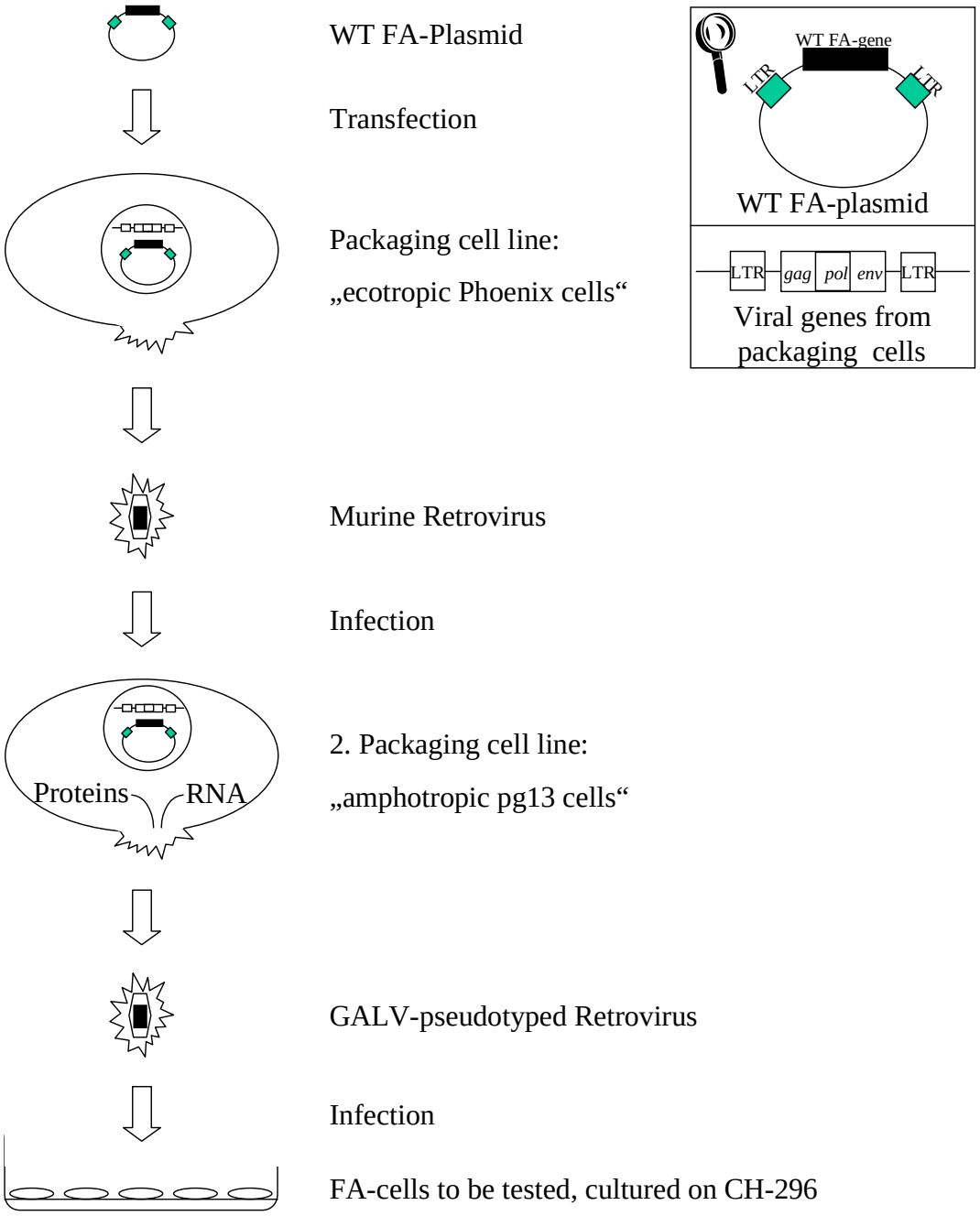


Figure 1.3: Packaging of recombinant retroviruses for complementation analysis and pathogenity testing of amino acid substitutions (chapter 3, chapter 5, chapter 6).

The viral transduction method to determine one´s complementation group is fast and reliable, but there are two limiting factors. First, the underlying FA gene

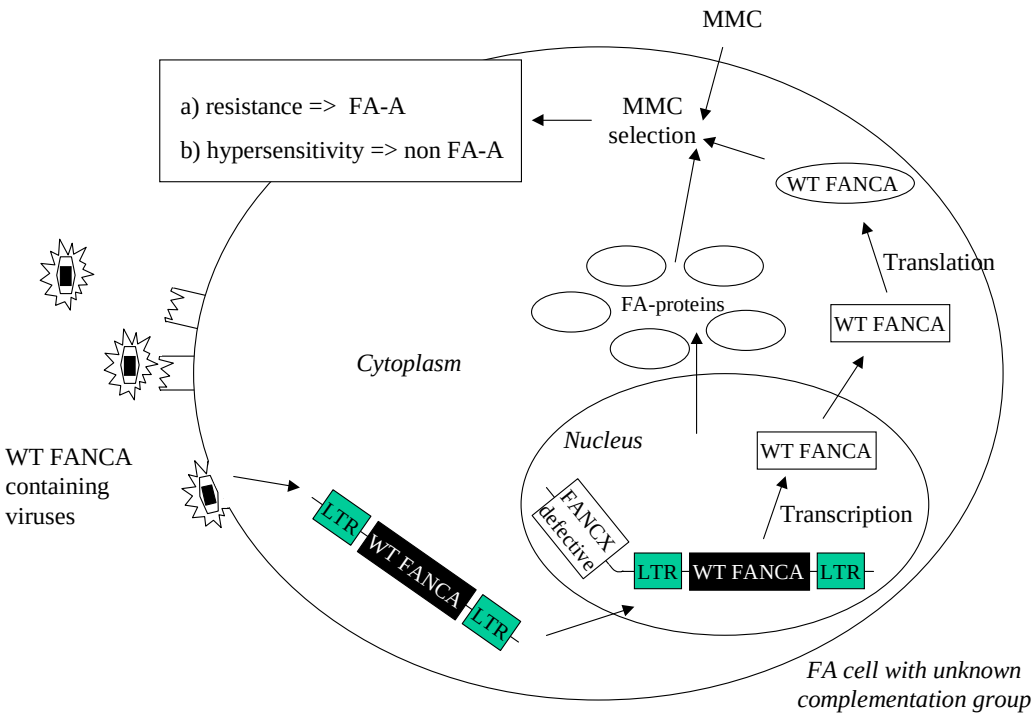


Figure 1.4: Principle of retroviral infection of FA cells with unknown complementation group for complementation analysis, examplary complementation group A.

has to be identified before, otherwise no virus can be constructed. Second, a lymphoblastoid cell line cannot be classified, if mosaicism has developed. In that case, skin fibroblasts have to be examined in order to get interpretable results.

In current gene therapy trials, peripheral blood leukocytes are enriched for hematopoietic stem cells. Then retroviral vectors which express WT FANCA or FANCC are used to transduce *in vitro* those blood cells, before they are given back into the patient´s bloodstream (Noll et al., 2001; Fu et al., 1997). The corrected stem cells are supposed to initiate and support proficient hematopoiesis. Actually, those attempts are not as successful as expected, because some important obstacles still have to be overcome. The most important problems to be solved are as follows: Generally, the transduction efficiency is poor, since the number of hematopoietic stem cells in the peripheral blood after enrichment still remains small. The transgene often is unstably expressed due to silencing and immunological attack of the transduced cells, which now express a protein that the body might recognize as being foreign. Last not least, even after successful gene therapy, the residual mutant cells might remain predisposed to cytogenetic abnormalities, which could lead to leukemia (Gregory et al., 2001).

1.8 Thesis outline

To date, several mutations have been identified in the FA genes which have provoked many interpretations, such as the occurence of founder mutations, genotype-phenotype-correlations, hotspot regions as well as the position of possible functional domains or protein-protein-interactions. In the context of this thesis, we tried to detect as many mutations as possible in order to confirm the complementation groups determined by our virus-based method for complementation analysis. Newly detected mutations should provide further information on mutation spectrum, hotspot regions or functional domains in the three FA genes (FANCA, FANCC and FANCG) which are routinely examined at our laboratory (chapter 2, chapter 3, chapter 4). The main focus is on FANCC (chapter 3), because there are only 10 known mutations accumulated in the 5´- and 3´-region and in exons 4-6, but so far no large deletions have been described. We therefore examined ten FA-C patients in order to get further information about the mutation spectrum and/or the position of possible functional domains. In the FANCA gene, there are more than 100 different mutations published to date, due to FA-A being the largest complementation group as well as FANCA being hypermutable carrying mostly "private" mutations. The main interest in that gene was in the detection of both mutations in patients with diagnosed mosaic status in their blood cells. The patients´ skin fibroblasts, lymphoblastoid cell lines and their peripheral blood cells were examined to detect the reverted allele and to find out the mechanism of reversion (chapter 5). In two of the FA-A mosaic patients the different blood cells were analyzed separately in order to find out when in the patients´ hematopoiesis the reversion took place, which cell lineages were affected and if there was hit a bone marrow stem cell (chapter 6).

Due to the screening method (SSCP) used for mutation analysis, mostly single base changes leading to amino acid substitutions are detected. The pathogenicity of these single base changes has to be proved by showing a causal relationship between genotype and phenotype. For this purpose, a virus-based method for the identification of pathogenic missense mutations described in chapter 3 and chapter 5 has been established during this work in cooperation with the group of Dr. H. Hanenberg at Düsseldorf, including site directed mutagenesis and MMC complementation of $FA^{-/-}$ cell lines by the constructed viruses (used in chapter 3, chapter 5 and chapter 6).

Chapter 2

Mutation analysis in the Fanconi anemia genes, especially FANCA

Michaela Gross

Department of Human Genetics, University of Würzburg, Biozentrum, Am Hubland, D-97074 Würzburg, Germany

Translation from the German article: Michaela Gross (2002) Mutationsanalyse in den Fanconi Anämie Genen unter besonderer Berücksichtigung von FANCA. *medgenetik* edition 2 / 2002 Fanconi Anämie, in press

2.1 Abstract

Recent evidence concerning severity and hematological course of Fanconi anemia demonstrated that assignment to one of the complementation groups is not as crucial for the clinical outcome of the disease as two other important factors (Faivre et al., 2000): on the one hand the type of mutation and on the other hand the development of somatic mosaicism in hematopoietic cells. For both aspects mutation analysis is necessary. In addition, mutation analysis is a helpful tool for FA diagnosis, including prognostication of the clinical course and the possibility of prenatal diagnosis.

2.2 Introduction

Fanconi anemia is a genetically heterogenous disorder with at least eight different complementation groups (FA-A, FA-B, FA-C, FA-D_1 (BRCA2), FA-D_2, FA-E, FA-F, FA-G). Because of the rarity of the complementation groups FA-E, FA-F, FA-D_2 and BRCA2 in our patients, mutation analysis in those genes has not been performed. Although FANCA is the largest and furthermore the most polymorphic FA gene, mutation analysis in patients with undefined complementation group is always started in that gene. The reason being that FA-A with about 60% is the most prevalent complementation group in Germany. If there are no mutations detected in FANCA, the second gene screened is FANCG, because about 15% of the German FA-patients belong to that complementation group. The last FA gene routinely examined is FANCC, but regional or ethnic origin of the patient can modify that sequence. Recently, a rapid method for defining one´s complementation group using recombinant diagnostic retroviruses (Dr. H. Hanenberg, University of Düsseldorf) renders complementation analysis possible before mutation analysis is done.

Mutation analysis is performed on genomic DNA as well as on cDNA. For genomic DNA, SSCP analysis ("Single strand conformational polymorphism"), which detects single base changes, small deletions and small insertions, is used for prescreening. On the basis of cDNA another prescreening method is used, called PTT ("Protein Truncation Test"), which detects all types of truncating mutations in an *in vitro* translated protein. DNA sequencing is performed on an ABI Prism 310, which was provided by the "Schroeder-Kurth-Fonds" and the "Deutsche FA-Hilfe".

2.3 Results and discussion

2.3.1 Spectrum of mutations in FANCA

The mutation spectrum in the FANCA gene is quite different from those in FANCC and FANCG because of its heterogeneity. There have been described more than 100 different mutations in FANCA (Wijker et al., 1999; Morgan et al., 1999; Savino et al., 1997; Centra et al., 1998; Ianzano et al., 1997; Levran et al., 1997; Levran et al., 1998; Tachibana et al., 1999). These mutations are dispersed throughout the entire gene and most of them can be found online under www.rockefeller.edu/fanconi/mutate/default.html. In contrast to FANCC and FANCG, the FANCA gene exhibits all kinds of mutations. The most frequent mutation type represents large deletions, which can span 1 to 31 exons. In some cases deletions spanning the entire FANCA gene have been reported (Centra et al., 1998). To date, large deletions have neither been published in FANCC nor in FANCG. Almost the same percentage as large deletions represent microdeletions and microinsertions, which can - but don´t necessarily have to - result in a frameshift. Other reported changes involve splice site mutations, nonsense mutations as well as some missense mutations. Because of the great variety of alterations, patients carrying the same mutations and homozygosity are rarely found. Most FANCA mutations are so-called "private mutations", with the exception of a few common mutations, such as 1263delF (Levran et al., 1997). The percentage of compound heterozygous patients is about 50-60% (Savino et al., 1997; Tachibana et al., 1999; Wijker et al., 1999; Morgan et al., 1999). Concerning "private mutations" and compound heterozygosity , genotype/phenotype correlations are difficult, because there exist only very few individuals carrying the same mutations, and the phenotype of patients with only a single identical mutation is difficult to compare to each other. According to Faivre (Faivre et al., 2000), genotype/phenotype correlations can at best be assumed by the occurence of milder and more severe mutations, but not by complementation group or by single mutations. Generally, amino acid substitutions are thought to be the mildest type of mutations, whereas such alterations without any residual protein usually seem to provoke a more severe phenotype. The severity of the phenotype is generally characterized by criterions like the age of onset of aplastic anemia, the time of survival after diagnosis and the occurence of AML or MDS. But even the distinction between so-called milder and more severe mutations has to be done with care, because there are siblings with obvious variations in their phenotypes, even though they carry inherited mutations. Some of this variation might be due to the emergence of mosaicism in one of the siblings.

The identified mutations in the FANCA gene seem to be dispersed throughout the entire gene. There are only few exons where no mutation has been described to date (figure 2.1).

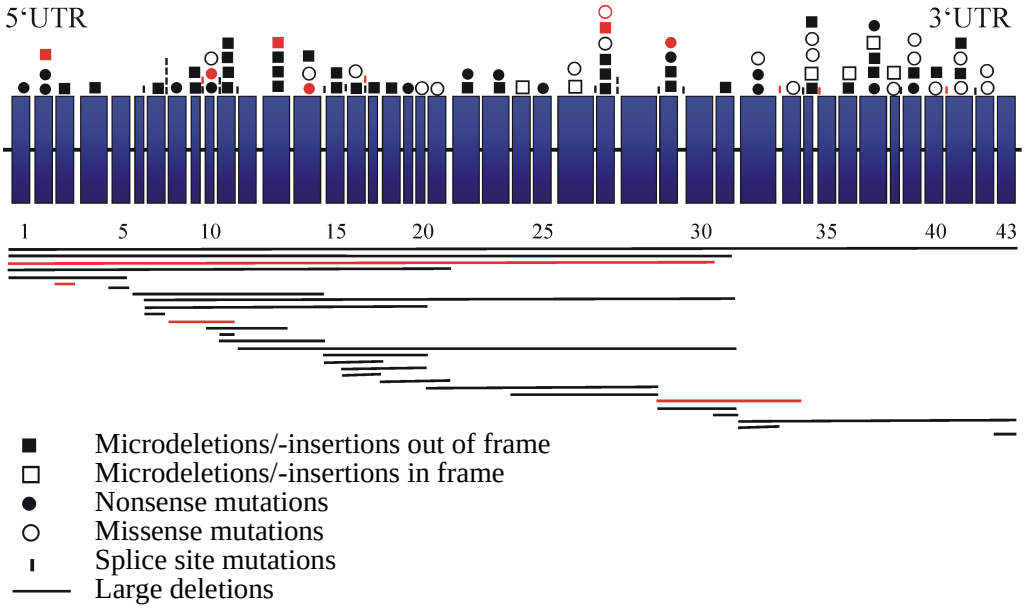


Figure 2.1: Distribution of the mutations in the FANCA gene. The figure combines unpublished mutations found at Würzburg with those detected by other groups up to 2000 (Wijker et al., 1999; Morgan et al., 1999; Savino et al., 1997; Centra et al., 1998; Ianzano et al., 1997; Levran et al., 1997; Levran et al., 1998; Tachibana et al., 1999).

The hypermutability of FANCA appears to be caused by the large number of repetitive elements spread throughout the gene. Repetitve elements include short direct repeats, homonucleotide tracts, *Alu*-repeats, CpG-islands as well as several hotspot-motifs, for example CCTG/CAGG. As shown in table 2.1 microdeletions and -insertions as well as amino acid substitutions are frequently found (73%) next to short direct repeats and homonucleotide tracts (Levran et al., 1997). These types of repetitive elements are thought to trigger slipped-strand-mispairing, leading to microdeletions/-insertions and base changes. Base changes are also found to be located next to CpG-motifs, which are known to trigger methylation-mediated mutations. A possible reason for the relative frequency of large intragenic deletions might be *Alu*-mediated recombination between the numerous intronic *Alu*-repeats interspersing FANCA. A highly significant correlation between the number of deletion breaking points in a specific intron and the number of *Alu*-repeats has been reported (Morgan et al., 1999). The relationship between the location of *Alu*-repeats and deletion breakpoints is illustrated in figure 2.2 and corresponds with the deletion breakpoints detected at Würzburg (figure 2.1).

Mutation type	Short direct repeats	Homo-nucleotide tracts	CpG-motifs	*Alu*-repeats	Mutation mechanism
Microdeletions/ -insertions	+	+			Slipped-strand-mispairing
Base substi-tutions	+	+	+		Slipped-strand-mispairing, DNA-methylation
Large deletions				+	*Alu*-mediated recombination

Table 2.1: Repetitive domains described for FANCA and the mutation types triggered by them.

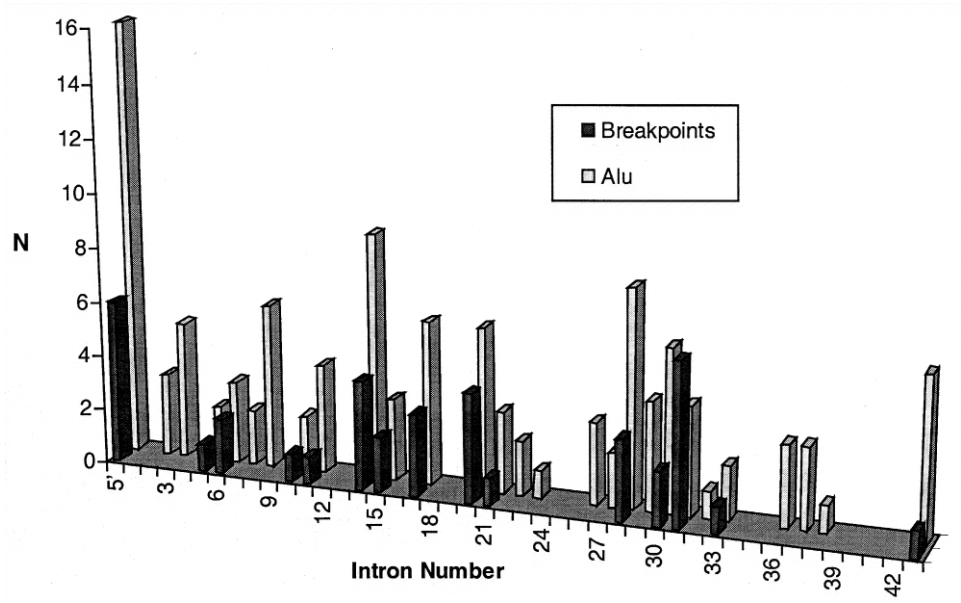


Figure 2.2: Connection between FANCA deletion breakpoints and Alu-repeats. (from: (Morgan et al., 1999))

In spite of the generally widely dispersed distribution of mutations in FANCA, there are some regions where changes seem to cluster. For example, the 5´-region contains mostly truncating mutations, such as microdeletions or microinsertions resulting in a frameshift. In the 3´-region (especially from amino acid 1046 to 1320), however, there are many microdeletions/-insertions which retain the reading frame as well as many missense mutations, suggesting that this region of FANCA might be critical for protein function. This notion is supported by the

fact that interactions with other FA proteins like FANCG have been assigned to this region (Ianzano et al., 1997; de Winter et al., 2000).

2.3.2 Polymorphisms in FANCA

As has been discussed for its pathogenic mutations, the FANCA gene is also hypervariable in its spectrum and distribution of polymorphisms. Sequencing reveals numerous single base substitutions as well as small insertions or deletions in its intronic sequence. In particular, introns 19 and 20, but also introns 23, 24, 31, 35 and 39 are found to be highly mutable and they show several different types of gene variation. In the coding region the reported polymorphisms are spread all over the gene just as described for mutations. However, there exist some areas in the gene where the density of identified alterations is more intense than elsewhere in FANCA. Such highly polymorphic regions include exons 8-11 and exon 32 compared to relative constant regions like exons 2-7 or exons 16-21. The distribution of polymorphisms found in the coding sequence are shown in figure 2.3.

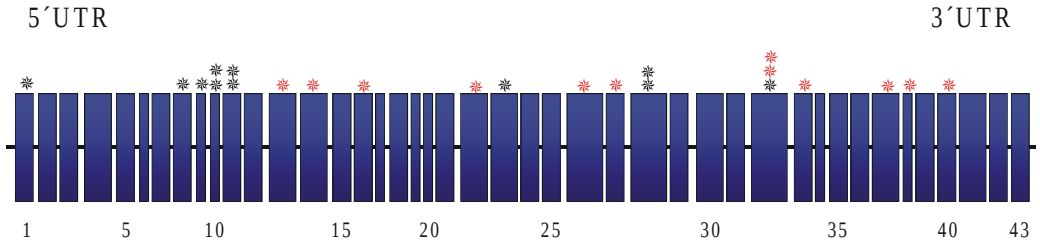


Figure 2.3: Distribution of the polymorphisms found in the coding sequence of FANCA. Polymorphisms that have been detected in Würzburg are shown light grey.

To distinguish polymorphisms from pathogenic mutations, the following strategies must be applied:

1. Detection of further, conclusively pathogenic mutations
2. Screening of at least 100 control alleles
3. Cosegregation with the clinical phenotype
4. Complementation analysis

Polymorphisms characterized at Würzburg have been confirmed by method one and/or two. In addition, there are ongoing trials to establish confirmation by retroviral complementation analysis (see chapter 3, chapter 5, chapter 6).

2.3.3 Spectrum of mutations in FANCG and FANCC

In contrast to FANCA, the mutation spectra of FANCG and FANCC are less variable. On the one hand, both genes are much smaller than FANCA (about 500 amino acids each compared to 1500 amino acids in FANCA), and on the other hand both the exon and the intronic sequences seem to be less polymorphic. The lack of repetitive elements is thought to be the reason for the relative stability of these genes. For both genes founder mutations have been described. A very common founder mutation in FANCG is the nonsense mutation E105X, because 44% of the German FA-G-patients carry it. For FANCC there exist two common founder mutations, the microdeletion 65delG with high prevalence in the Netherlands and in Germany, as well as the splice site mutation IVS4+4A>T, described as a founder mutation for Ashkenazi Jews. The distribution of mutations and polymorphic changes in those two genes is illustrated in figure 2.4 (see also chapter 4) and figure 2.5 (see also chapter 3), respectively.

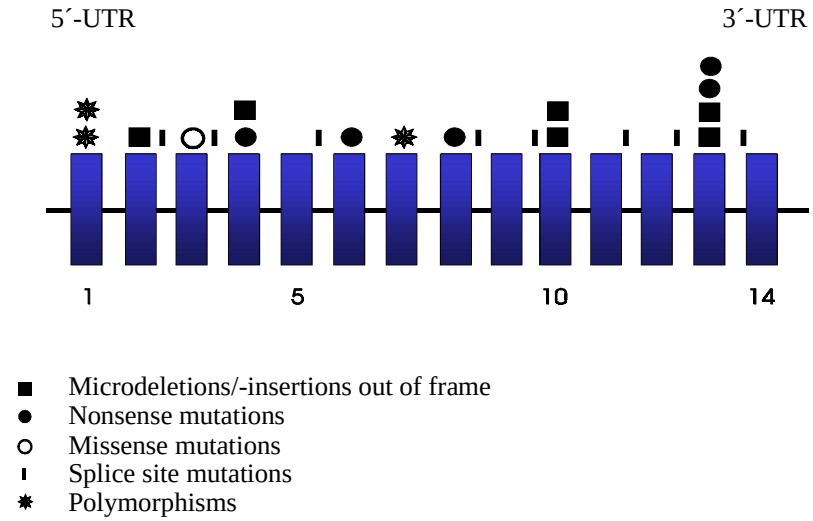


Figure 2.4: Distribution of the mutations and the polymorphisms found in the coding sequence of FANCG (Demuth et al., 2000; Yamada et al., 2000).

The distribution of mutations in FANCG (figure 2.4) is similar to that in FANCA, but nevertheless there are some significant differences between these two genes. To date there has been no report of any large intragenic deletion in FANCG, which seems to represent the most frequent kind of mutation in FANCA. Another significant difference is the high proportion of truncating mutations (94%) compared to only 81% in FANCA. In addition, there is only a single confirmed missense mutation, which has not been found in 100 ethnical matched control alleles and, moreover, is located in a leucine-zipper-motif of presumptive functional significance. Three further amino acid substitutions in exons 1 and 7 have been

classified as polymorphisms. Interestingly, these are all located in exons without any described pathogenic mutation. Changes of the latter type might therefore be lethal if they occur in these exons.

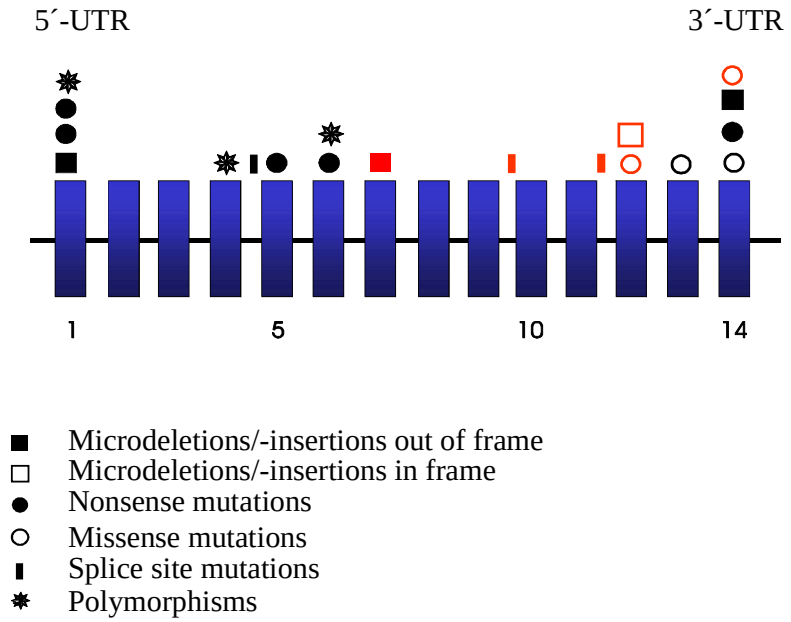


Figure 2.5: Distribution of the mutations as well as the polymorphisms found in the coding sequence of FANCC (Strathdee et al., 1992; Gibson et al., 1993b; Gibson et al., 1996; Whitney et al., 1993; Verlander et al., 1994; Lo Ten Foe et al., 1996c; Lo Ten Foe et al., 1996a; Lo Ten Foe et al., 1998). Those alterations that have newly been detected at Würzburg are coloured in red (see also chapter 3).

In FANCC, the distribution of the mutations differs considerably from that in FANCA. Mutations are not found to be dispersed throughout the entire gene, but they appear to cluster in certain regions. Such regions are exon 1, exons 5-7 and the C-terminus of FANCC. Based on the relative prevalence of mutations in exon 14 and the high conservation of this exon across species, the carboxy terminal region of FANCC is likely to contain a critical functional domain. The spectrum of mutations can generally be compared with that of FANCG, and there are also no large deletions. However, the proportion of truncating mutations (73%) is smaller than for FANCG and still smaller than for FANCA. Similar to FANCA, pathogenic missense mutations are found to be located in the 3´-area, suggesting the presence of either domains for protein interactions or for protein folding.

2.3.4 Mosaicism

The phenomenon of somatic mosaicism is found in about 15-25% of FA-patients. To date, mosaicism has only been described in patients carrying mutations in the FANCA and for the FANCC genes. After developing mosaicism, patients exhibit two subpopulations of cells in their blood, the mutated as well as the corrected one. The proportion of corrected blood cells can vary from poorly detectable (in an early stage of mosaicism) to almost 100%. If 100% of the cells are corrected there are hardly any MMC-sensitive cells in the patient´s blood, rendering FA diagnosis on lymphocytes difficult to impossible. In this case, the diagnosis has to be confirmed by means of skin fibroblasts.

There are several mechanisms thought to be responsible for triggering mosaicism. Generally, all of them create a WT allele resulting in normal chromosomal breakage of the patient´s lymphocytes. In compound heterozygous patients, one possible mechanism is **intragenic mitotic recombination**, where afterwards both mutations are carried on one allele. Consequently, the progeny of that single corrected cell presents a heterozygous phenotype, although still carrying both mutations in its genome (Lo Ten Foe et al., 1997). The mechanism of **gene conversion** is different, because one mutation completely disappears and therefore all progenitor cells are heterozygous. **Compensatory mutations** have been described as another reversion mechanism (Waisfisz et al., 1999b). Compensatory mutations have been described for homozygous FA-patients, where frameshift mutations have been corrected by microdeletions or -insertions a few basepairs downstream restoring the reading frame thereby partially restoring protein function. A missense mutation can also be corrected by a further base change in the same codon leading to a third amino acid, which also goes along with restoration of protein function. A fourth, hypothetical mechanism could be **polymerase slippage**, where an intact residual protein might be present in addition to the mutated protein.

The clinical consequences of mosaicism are not fully understood. Some of the older FA-patients have developed a somatic mosaic, which might be responsible for improved survival and a mild hematological course of the disease. But not all mosaic patients have stable blood counts and some of them even have very severe clinical course of disorder. Furthermore, the development of mosaicism may have a negative effect on bone marrow transplantation. Severe complications may arise, as the corrected blood cells are able to survive chemotherapeutical treatment with immunosuppressive agents, such as cyclophosphamide or cisplatin, used to prevent graft-versus-host disease. Altogether, the diagnosis of mosaicism may alter the clinical course of the disease and needs careful evaluation prior to therapeutic interventions. A positive aspect of mosaicism can be seen in the fact that self-corrected cells may obtain a proliferative advantage and replace the patient´s defective cells. However, this process may take several years, and com-

plete restoration of bone marrow function requires that the reversion takes place in a putative bone marrow stem cell. In these cases, somatic cell mosaicism can be regarded as an example of "natural" gene therapy, which implies that artificial gene therapy by replacing the patient´s defective gene in bone marrow cells may ultimately be successful.

Chapter 3

Novel mutations, including a large deletion, in the FANCC gene

Michaela Gross (1), Reinhard Kalb (1), Detlev Schindler (1) , Helmut Hanenberg (2), Johannes Rischewski (3)

(1) Department of Human Genetics, University of Würzburg
(2) Department of Pediatrics, University of Düsseldorf
(3) Department of Pediatrics, University of Hamburg

To be submitted to *Human Mutation*

3.1 Abstract

Fanconi anemia (FA) is a genetically heterogenous autosomal recessive disorder associated with chromosomal instability, progressive bone marrow failure, congenital abnormalities, predisposition to cancer and marked hypersensitivity to DNA crosslinking agents such as mitomycin C (MMC). The FA proteins are thought to cooperate in a multiprotein pathway instrumental in the cellular response to DNA damage. Among the currently known seven FA genes, the FANCC gene stands out both in terms of genotype/phenotype correlations and presumptive function. We complement and expand the mutation spectrum reported for FANCC by analyzing ten additional FA-patients belonging to complementation group C. Five of these ten patients were compound heterozygotes, and eight carried at least one previously unknown mutation. We also describe the first large deletion of FANCC which was found in a homozygous patient with consanguinous parents. A DHPLC-based screening method has been developped to identify heterozygous large deletions in FANCC.

Keywords: Fanconi anemia, FANCC, mutation screening, DHPLC, complementation

3.2 Introduction

Fanconi anemia is a genetically heterogenous autosomal recessive disorder characterized by chromosomal instability, progressive bone marrow failure, congenital abnormalities, predisposition to cancer and marked hypersensitivity to DNA crosslinking agents (Alter, 1996; Schroeder et al., 1964; Sasaki and Tonomura, 1973; Auerbach et al., 1997). To date, seven FA genes have been cloned: FANCA (Lo Ten Foe et al., 1996b; The Fanconi anemia/Breast cancer consortium, 1996), FANCC (Strathdee et al., 1992), FANCD2 (Timmers et al., 2001), FANCE (De Winter et al., 2000a), FANCF (De Winter et al., 2000b), FANCG (De Winter et al., 1998) and FANCD1 which has recently been shown to be identical with the breast cancer gene BRCA2 (Wooster et al., 1995; Tavtigian et al., 1996; Witt and Ashworth, 2002; Howlett et al., 2002; Stewart and Elledge, 2002; Daniel, 2002). All FA proteins together constitute a multiprotein pathway which is involved in cellular responses to DNA damage and, presumably, in other as yet unknown cellular processes (Joenje and Patel, 2001; Grompe and D´Andrea, 2001; Dronkert and Kanaar, 2001).

The FANCC gene was the first FA gene to be isolated by expression cloning (Strathdee et al., 1992), and the first convincing genotype/phenotype correlation was established for mutations in FANCC (Kutler et al., 2002). While in response to DNA damage five of the seven known FA genes, including FANCC, form a nuclear complex that activates the downstream FANCD2 effector gene (Joenje and

Patel, 2001), the FANCC gene product appears to exert a number of additional functions that are largely independent of its participation in the FA multimeric complex. These additional functions include the apparent involvement of FANCC in detoxification, anti-oxidant and anti-apoptotic pathways (Kruyt et al., 1998b; Cumming et al., 2001; Pang et al., 2000). In order to understand the pleiotropic nature and its functions, it is important to take note of the full range of naturally occurring alterations in the FANCC gene.

By analyzing the mutations in ten additional FA-C patients, we here complement and expand the published record of viable alterations in FANCC. We show that the spectrum of FANCC mutations is much more heterogenous than previously assumed and may include large deletions. We further describe the establishment of a quantitative screening method for the detection of large deletions in FANCC by use of denaturating HPLC (DHPLC). This PCR based method has been developped to exclude large deletions, especially if they start upstream or end downstream of the FANCC gene, on genomic DNA avoiding the pitfalls due to mRNA instability.

3.3 Material and methods

3.3.1 Patients, cell culture and FANCC diagnosis

The ten patients we describe were referred to our laboratory in order to confirm or rule out the clinical suspicion of Fanconi anemia. Whenever possible lymphoblastoid cell lines and/or, especially in the case of mosaicism, fibroblast cultures were established with informed consent. Mononuclear blood cells isolated by Ficoll separation were used for direct transfections, for 72 h PHA lymphocyte cultures and for the establishment of EBV-transformed lymphoid cell lines (LCL). LCL were maintained in RPMI-media (Gibco) with 16% fetal bovine serum. Fibroblast cultures were established using standard cell culture procedures and MEM-media supplemented with glutamine and 16% fetal bovine serum. All cell cultures were kept in high humidity incubators equipped with CO_2 and O_2 sensors in an atmosphere of 5% (v/v) CO_2 and 5% (v/v) O_2 by replacing air through nitrogen. The clinical suspicion of FA was confirmed by flow cytometric evaluation of lymphocyte cell cultures according to standard-techniques (Poot et al., 1994; Seyschab et al., 1995). Assignment of patient cells to complementation group C was achieved via complementation analysis using a retroviral vector containing the full-length cDNA of FANCC (Hanenberg et al., 2002; Freie et al., 1996) and (Lobitz et al, manuscript in preparation). Nine of 146 tested FA-lymphocyte cultures were shown to belong to complementation group C on the basis of retroviral transfection studies, one further patient was identified to belong to FANCC on the basis of molecular analysis of genomic DNA. We describe the mutations

detected in these ten FANCC-patients, nine of which represent Caucasians and one is from central Asia.

3.3.2 Mutation analysis

PCR, SSCP and sequencing: DNA and mRNA were extracted using standard protocols (Maniatis et al., 1982). PCR conditions were established to amplify the exons one to 14 of FANCC according to table 3.1 (Saiki et al., 1988). PCR was performed in 50 μl reactions. Prescreening was performed using SSCP-analysis with commercial acrylamide gels (Amersham pharmacia, Freiburg, Germany) and chambers (Pharmacia Biotech, Freiburg, Germany) at 14°C followed by silver staining. Direct sequencing of aberrant fragments was done by fluorescent end labeling on an ABI Prism 310.

RT-PCR: cDNA was produced using SuperScriptII (Invitrogen, Karlsruhe, Germany) according to the manufacturer´s instructions. For PCR and sequencing, primers and conditions were according to table 3.1.

Strategies revealing the large deletion of patient BAFO: In one patient we were not able to amplify exons one and two, suggesting a homozygous deletion of these two exons. The existence of a correct mRNA of the dowstream exons suggested an intact promoter region. In order to amplify a product across the putative deletion region, the following primers were used: ACTGCTGACACGTGTGCGC (255 bp upstream of ATG) and ATGATTCTCTCTGAGTTCAGACG (exon 5). The reaction revealed two different PCR products in the heterozygous mother of BAFO (750 bp and 407 bp), a single short fragment (407 bp) in patient BAFO and the homozygous long variant (750 bp) in a healthy control. In order to identify the deletion breakpoints, the PCR products were cloned into the TOPO Cloning vector using TopoTA cloning kit for sequencing (Invitrogen, Karlsruhe, Germany) according to the manufacturer´s instructions. After preparation, the plasmids were sequenced on the ABI310 sequencer using the above-mentioned primers.

3.3.3 Quantitative PCR analysis

The genomic PCR fragments were analyzed by denaturating HPLC (DHPLC) according to the method described by Oefner and Underhill on a WAVE detector (Transgenomic, USA). 10 μl of the sample were injected into a preheated C_{18} reversed-phase column with nonporous polystyrene-divinylbenzene particles (DNASep®, Transgenomic, USA). Analysis was performed using non-denaturing conditions with a column temperature of 50°C. The sample was eluted with a linear acetonitrile (ACN) gradient consisting of buffer A (0.1M triethylammonium acetate, TEAA) and buffer B (0.1M TEAA, 25% ACN) with a 2% increase of

Gen	Exon	forward-Primer	reverse-Primer	Annealing	Cycles
FANCC	1	accatttccttcagtgctgg	accacaagtcccgattctggg	51,8°C	40
FANCC	2	ccctcaatctataaatgtccag	gtaagcctctgtgaaacaatg	55,3°C	40
FANCC	3	tagtgatttgagatttttcc	gcagcacttttaaataatc	53,4°C	40
FANCC	4	gtaggcattgtacataaaag	tggcacattcagcatttaaac	56,8°C	40
FANCC	5	ctgatgtaatcctgtttgcag	cctctcataaccaaactgatac	62,4°C	40
FANCC	6	gtccttaaattatgcatggctc	caacacaccacagccttctaag	63,5°C	40
FANCC	7	ttttcagtgagccatttctg	aaatgagtccaagcatgtcc	56,8°C	40
FANCC	8	ctcctttggctgataatagc	cccatgatacagccagagac	53,4°C	40
FANCC	9	tttcccttatacagtgcagg	gtgctcttgtccaaaatactc	63,5°C	40
FANCC	10	ttcctgacccgtttcaatc	ttgacaatgctcttcccagg	61,1°C	40
FANCC	11	gtgaaccagaagtaaagggc	aggatctagggaaaccatg	51,8°C	40
FANCC	12	cccaaaggaagaagaatttag	ctctagccccccagagcc	51,8°C	40
FANCC	13	cctagaagtatgtctgtcctg	ctctccttgactaggatgctg	53,4°C	40
FANCC	14	ggatagggcttctttcaggg	tcccaagatgtgtacagctc	61,1°C	40
PCR 1	1	accatttccttcagtgctgg	accacaagtcccgattctggg	61°C	28
	6	gtccttaattatgcatggctc	caacacaccacagccttctaag		
	vWJ3	tgagatcaccagcccaacc	cagccctccctctgaagtcc		
PCR 2	14	ggataggggcttctttcaggg	tcccaagatgtgtacagctc	61°C	28
	vWJ3	tgagatcaccagcccaacc	cagccctccctctgaagtcc		
FANCC	Fr. A, E. 1-4	gctgctgtgaagggacatc	attcgcctttgagtgttaaatcc	58°C	35
FANCC	Fr. B, E. 4-9	tctcatatactttcagcactcag	agaagtgtaaggaaagtaggtc	58°C	35
FANCC	Fr. C, E. 8-11	atgctgcatctttttgaaaagc	caggacccatgagtctggtc	58°C	35
FANCC	Fr. D, E. 11-14	agctgcggtttgcactcaag	ctagacttgagttcgcagctc	58°C	35

Table 3.1: PCR-primers and conditions for FANCC: a) Primers/conditions on genomic DNA b) Primers/conditions for the multiplex-PCRs used for DHPLC c) Primers/conditions on cDNA

buffer B/min. PCR products were separated with a flow rate of 0,9 ml/min. The retention time was measured online via UV-absorbance in the eluate. Resulting diagrams showed the absorbance intensity (mV) over the retention time (mV/ml) after injection into the column. For gene dose analysis of FANCC, a triplex (PCR 1) and a duplex (PCR 2) PCR reaction of genomic material were analyzed. PCR 1 contained exon 1 and exon 6 of the FANCC gene as well as vWJ3 (von Willebrand Jürgens disease gene exon 3) as an external non-FA dosage reference; PCR 2 consisted of FANCC exon 14 and vWJ3. Reactions were optimized such that the cycle number was within the exponential product region. Product length was chosen such that the retention peaks as measured by DHPLC could clearly be separated from each other. PCR conditions are given in table 3.1. The product amount (= area under the eluted retention peak of a specific exon) was measured relative to the amount of vWJ3 (= area under the curve of the vWJ3 peak) as a biallelic control. Reliability of the method was tested by injecting PCR products from an artificial hemizygous individual (patient BAFO, who misses exon 1 and 2 on both alleles of FANCC, mixed with wt-DNA, see results) and the patient´s obligatory heterozygous mother.

3.3.4 Site directed mutagenesis

In order to confirm the pathogenic status of our two new amino acid exchanges, they were functionally analyzed by transducing the mutations L423P and T529P into the pCMV/FANCC-vector (courtesey of Dr. Matthias Wagner, Würzburg, Germany) by the method of site directed mutagenesis. The sequences of the phosphorylated mutagenic primers were designed according to the manufacturer´s instructions (QuikChange Site-Directed Mutagenesis Kit, Stratagene, Amsterdam, Netherlands) and were as follows:
L423P/for 5´-CCGAACCCCCCACGGCCCTGC**C**GTGGCTCTTGGCCT,
L423P/rev 5´-GTAGTAGAAGGCCAAGAGCCAC**G**GCAGGGCCGTGGG,
T529P/for 5´-CATTGGCTTTCTTGACCAG**C**CCTTGTACAGATGGAA and
T529P/rev 5´-GACGATTCCATCTGTACAAGG**G**CTGGTCAAGAAAGC
(modified nucleotides are written in bold letters). The reaction was performed in 50 μl-reactions containing 20 ng of plasmid DNA, 125 ng of each mutagenesis primer, 1,5 mM $MgCl_2$, 1x reaction buffer, 200 nM dNTPs and 2,5 Units *Pfu*-Turbo DNA Polymerase (Stratagene, Amsterdam, Netherlands). An initial denaturation for 30 s at 95°C was followed by amplification for 12 cycles, each with denaturation for 30 s at 95°C, annealing for 60 s at 55°C and extension for 13 min at 68°C. After placing the reactions on ice for 2 min, 10 Units DpnI were added and the reaction was incubated at 37°C for 1 h to digest the parental supercoiled dsDNA. The FANCC coding sequence carrying the desired mutations were full-length amplified using primers carrying a NotI- (forward-primer) and a BamHI- (reverse-primer) tail and afterwards cloned as 1.9 kb NotI/BamHI DNA

fragments into the S11IEG expression vector (subsection 3.3.5) and sequenced to show the integrity of the entire coding sequence except for the presence of the targeted mutations. The FA-C cell line HSC536 (reference cell line for FA complementation group C) was stably transfected with the targeted cDNAs and tested for correction of MMC hypersensitivity as described in the following section.

3.3.5 Retroviral vectors

The plasmid LFCPEG, a FANCC-WT and EGFP (enhanced green fluorescent protein) containing derivative from MSCV2.1 (Hawley et al., 1994), was used for complementation studies. For site directed mutagenesis testing, the FANCC coding sequence carrying the amino acid substitutions L423P and T529P respectively were cloned into S11IEG, a vector derived from S11 (Hildinger et al., 1998; Baum et al., 1995). In both vectors the FANCC cDNA was expressed off the retroviral LTR, the enhanced green fluorescent protein (EGFP) cDNA was under control of the internal murine PGK promotor from MSCV2.1. In LEG (mock transfection), EGFP was directly expressed off the LTR. The first packing cell line, 293T-derived ecotropic Phoenix cells (Nolan 1996), was transfected with 10-20 μg of the FANCC plasmid DNA using FuGene6 (Roche, Mannheim, Germany). After two days retrovirus containing supernatants (SNs) were harvested, filtered with 45 μm and used for transducing a second packaging cell line, NIH/3T3-derived pg13 cells (Miller et al., 1991), three times in the presence of 7.5 μg/ml protamin (Roche, Mannheim, Germany). GALV-pseudotyped retroviruses were harvested from pg13 cells as previously described (MacNeill et al., 1999). For complementation analysis, the cell lines of our patients were cultured on the fibronectin fragment CH-296 as described previously (MacNeill et al., 1999; Pollok et al., 1998; Hanenberg et al., 1996; Hanenberg et al., 2002) and exposed to the harvested viruses containing FANCC wt plasmids. An identical protocol was used for the pathogenicity testing of the amino acid exchanges L423P and T529P: HSC536 complementation group C reference cells were cultured on fibronectin and exposed to viruses containing FANCC plasmids carrying one of the above base changes. Cells were grown for at least two days after the last exposure to viruses to ensure stable expression of cDNAs from the integrated proviruses. For cell cycle analysis, cells were stained with Hoechst 33342 (Hoechst, Frankfurt, Germany). The forward/side scatter signals and the EGFP signals were used on a BD-LSR flow cytometer (Becton Dickinson, Heidelberg, Germany) to define a region of intact cells transduced with retroviruses. Cell cycle distributions of these cells were recorded using Hoechst 33342 emission and analyzed with Multi2D and MCYCLE software (Phoenix Flow Systems, San Diego, USA).

3.4 Results

Results of the mutation analysis of the ten FANCC patients are given in table 3.2. Nine are Caucasians and one is from central asia (HAM). In all patients the mutations on both alleles could be identified. Five carried homozygous, the other five compound heterozygous mutations. All German patients carried the 67delG mutation on at least one allele; a single patient with no known Askhenazy ancestry (K633) was compound heterozygous for the Ashkenazy founder mutation IVS4+4G>A, and three of five German patients carried a previously unknown mutation. Three of the nine Caucasian patients carried previously reported mutations.

We discovered seven new allelic alterations, in addition to four mutations that had been previously reported (67delG by (Strathdee et al., 1992), R548X by (Gibson et al., 1993b), Q13X by (Verlander et al., 1994) and IVS4+4A>T by (Whitney et al., 1993)). Two of the newly detected mutations (patients RNT, BAPA) were splice-site mutations, one a C to A transition in intron 9 (IVS9+1) and the other an A to G transition in intron 11 (IVS11-2). Both were detected in compound heterozygous patients, and both led to skipping of the corresponding exon. Two further novel mutations were a small deletion 428delF (patient MRCN) and a small insertion 727insCT (patient HLS). The latter introduces the two bases (CT) into the coding sequence of exon 7 at base position 727. The resulting frameshift leads to a premature stop-codon (262X) 20 amino acids downstream. 428delTTC represents an inframe triplet deletion, resulting in a protein lacking a single amino acid, namely phenylalanine. The other novel mutations were the missense mutations L423P (patient HAM) and T529P (patient MSS1) the pathogenicity of which had to be established. After sequencing of the entire gene without detecting any other pathogenic variants, pathogenicity of these two amino acid changes was proven by site directed mutagenesis and viral transfection into the FANCC$^{-/-}$ cell line HLS. As illustrated in figure 3.1, mock transfection with a virus containing only EGFP but no wt-FANCC cDNA did not eliminate the FA-typical G2 phase arrest. However, transduction with wt-FANCC cDNA caused a significant reduction of G2 phase blockage proving complementation. Failure of complementation (meaning persistence of G2 phase blockage) was observed after transduction with FANCC-cDNA containing the mutations to be tested. Both base changes were homozygous, and neither of the two patients carreid any futher alterations as evidenced by sequencing of the complete FANCC gene.

Comparison to the mouse FancC protein indicates conservation of the corresponding protein regions (figure 3.2). Both mutations introduce a proline into the coding region that may have consequences on the folding of the protein. Moreover, both mutations are located next to the previously reported amino acid substitutions L554P and L496R in the 3´-region of FANCC, suggesting the impairment of a functional domain.

Patient code	Ethnic origin	Detected mutations (allele 1/2)	Confirmation test	Consequence of the mutation	Reference
RNT	German	67delG	cDNA-sequ.	Frameshift, Stop after 23 aa	(Strathdee et al., 1992)
		IVS11-2A>G	cDNA-sequ.	Splice-site mutation, skipping of exon 12	Present paper
NDAO	German	67delG	cDNA-sequ.	Frameshift, Stop after 23 aa	(Strathdee et al., 1992)
		67delG	cDNA-sequ.	Frameshift, Stop after 23 aa	(Strathdee et al., 1992)
HLS	German	67delG	cDNA-sequ.	Frameshift, Stop after 23 aa	(Strathdee et al., 1992)
		727insCT	cDNA-sequ.	Frameshift, Stop after 20 aa	Present paper
MRCN	German	67delG	cDNA-sequ.	Frameshift, Stop after 23 aa	(Strathdee et al., 1992)
		428delTTC	cDNA-sequ.	Del F	Present paper
MSS1	Spanish	1582A>C	cDNA-sequ., s.dir.mut.	T529P	Present paper
		1582A>C	cDNA-sequ., s.dir.mut.	T529P	Present paper
BAPA	Bulgarian	37C>T	cDNA-sequ.	Q13X	(Verlander et al., 1994)
		IVS9+1C>A	cDNA-sequ.	Splice-site, skip of exon 9	Present paper
HAM	Uzbekistanian	1268T>C	cDNA-sequ., s.dir.mut.	L423P	Present paper
		1268T>C	cDNA-sequ., s.dir.mut.	L423P	Present paper
HAAY	Dutch	67delG	cDNA-sequ.	Frameshift, Stop after 23 aa	(Strathdee et al., 1992)
		67delG	cDNA-sequ.	Frameshift, Stop after 23 aa	(Strathdee et al., 1992)
BAFO	Italian/ Sicilian	del exons 1-2 [a]	Parental screening	Deletion of exons 1-2	Present paper
		del exons 1-2 [a]	Parental screening	Deletion of exons 1-2	Present paper
K633	German	IVS4+4A>T	Sibling screening	Frameshift, Stop after 23 aa	(Whitney et al., 1993)
		67delG	Sibling screening	Frameshift, Stop after 23 aa	(Strathdee et al., 1992)

Table 3.2: Results of mutation analysis in 10 FANCC-patients (cDNA-sequ.= cDNA-sequencing; s.dir.mut. = site directed mutagenesis).

[a] deletion breakpoints determined at cDNA level only

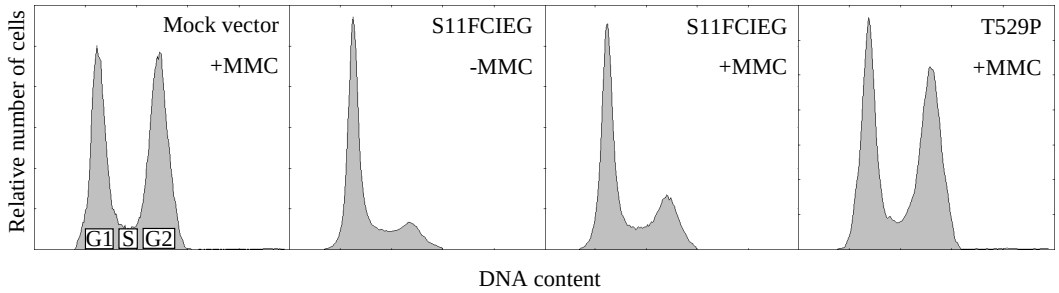


Figure 3.1: Testing for pathogenicity of the missense mutation 1585A>C, T529P of patient MSSI, using the FANCC lymphoblastoid cell line HLS. The mock-transfected HLS-cells show the typical FA G2 phase arrest, whereas G2 phase level of HLS-cells with the WT-FANCC vector (S11FCIEG) +/- addition of MMC has returned to WT-level. The rightmost cell cycle distribution shows the lack of complementation after transfection with the S11FCIEG vector containing the T529P-alteration, which proves the pathogenicity of this amino acid substitution.

```
L423P
murine   391  PFESWFLFVHFGGWVDLAVAELLLREEAEPPAGLLWLLVFYYSPQDGSQQREQSMV
human    390  PFESWFLFIHFGGWAEM-VAEQLLMSAAEPPTALLWLLAFYYGPRDGRQQRAQTMV
              **************    *** **   ****  ***** *** * ** *** * **

T529P
murine   545  TEMAHTDAVIHEIIGFLDQTLYRSQHLCVEASR
human    510  TLMAHTAEITHEIIGFLDQTLYRWNRLGIESPR
              * ****    *************   *  *  *
```

Figure 3.2: Comparison of the human FANCC protein sequence and the murine FancC demonstrates the conserved character of the two amino acids L423 and T529. Conserved amino acids are marked with asterisks and the mutated amino acids are underlined.

A single patient (BAFO) carried homozygous large deletions in the FANCC gene - a type of mutation not described previously for any FA-C patient. PCR on genomic DNA yielded no product for exons 1 and 2. Homozygosity of the deletion was suspected due to consanguinity of the patient´s parents. Sequencing of the remainder of the gene revealed only a single non-pathogenic sequence variation to be present in homozygous state (IVS11-37T>C). Using the fragment primers Fr. A forward and Fr. D reverse, no cDNA full-length PCR product was obtained. Likewise, the pair of primers for the first cDNA fragment (Fr. A forward and Fr. A reverse, spanning exons 1-4, table 3.1) revealed no product, but there were normal fragments using primer pairs B to D. This led to the assumption that

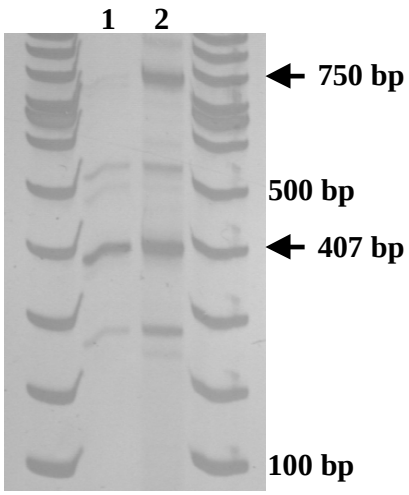


Figure 3.3: cDNA PCR products of BAFO (1) and his heterozygous mother (2) using a sense-primer 250 bp upstream of ATG and an antisense-primer lying in exon 5. PCR with BAFO cDNA revealed one single specific band at 407 bp, his mother exhibited two different fragments at 407 bp and 750 bp.

the promotor region had to be intact because of an intact downstream mRNA. As a consequence, forward primers positioned upstream of the ATG start codon were tested in combination to a reverse primer covering exon 5. The resulting PCR fragment was 407 bp in length, and sequencing revealed cDNA breakpoints 79 bp upstream from ATG and at base position 251, which represents the first base of exon 3. Since exon 3 starts with an out of frame ATG, this ATG is used as new ATG start codon leading to a premature stop codon six amino acids downstream. The fragment length of a healthy control was 750 bp, and the heterozygous status of the patient´s mother revealed two products of the corresponding fragment lengths of 750 and 407 bp (figure 3.3).

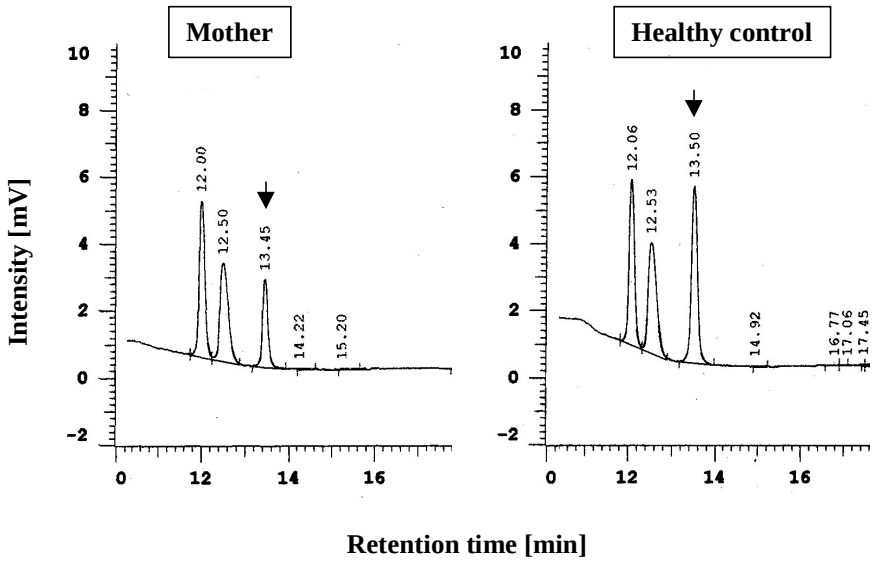
Notwithstanding the consanguinity of the parents, quantitave PCR analysis was used to exclude hemizygosity. We performed two multiplex PCR reactions. PCR 1 contained FANCC exons 1 and 6 as well as von Willebrand Jürgens disease gene exon 3 (vWJ3) as an external non FA dose reference; PCR 2 consisted of FANCC exon 14 and again vWJ3. The ratios of the FANCC PCR products and the vWJ3 of the patient and of his obligatory heterozygous mother were compared to that of a healthy control (figure 4). Because of no visible PCR product of exon 1 in patient BAFO, there was no peak for exon 1, but a biallelic peak for exons 6 and 14 (data not shown). The patient´s mother also showed biallelic gene dosage for exons 6 and 14, but a monoallelic status for exon 1. This result is consistent with obligate heterozygosity of the patients mother. If the patient´s DNA was mixed 1:1 with wildtype DNA in order to produce artificial heterozygosity, both

exon 6/vWJ3 and exon 14/vWJ3 ratios were normal, whereas exon 1/vWJ3 was aberrant due to the relative lack of an exon 1 product. This finding excludes a hemizygous deletion of one entire FANCC allele in our patient, because in this case the exon 6 and exon 14 to vWJ3 ratios would be likewise aberrant. The existence of a different large deletion on the second allele, including exons 1 and 2 plus exons 3/4/5 could also be excluded because we observed a single full length PCR fragment (forward primer: 255 bp upstream of ATG, reverse primer: Fr. D reverse) containing exons 3 to 14.

3.5 Discussion

In contrast to the large FANCA gene, the published spectrum of mutations in the relatively small FANCC gene appears much less variable. Pathogenic alterations described include a single microdeletion, a single microinsertion, a single splice-site mutation, five nonsense and two missense mutations. The low number of different types of mutations reported so far seems to be due to the prevalence of typical founder mutations in certain populations or ethnic groups, such as the Ashkenazi founder mutation IVS4+4A>T and the Dutch founder mutation 65delG (Verlander et al., 1994). The pathogenic alterations published for FANCC seem to be clustered in both the amino- and the carboxy-terminal region as well as around exons 5-6, suggesting mutation hot spot regions (Strathdee et al., 1992; Gibson et al., 1993b; Gibson et al., 1996; Whitney et al., 1993; Lo Ten Foe et al., 1996c; Lo Ten Foe et al., 1996a; Lo Ten Foe et al., 1998). In the present study, we detected new mutations in exons 7, 12 and 14 as well as in introns 9 and 11, indicating a more heterogeneous distribution of mutations throughout the gene. Moreover, the specific types of alterations detected in our patient cohort seem to differ from those of previous publications as, for example, we did not find any nonsense mutations and identified the first large intragenic deletion in FANCC. Given the predominant central European origin of our patients, the Dutch founder mutation 67delG was the most frequent mutation, but out of the remaining alleles more than 80% contained previously unknown mutations. The newly described alterations include, in addition to the large deletion, two missense mutations (L423P and T529P) in the 3´-area of the FANCC gene, one small 2bp-deletion and an in frame 3bp-insertion, respectively, as well as two splice-site mutations. The two splice-site mutations both lead to skipping of the corresponding exons. A single patient (without any known Ashkenazi ancestry) carried the the Ashkenazi founder mutation IVS4+4A>T.

We identified seven new mutations that appear to be spread out over the entire gene (figure 3.5). Seven out of 20 mutated alleles represents a fairly high percentage of novel alterations, whereas most of the remaining 13 constitutional mutations were due to the Dutch (8/20) and the Ashkenazi (1/20) founder mutations.



	RT	Area Mother	Ratio	Area Control	Ratio
vWJ3	**12.00 min**	**36376**		**36436**	
E. 6	**12.50 min**	**34618**	**0,95**	**39411**	**1.08**
E. 1	**13.45 min**	**19288**	**0,53**	**45166**	**1,23**

Figure 3.4: DHPLC diagrams of PCR 1 from a healthy control and the patient´s obligate heterozygous mother. The upper panel shows the peak intensity over the retention time, where vWJ3 elutes at 12.0-12.1 min, FANCC exon 6 at 12.5-12.55 min and FANCC exon 1 at 13.45-13.5 min. The lower panel shows the corresponding peak areas and the ratio between the FANCC exons and vWJ3, indicating the mono- or biallelic status of the samples. Compared to the control sample, the patient´s mother also contains two alleles of exon 6, but obviously only one allele of exon 1.

These numbers suggest that a high proportion of European FA-C patients may carry at least one previously undescribed mutation. With respect to screening strategies this means that if there is no 67delG founder mutation in an European FA-C patient, the entire gene has to be analyzed in order to detect these "private" alterations.

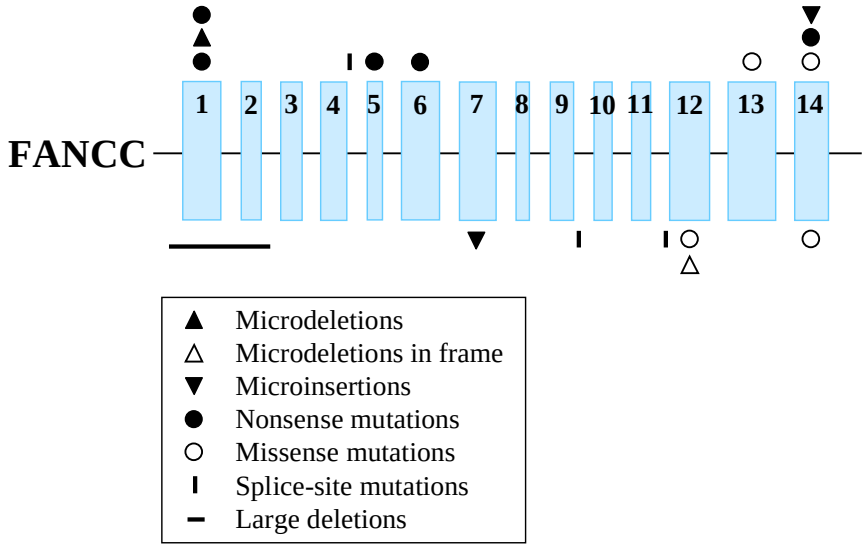


Figure 3.5: Mutation spectrum of FANCC. Mutations published to date are marked on top of the schematic exon-intron-structure. Our newly detected mutations are indicated at the bottom of the gene and contain a large deletion of exons 1-2, two splice-site mutations, a microinsertion, a microdeletion in frame as well as two amino acid substitutions. The distribution of the mutations now seems to be spread out over the gene, e.g. mutations concerning exons 1, 2, 7, 12 and 14 are described.

Looking at the different types of mutation found in our study, the number of microinsertions/-deletions and amino acid substitutions is similar to that in the previous publications. A striking difference to the literature is the lack of nonsense mutations in our study of ten patients. Instead, our study adds two new splice-site mutations and reports, for the first time, a large intragenic deletion involving exons 1 and 2. Screening methods taking the possibility of such large deletions into consideration should be used if there are difficulties in finding both mutations in a given patient. For this purpose we employed a sensitive screening method to verify heterogenous deletions. By measuring the dosage of FANCC exons 1, 6 and 14, deletions spanning these exons can be identified. We have chosen these three exons for routine screening in order to cover the entire gene region. Deletions beginning 5´ of FANCC and ending within the gene as well as those beginning in FANCC and ending 3´ of the gene can be found. Deletions that span only a few exons within the gene, e. g. exons 2-5 or exons 7-13, might be detected by conventional RNA/cDNA-screening.

The two newly described amino acid substitutions L423P and T529P were found in exons 12 and 14, both belonging to the C-terminal region of FANCC. Since these alterations are both unable to abolish MMC-hypersensitivity and to comple-

ment FA-C cells after retroviral transfection, their pathogenicity has been firmly established. They are located close to the two previously published missense mutations, L554P and L496R, suggesting the presence of a functional domain(s) in that region. Binding of FANCC to FANCA is mediated by carboxyl-terminus of FANCC, which appears to be a critical region for FANCC function *in vivo* (Gavish et al., 1993; Yamashita et al., 1994). This region is predicted to form an α-helical structure (Yamashita et al., 1994), and L554P disrupts this structure, leading to loss of FANCC function and FANCA binding. In L423P, the same amino acids are changed as in L554P. Even though the polarity of the terminal region is not likely to be altered by the mere exchange of two non-polar amino acids, there is a local decrease in hydrophobicity due to proline, and a likely conformational change (Gavish et al., 1993). In the case of T529P, polarity decreases because of the alteration of the polar amino acid threonine to the non-polar proline. In addition, hydrophobicity increases after the substitution of threonine by proline. These biochemical alterations might account for the loss of function of the resulting FANCC protein.

The mutant L554P fails to bind cdc2 (cyclin-dependent kinase 2) which was postulated to be correlated with the functional activity of the FANCC protein, and binding of FANCC to cdc2 might be required for normal G2/M progression in mammalian cells (Kupfer et al., 1997b). Moreover, the binding of FANCC to cdc2 seems to be mediated by the carboxy-terminal 50 amino acids of FANCC. T529P is located within these 50 amino acids and might therefore disrupt cdc2 binding and cell cycle progression.

In conclusion, our study shows that the mutation spectrum of European FANCC patients is much more variable than assumed to date. Missense mutations with proven pathogenicity do not appear to be rare, and the existence of large deletions that escape detection with conventional screening methods must be taken into account.

3.6 Acknowledgements

The authors gratefully acknowledge the kind cooperation of the Fanconi anemia patients and their families. Financial support was provided by the "Deutsche Fanconi-Anämie Hilfe e.V." and the Schroeder-Kurth Fonds of the University of Würzburg. We also thank Gitta Emmert, Ariane Klobe and Richard Friedl for technical assistance.

Chapter 4

Spectrum of mutations in the Fanconi anemia group G gene, FANCG/XRCC9

Ilja Demuth[1], *Marcin Wlodarski*[1], *Alex J Tipping*[2], *Neil V Morgan*[2], *Johan P de Winter*[3], *Michaela Thiel*[4], *Sonja Gräsl*[4], *Detlev Schindler*[4], *Alan D D'Andrea*[5], *Cigdem Altay*[6], *Hülya Kayserili*[7], *Adriana Zatterale*[8], *Jürgen Kunze*[1], *Wolfram Ebell*[9], *Christopher G Mathew*[2], *Hans Joenje*[3], *Karl Sperling*[1] *and Martin Digweed*[1]

[1]Institute of Human Genetics, Charité, Campus Virchow, Humboldt University, Berlin, Germany;

[2]Division of Medical and Molecular Genetics, Guy's, King's and St Thomas' School of Medicine, London, UK;

[3]Department of Clinical Genetics and Human Genetics, Free University Medical Center, Amsterdam, The Netherlands;

[4]Institute of Human Genetics, University of Würzburg, Germany;

[5]Department of Pediatric Oncology, Dana-Faber Cancer Institute, Boston, USA;

[6]Department of Pediatrics, Hematology Unit, Hacettepe University, Ankara;

[7]Institute of Child Health, Istanbul, Turkey;

[8]Servizio di Citogenetica, Ospetale Elena d'Aosta, Napoli, Italy;

[9]Children's Hospital,Charité, Campus Virchow, Humboldt University, Berlin, Germany

European Journal of Human Genetics (2000) 8,861-868.

4.1 Abstract

FANCG was the third Faconi anaemia gene identified and proved to be identical to the previously cloned XRCC9 gene. We present the pathogenic mutations and sequence variants we have so far identified in a panel of FA-G-patients. Mutation screening was performed by PCR, single strand conformational polymorphism analysis and protein truncation tests. Altogether 18 mutations have been determined in 20 families - 97% of all expected mutant alleles. All mutation types have been found, with the exception of large deletions, the large majority is predicted to lead to shortened proteins. One stop codon mutation, E105X, has been found in several German patients and this founder mutation accounts for 44% of the mutant FANCG alleles in German FA-G-patients. Comparison of clinical phenotypes shows that patients homozygous for this mutation have an earlier onset of the haematological disorder than most other FA-G-patients. The mouse FancG sequence was established in order to evaluate missense mutations. A putative missense mutation, L71P, in a possible leucine zipper motif may affect FANCG binding of FANCA and seems to be associated with a milder clinical phenotype.

4.2 Introduction

The autosomal recessive genetic disorder Fanconi anaemia (FA; MIM 227650) is genetically highly heterogeneous with seven complementation groups so far established and thus seven causative genes anticipated (Joenje et al., 1997) and (Joenje et al., personal communication). The identification of these genes is an important goal for understanding the pathophysiology of the disease since the basic defect is still poorly understood. The characteristic chromosomal breakage observed in patient cells, particularly after treatment with a bifunctional alkylating agent capable of forming DNA interstrand crosslinks, has led to the assumption that FA cells are deficient in the repair of this DNA lesion. However, alternative theories implicating cell cycle regulation, oxygen detoxification and apoptosis have been proposed (Digweed and Sperling, 1996; Schindler and Hoehn, 1988).

The clinical picture of FA is characterised by progressive bone marrow failure and an increased risk of neoplasia, particularly leukemia. In addition, a range of variable congenital defects such as growth retardation, skeletal abnormalities including radial aplasia and hyperpigmentation of the skin are observed in some patients (Glanz and Fraser, 1982). Whilst increased chromosomal breakage remains the critical diagnostic test for FA, recent studies have demonstrated reverse mosaicism in FA leading to a population of undamaged cells in peripheral blood lymphocytes (Lo Ten Foe et al., 1997; Waisfisz et al., 1999b).

Of the seven FA genes, four have been identified: FANCA (Lo Ten Foe et al., 1996b; The Fanconi anemia/Breast cancer consortium, 1996), FANCC (Strathdee

et al., 1992), FANCF (De Winter et al., 2000b) and FANCG (De Winter et al., 1998). The localisation of FANCG on chromosome 9p13 was established by homozygosity mapping and linkage analysis in FA-G-families (Saar et al., 1998), its identification was achieved by functional complementation of FA-G lymphoblastoid cells after cDNA transfer (De Winter et al., 1998). The gene thus identified was identical to XRCC9, localised on 9p13, and previously identified by virtue of its ability to complement a crosslinker sensitive hamster cell mutant, UV40 (Liu et al., 1997). The gene covers some 6 kb with 14 exons and has a 2.5 kb mRNA encoding the FANCG protein of 622 amino acids and a calculated molecular weight of 68 kD. The protein shows no homology to known proteins and its function is thus unclear. However, several studies have shown that FANCG is found in the cells as a nuclear complex with the FANCA protein (Waisfisz et al., 1999a; Kruyt et al., 1998a).

Pathogenic mutations in FANCG were previously reported in four FA-G-patients, we report here further mutations in 16 FA-G-patients, including common German and Turkish founder mutations.

4.3 Material and methods

4.3.1 Patients and samples

Patients were diagnosed on the basis of chromosomal breakage tests and clinical features. Many patients were recruited as part of the European Concerted Action on FA Research (EUFAR), others were referred to the contributing laboratories for diagnosis. Clinical data were collected on a standard EUFAR questionnaire. Where possible, lymphoblastoid cell lines were established by EBV transformation and fused to the 7 FA reference cell lines to establish complementation group (Joenje et al., 1997; Joenje et al., 1995a). In some cases, assignment to FA group G was indicated by the absence of detectable FANCG protein in immunoblots. DNA and RNA were extracted from LCLs and/or peripheral blood samples by standard techniques.

4.3.2 Mutation screening

Mutations in the FANCG gene were screened by amplification of all 14 exons from genomic DNA, using primers flanking the exon/intron boundaries as described previously (De Winter et al., 1998), or by amplification of FANCG cDNA from RNA. SSCP analysis under four different conditions was used to identify exon PCR products with an aberrant mobility pattern; bis-acrylamide: acrylamide ratios of 1:30 and 1:50, gel runs at 4°C and at 15°C. Fragments showing an aberrant SSCP pattern were sequenced using the ABI 310 (Foster City, CA,

USA) sequencer to identify the mutations. Most patients showed mobility shifts in one or more exons, those mutant alleles not detectable by SSCP were sequenced directly. Some patient samples were screened by RT-PCR and *in vitro* translation using standard methods (Roest et al., 1993). Appropriate FANCG exons were then amplified and sequenced to establish the underlying mutation.

Where possible, the identified mutations were confirmed by restriction enzyme digest of appropriate PCR products bearing the mutation, by RT-PCR and/or by examination of DNA from the patients' parents.

4.3.3 Mouse FancG sequence

A BLAST v2.0 search with the entire FANCG cDNA sequence revealed high homology (P = 7e-37) to an anonymous murine BAC clone in the GenBank database (Lamerdin et al., accession no. AC005259). The murine coding sequence was verified by the amplification of the FancG cDNA from mouse total RNA by nested RT-PCR using specific primers. The amplified product was 1.9 kb long, in agreement with estimates for the size of the FancG transcript encoded by the BAC clone. The RT-PCR product was sequenced and the completed FancG cDNA sequence has been submitted to GenBank with accession number AF112439.

4.3.4 Haplotype analysis at the FANCG locus

Examination of the sequence of the P1 clone, 11659 (Lamerdin et al., accession no. AC004472), containing the entire FANCG gene revealed the presence of a (CA) dinucleotide repeat at position 5531 of the clone, 48 kb from the 3' end of the FANCG coding sequence. Primers were designed which flank this repeat and 65 unrelated individuals were examined for polymorphism in the size of the CA repeat sequence; 81% of the individuals were heterozygous. This genetic marker has been given the assignment D9S2176.

DNAs from families in which the common 313G>T or 1649delC mutations segregate were examined for microsatellite markers D9S2176, D9S1853, D9S1874, D9S1817, and D9S165 located at the FANCG locus. PCR products were analysed on Pharmacia ALF (Freiburg, Germany) sequencing apparatus.

4.4 Results

The FANCG mutations found in the 16 new FA-G-patients examined here and for the four patients previously described (De Winter et al., 1998) are detailed in table 4.1. The majority of mutated alleles (94%) are expected to result in protein

truncation. Six mutations are small deletions (1, 2, or 10 bp) or insertions (2 bp) leading to frame shifts and premature termination of translation after, between 4 and 81 novel codons. Six mutations affect the invariable intronic bases of mRNA splice sites (donor sites of introns 2, 5 and 11; acceptor sites of introns 8, 9 and 13), and would be predicted to result in false splicing, frame shift and truncated proteins. The loss of exon 2 due to the IVS2+1G>A mutation in patient FA1BER was verified by RT-PCR and sequencing (figure 4.1). Interestingly, the second mutant allele, which has a dinucleotide deletion in exon 4, was not detectable in this RT-PCR, suggesting that the mutated mRNA is unstable. One further splice mutation affected the last base of exon 12 and was first detected as an aberrant RT-PCR product lacking the entire exon 12.

The splice mutation IVS8-2A>G leads to two aberrant mRNAs. One RNA species has exon 8 joined to exon 10, as expected if the acceptor of intron 8 is inactivated. This is predicted to result in protein truncation after 20 amino acids. The other RNA has exon 8 joined to a cryptic acceptor site 25 bases into exon 10, leading to truncation after 2 amino acids (data not shown).

Four base change mutations lead to premature stop codons, in exon 4, exon 6 and exon 13, but only one missense mutation has been found so far. The transition 212T>C, for which the patient EUFA569 is homozygous, leads to the non conservative amino acid substitution of proline for leucine at position 71. This mutation was not found in 100 ethnically matched chromosomes and no other sequence variation was found in this patient´s DNA. Examination of the mouse cDNA sequence presented here (figure 4.2) shows that the amino acid leucine is conserved at this position in the mouse sequence. The 18 mutations found so far show no obvious clustering within the gene (figure 4.3).

Three sequence variants, all affecting amino acid coding, were found in the FANCG gene of FA-G-patients. These are not considered to be disease related, since in each case a truncating mutation was found in the same allele. Analysis of 100 ethnically matched chromosomes resulted in the frequencies for these polymorphisms given in table 4.2. The base change, 77A>G, leading to Q26R was not found in 70 ethnically matched chromosomes and affects an amino acid conserved in the mouse sequence, however, it does not disturb complementation of MMC-sensitivity after transfection into FA-G cells (data not shown) and thus presumably represents a rare variant.

As with other FA genes, most mutations have been found only once, however, the truncating mutation, 313G>T, was found in six of nine unrelated German patients examined. Of the 18 alleles expected amongst these German patients, 8 carry 313G>T. Analysis of microsatellite markers linked to FANCG indicated a common ancestral haplotype on which the 313G>T mutation occured table 4.3. The most tightly linked markers, D9S2176 (approx. 50 kb from FANCG) and D9S1817, each have the same size repeat on the mutant haplotype in all patients. The D9S2176 allele 335 bp has a population frequency of 0,038. D9S1853 and

Patient code	Ancestry	Consan-guineous	Mutations [a]	Predicted effect	Exon	State [b]
EUFA313	Germany	no	IVS9-1G>C/ 1310-1311insGA	R359S+20X/ D437E+80X	i9/ 10	HET
F99/112	Germany	no	313G>T/ not determined	E105X	4	HET
FA1BER	Germany	no	IVS2+1G>A/ 346-347del	V29G+11X/ Q116G+36X	i2/ 4	HET
EUFA1093	Germany	no	313G>T	E105X	4	HOM
EUFA282	Germany	no	652C>T/ 1183-1192del	Q218X/ E395W+3X	6/ 10	HET
F00/49	Germany	no	313G>T/ 1183-1192del	E105X/ E395W+3X	4/ 10	HET
F99/186	Turkey	yes	1649delC	T550I+7X [c]	13	HOM
EUFA636	Turkey	no	1649delC	T550I+7X	13	HOM
EUFA624	Turkey	no	1642C>T	R548X	13	HOM
F99/121	Turkey	yes	IVS5+1G>T	S171V+3X [c]	i5	HOM
EUFA569	Turkey	yes	212T>C	L71P [d]	3	HOM
DF3	Arabia	yes	1636G>C	A495-G546del	12	HOM
PRU63622	Arabia	yes	1749delA	D584M+8X	13	HOM
EUFA0334	Italy	no	1715G>A	W572X	13	HOM
EUFA0872	Portugal	?	IVS8-2A>G	R359S+20X and G361L+2X [e]	i8	HOM
PRU104210	Iran	yes	109-110del	L37E+17X	2	HOM
FA15BER [f]	Lebanon	yes	IVS13-1G>C	truncated protein	i13	HOM
EUFA143 [f]	Germany	no	313G>T	E105X	4	HOM
EUFA349 [f]	Germany	no	313G>T/ IVS11+1G>C	E105X/ C479G+4X	4/i11	HET
EUFA316 [f]	Germany	no	313G>T/ 1183-1192del [g]	E105X/ E395W+3X	4/ 10	HET

Table 4.1: FANCG mutations detected in Fanconi anemia patients.

[a] Numbering of the mutations from the initiation codon of the cDNA [b] HOM = homozygous mutation; HET = compound heterozygous mutation [c] Confirmed by *in vitro* translation of RT-PCR products (data not shown) [d] Not confirmed experimentally [e] Two aberrantly spliced mRNAs differing in length by 25 bases are detected by RT-PCR (data not shown) [f] Previously published (De Winter et al., 1998)
[g] This is a correction of the mutation previously published as 1184-1194del

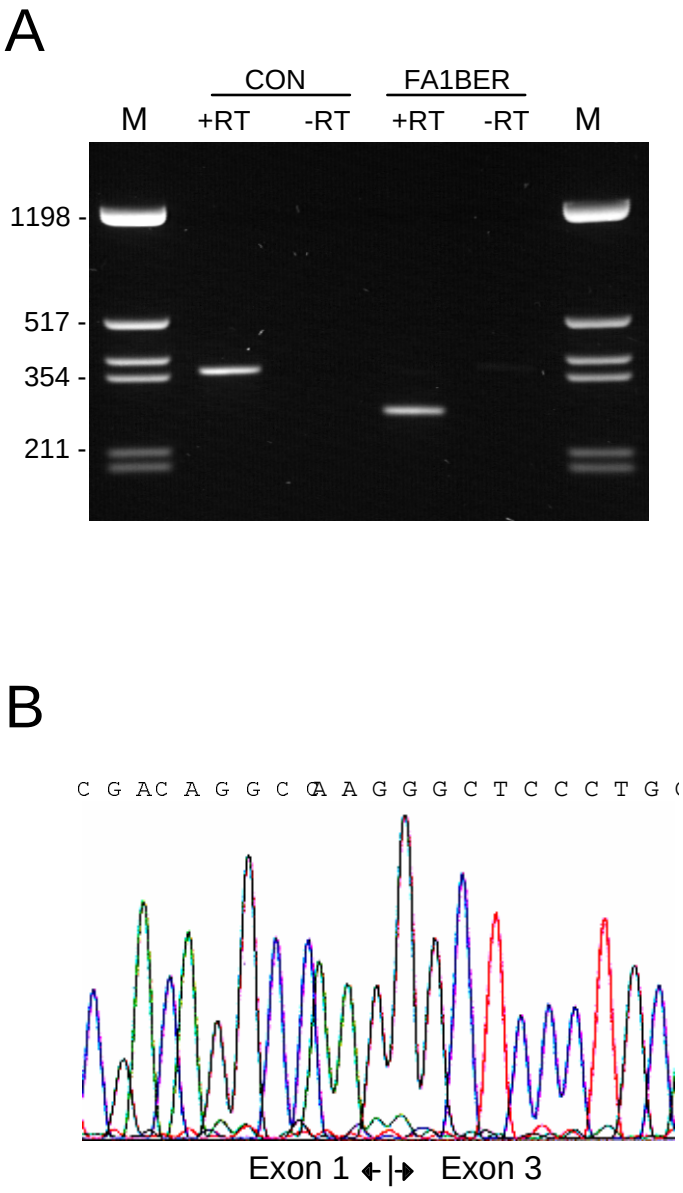


Figure 4.1: Detection of an aberrantly spliced mRNA in patient FA1BER. (a) RT-PCR was used to amplify a cDNA fragment of 365 bp extending from exon 1 to exon 3 from FA1BER RNA and control RNA. The PCR product from FA1BER is ca. 90 bp shorter, compatible with the loss of exon 2 due to the splice site mutation, IVS2+1G>A. (b) Sequence analysis of the aberrantly spliced mRNA in FA1BER showing the loss of sequence from exon 2.

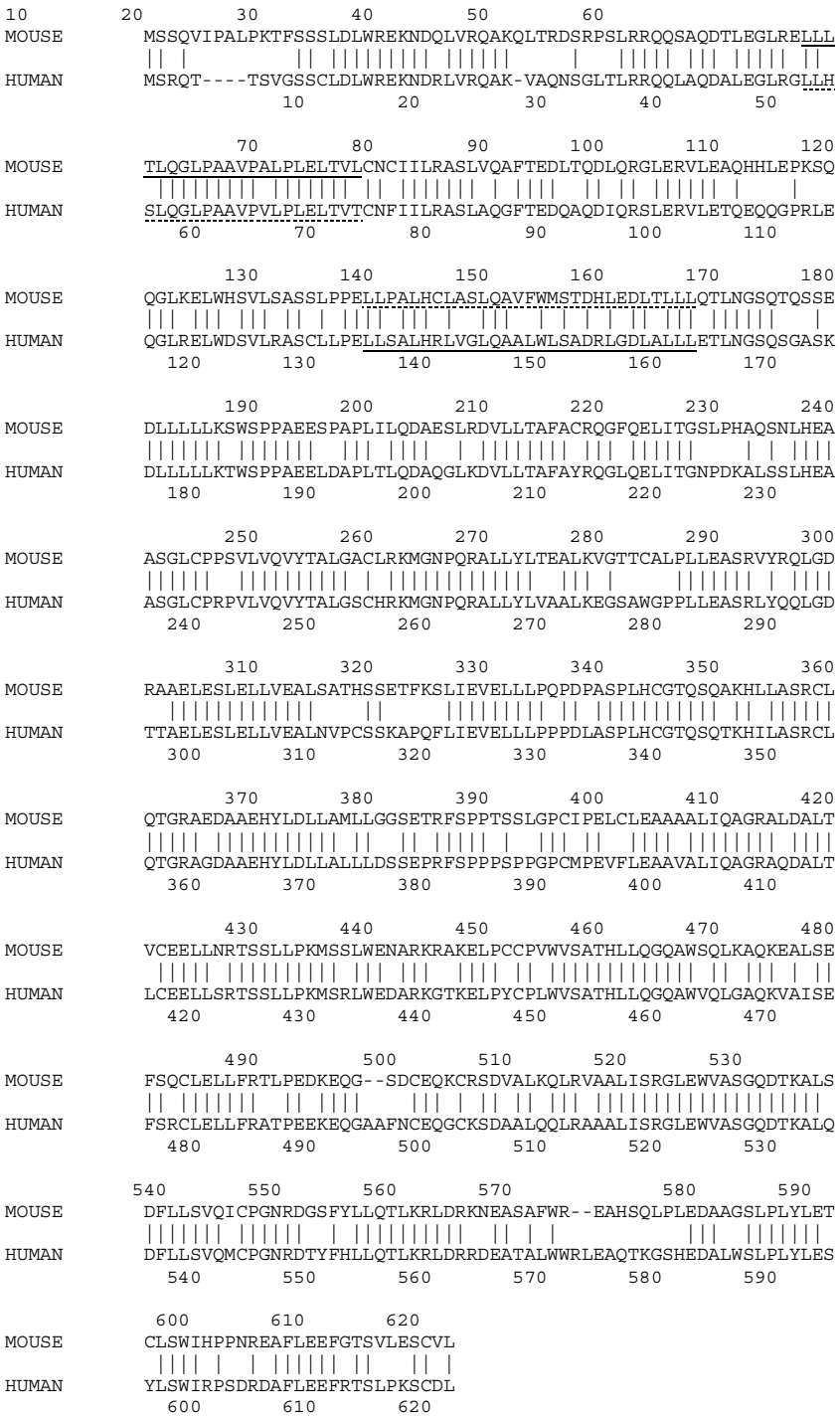


Figure 4.2: Mouse FancG sequence and alignment to the human FANCG. Conserved residues are marked by |. The positions of the two possible leucine zippers are underlined.

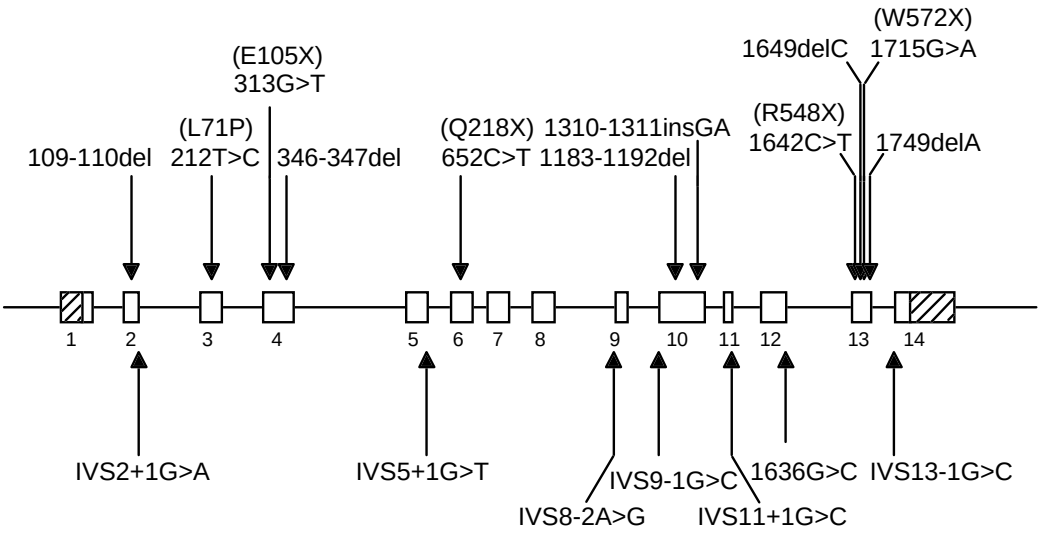


Figure 4.3: Positions of the mutations detected in FANCG. The exons and introns of the FANCG gene are shown together with the positions of the mutations presented here. Splice mutations are shown below the gene, insertions, deletions and amino acid substitutions, above the gene.

Patient code	Variant	Effect	Mouse sequence	Frequency	Exon	State [a]
EUFA313	890C>T	T297I	A	<0,01	7	HET
EUFA636 F99/186	20C>T	S7F	K	0,01	1	HOM
PRU63622	77A>G	Q26R	Q	-	1	HOM

[a] HET = compound heterozygote; HOM = homozygous

Table 4.2: FANCG sequence variants detected in Fanconi anemia patients.

	EUFA 143mat	EUFA 1093mat	EUFA 316	EUFA 143pat	F99/ 112	EUFA 1093pat	EUFA 348	cM [a]
D9S1853	**249**	**249**	**249**	**249**	251	261	257	53,60
D9S165	**212**	**212**	**212**	**212**	**212**	**212**	216	58,26
D9S2176	**335**	**335**	**335**	**335**	**335**	**335**	**335**	
D9S1817	**257**	**257**	**257**	**257**	**257**	**257**	**257**	59,34
D9S1874	**195**	**195**	**195**	197	197	197	**195**	61,38

[a] Genetic distance in cM from 6pter taken from the Marshfield Medical Research Foundation (http://www.marshmed.org/genetics/).

Table 4.3: Haplotypes at the FANCG locus on 313G>T alleles. Sizes of PCR products containing $(CA)_n$ repeats at the listed loci are given in bp. Conserved alleles are printed in bold letters.

D9S165 have the same alleles in most patients but also show evidence of haplotype erosion due to mutation and/or recombination. The transversion 313G>T thus represents a common FANCG founder mutation in Germany. A further common mutation amongst German patients is the 10 bp deletion, 1183-1192del. This is present only together with the sequence variant 1182T>C, not found amongst 100 matched chromosomes (data not shown) thus indicating a common origin.

Evidence for a founder chromosome amongst Turkish patients is given by two patients homozygous for the deletion 1649delC. Again there is a common haplotype at D9S2176 (327 bp) and D9S1817 (267 bp) in these patients (data not shown). These patients also share the sequence variant, S7F (table 4.2).

The phenotypes of 23 of the patients for whom FANCG mutations have been established here are given in a standardised format for a selection of particularly common clinical features in table 4.4 and table 4.5. The heterogeneity characteristic for FA is clearly demonstrated, even the sibling pairs show discordance for several symptoms. The average age of haematological onset in this group of patients is 6.1 years (range 0 to 15 years), not significantly different from that found in other FA complementation groups (Faivre et al., personal communication); growth retardation and café au lait spots are common (78% and 75% of patients, respectively). Thumb malformations and microphthalmia are present in less than half the patients (36% and 36%, respectively).

4.5 Discussion

Mutation analysis by SSCP of amplified exons has proved to be highly efficient for detection of mutations in FANCG. In the 20 patients screened for mutations in the FANCG/XRCC9 gene, a total of 18 different mutations which are likely to

	Patient code	Sex	Age at present	Hematological onset (years)	Thumb malformation	Microcephaly	Microphthalmia
{	EUFA0281	F	12.4	5.5	-	+	-
	EUFA0313	F	17.4	10.5	-	+	+
	F99/112	F	10.0	9.5	-	-	+
	FA1BER	M	10.8 [a]	6.0	-	-	-
	EUFA1093	M	5.8	2.4	+	+	+
	F99/186	M	0.3	0.0	-	-	-
	EUFA636	M	19.0 [a]	15.0	-	+	-
{	10981-95-3	F	9.0 [a]	5.0	+	+	+
{	10981-95-2	M	10.0 [a]	9.0	+	-	-
	EUFA569	F	26.0	13.0	-	+	+
	EUFA624	M	11.0 [a]	7.0	-	+	+
	F99/121	M	5.3	1.5	-	-	-
	DF3	M	9.4	7.0	-	+	-
	PRU63622	F	n.a.	3.0	n.a.	n.a.	n.a.
{	EUFA0334	F	16.0 [a]	7.0	-	-	-
{	SdC01	F	9.0 [a]	5.0	+	+	-
	EUFA143	M	14.2 [a]	1.4	+	-	-
{	FA15BER	M	17.3 [a]	7.1	-	+	-
{	FA23BER	F	10.6 [a]	8.8	-	+	-
{	EUFA316	F	9.3	3.5	+	+	+
{	FA26BER	F	2.8	-	-	-	-
{	EUFA348	M	10.0	4.8	+	+	+
{	EUFA349	M	8.3	3.0	+	+	-

[a] age at death

Table 4.4: Clinical data on FA-G-patients. Affected siblings are shown in parenthesis ({); n.a. = No data available. (to be continued in table 4.5)

be pathogenic were identified. Of these, >90% were initially detected by SSCP or similar indirect methods. Thirteen of the patients were proved to have homozygous mutations and this was expected in seven of these due to consanguinity. For the compound heterozygous patients, only one mutation could not be determined, although this allele is expressed at the mRNA level as shown by RT-PCR (data not shown). This equates to a detection rate of 97% using the methodology described here, contrasting strongly with the situation in FANCA, where frequent partial deletions make mutation analysis difficult (Wijker et al., 1999; Morgan et al., 1999).

The majority of the mutations described are expected to yield truncated FANCG proteins varying from 7% to 95% of the wild type 68 kD. FANCG is known to

Patient code	Cutaneous symptoms, café au lait/ hypopigmentation	Growth retardation	Organ abnormalities	MDS/ AL
EUFA0281	+/-	+	none	-/-
EUFA0313	+/-	+	none	-/-
F99/112	+/+	-	none	+/-
FA1BER	-/-	+	liver	+/+
EUFA1093	+/+	-	GIT, kidney	-/-
F99/186	-/-	+	heart	-/-
EUFA636	+/-	+	hypogonadism	+/-
10981-95-3	n.a.	+	kidney	-/+
10981-95-2	n.a.	-	n.a.	-/+
EUFA569	+/+	+	none	-/-
EUFA624	+/+	+	none	-/+
F99/121	-/-	+	esophagus	-/-
DF3	+/-	+	kidney	+/-
PRU63622	n.a.	+	n.a.	n.a.
EUFA0334	+/-	+	none	-/-
SdC01	+/-	+	kidney	-/-
EUFA143	+/-	+	hypogenitalia	-/-
FA15BER	+/-	+	none	+/-
FA23BER	-/-	-	CNS	+/+ [a]
EUFA316	+/+	+	heart, kidney	+/-
FA26BER	-/-	-	none	-/-
EUFA348	+/-	+	none	-/- [a]
EUFA349	+/-	+	kidney	-/-

[a] Patient received transplant

Table 4.5: Continuation of table 4.4. (GIT = Gastro intestinal tract; CNS = Central nervous system; n.a. = No data available; MDS = Myelodysplastic syndrome; AL = Acute leukemia)

form a complex with FANCA, and it has been shown that this binding is due to sequences both in the amino terminal and the carboxy terminal ends of the protein (Huber et al., 2000) and (Kuang et al., personal communication). The carboxy terminal binding region has been shown to involve sequences coded by exon 10 and exon 14 (Kruyt et al., 1998a), thus offering a pathogenic mechanism even for those expected mutant proteins truncating in exons 13 and 14. The mutation in patient DF3 leads to an interstitial deletion of exon 12 and a deduced protein of 58 kD, it is likely that the relative positioning of the binding regions coded by exon 10 and exon 14 is thus disturbed.

Only one missense mutation has been found and its physiological relevance is revealed by comparison with the mouse cDNA sequence presented here. A comparison of the FancG sequence with that of FANCG is shown in figure 4.2. The two orthologues show 71.8% identity and 88% similarity at the amino acid level. Direct comparison of the murine and human protein sequences allows the identification of totally conserved and conservatively substituted residues which might be essential for biological function. Attention has been drawn to leucine zipper motifs in the FANCA and FANCG proteins (Lo Ten Foe et al., 1996b; The Fanconi anemia/Breast cancer consortium, 1996; Liu et al., 1997). In the case of murine FancG, this motif (a heptad repeat of leucine residues of the form L-X6-L) is not totally conserved (figure 4.2). Interestingly, the murine protein also contains another shorter leucine zipper motif from residues 58-79 which is not totally conserved in the human protein. The missense mutation L71P in patient EUFA569 is located directly in the second putative leucine zipper at a conserved leucine residue. Substitution of leucine by proline is likely to disrupt secondary structure in this region of the protein. It seems plausible that the disturbance of this leucine zipper prevents an essential protein:protein interaction required for FANCG function. Interestingly, LCLs from patient EUFA569 show neither FANCA nor FANCG signals in immunoblots (data not shown) suggesting that the L71P mutation destabilises the protein, perhaps due to a failure to complex with its partner, FANCA. We have observed interdependence of protein signals in immunoblots in other patients of both FA-A and FA-G complementation groups (Waisfisz et al., 1999a).

Of 35 assigned German families, nine belong to complementation group FA-G making this the second largest group in Germany after FA-A (68%). The 313G>T mutation accounts for 44% of the alleles in German FA-G-patients and is clearly due to a founder effect. Whilst there is generally no clear correlation between the mutations described here and the clinical symptoms, the two patients homozygous for the common 313G>T mutation do show a relatively early onset of the haematological disorder at 1.4 and 2.4 years in comparison with an average of 6.1 years for the 23 FA-G-patients as a whole (table 4.4). The putative missense L71P mutation found in patient EUFA569 would seem to lead to a disease with later onset (13 years) and milder clinical course. This patient is currently haematologically healthy at 26 years of age. The founder mutation, 1649delC, among Turkish patients is not obviously associated with a particular clinical phenotype.

One of the patients described here, FA1BER, was originally diagnosed with Estren Dameshek anaemia, considered to be a subtype of FA without malformations (Kunze, 1980; Schmid and Fanconi, 1978). However, this patient has mutations in the FANCG gene as do other patients with a more classical phenotype. There is, therefore, no genetic basis for the Estren Dameschek subtype. Fibroblasts from the patient FA1BER, which would have, if at all, a truncated FANCG protein of 4.6 kD, have been examined extensively in various laboratories and have been

shown to have several biochemical defects. Analysis of psoralen interstrand DNA crosslinks in these cells using isopyknic centrifugation of denatured genomic DNA provided one of the few demonstrations of a crosslink removal defect in Fanconi anaemia (Gruenert and Cleaver, 1985). Persisting DNA crosslinks are expected to prevent semiconservative DNA replication, and indeed a permanently reduced DNA synthesis rate after a crosslinking treatment has been shown for these cells (Digweed et al., 1988). Nevertheless, the repair of UV-induced thymine dimers is normal in these cells (Klocker et al., 1985), reflecting the fact that lesions affecting just one DNA strand are repaired by the independent pathways of base-excision and nucleotide-excision repair.

4.6 Acknowledgements

We are indebted to the Fanconi anaemia patients, their families and the Deutsche Fanconi-Anämie Hilfe e.V. for their co-operation. The excellent technical assistance of Gabriele Hildebrand, Susanne Rothe, Janina Radszewski, Carola van Berkel, Nicolle Cool, Ingrid Gruijs, Anneke Oostra, Martin Rooimans and Jurgen Steltenpool is gratefully acknowledged. AJT is supported by the Fanconi Anemia Research Fund, USA; NVM is supported by the European Union BIOMED2 Grant No. BMH4-98-3784. This work was supported by the Fritz Thyssen-Stiftung.

Chapter 5

Natural gene therapy: molecular self-correction in Fanconi anemia

Michaela Gross[1], *Stefan Lobitz*[2], *Richard Friedl*[1], *Sabine Herterich*[1], *Ralf Dietrich*[3], *Bernd Gruhn*[4], *Holger Hoehn*[1], *Detlev Schindler*[1], *Helmut Hanenberg*[2]

[1] Department of Human Genetics, University of Würzburg, 97074 Würzburg, Germany

[2] Department of Pediatrics, University of Düsseldorf, 40225 Düsseldorf, Germany

[3] Deutsche Fanconi Hilfe e.V., 59427 Unna-Siddighausen, Germany

[4] Department of Pediatrics, University of Jena, Germany

Human Molecular Genetics, in review

5.1 Abstract

Fanconi anemia (FA) is a genetically and phenotypically heterogenous autosomal recessive disease associated with chromosomal instability and hypersensitivity to DNA crosslinkers. Prognosis is poor due to progressive bone marrow failure and increased risk of neoplasia, but revertant mosaicism may improve survival. Mechanisms of reversion include back mutation, intragenic crossover, gene conversion and compensating deletions/insertions. We describe the types of reversion found in five mosaic FA-patients who are compound heterozygotes for single base mutations in FANCA or FANCC. Intragenic crossover could be proven as the mechanism of self-correction in the FANCC-patient. Restoration to wildtype via back mutation or gene conversion of either the paternal or maternal allele was observed in the FANCA-patients. The sequence environments of these mutations/reversions were indicative of high mutability, and selective advantage of bone marrow precursor cells carrying a completely restored FANCA allele might explain the surprisingly uniform pattern of these reversions. We also describe a first example of *in vitro* phenotypic reversion via the emergence of a compensating missense mutation 15 amino acids downstream of the constitutional mutation, which explains the reversion to MMC-resistance of the respective lymphoblastoid cell line. With one exception, our mosaic patients showed improvement of their hematological status during a 3 to 6 year observation period, indicating a proliferative advantage of the reverted cell lineages. In patients with Fanconi anemia, genetic instability due to defective caretaker genes sharply increases the risk of neoplasia, but at the same time increases the chance for revertant mosaicism leading to improved bone marrow function.

5.2 Introduction

The genome of long-lived, warm blooded species such as ours suffers continuous damage from a variety of exogenous and endogenous sources. In order to counteract this threat, a number of gene families have evolved that recognize DNA damage and initiate its repair and/or elimination. Some of these "guardian" functions are carried out by genes that are responsible for the maintenance of our somatic genome. Mutations in such caretaker genes lead to genetic instability in somatic cells and mutator phenotypes, whose most detrimental manifestations are neoplasia (Cahill et al., 1999) and premature aging (de Boer et al., 2001).

During the past few years it has been recognized that genetic instability may not only be detrimental but potentially beneficial for some of the affected patients. This is because genetic instability appears to increase the opportunity for correction of the constitutional genetic error in a proportion of body cells by reversion and/or compensation of the original mutation. For example, in

the case of compound heterozygous Bloom syndrome and Fanconi anemia (FA) patients, revertant mosaicism has been described in peripheral blood cells reflecting the side by side existence of defective and self-corrected cell lineages (Ellis et al., 1996; Lo Ten Foe et al., 1997). In situations where self-correction confers a proliferative advantage, the progeny of the self-corrected precursor cell can be expected to gradually replace the defective cell population (Hirschhorn et al., 1996; Stephan et al., 1996). Somatic cell mosaicism as the result of self-correction has important clinical implications. In the case of Fanconi anemia, clonal expansion of a corrected bone marrow stem or early progenitor cell may lead to complete reversal of the disease phenotype in the periphery such that the definitive laboratory diagnosis must employ other than peripheral blood cells (Joenje et al., 1998). With regard to gene therapy, the observed *in vivo* growth advantage of self-corrected cells is encouraging, and emergence of mosaicism may in fact mitigate and improve the natural course of the disease in at least some of the FA-patients (Lo Ten Foe et al., 1997). In this positive context, it may be justified to speak of "natural gene therapy" (Youssoufian, 1996b). A negative aspect concerns bone marrow transplantation where the co-existence of blood cells with different sensitivities towards cytotoxic agents complicates the conditioning regimen. Finally, the phenomenon of somatic cell mosaicism may contribute to the pronounced clinical heterogeneity encountered among patients with Fanconi anemia.

We here document somatic cell mosaicism due to self-correction in five FA-patients. We define the underlying mutations in different patient tissues, and test their functional significance by site-directed mutagenesis and complementation analysis using retroviral vectors. In addition to previously reported mechanisms of reversion of cellular phenotypes (such as back mutation, intragenic crossover, gene conversion, and compensating deletions/insertions; (Lo Ten Foe et al., 1997; Waisfisz et al., 1999b; Gregory et al., 2001)) we describe a compensating missense mutation downstream of the original base change as an *in vitro* mechanism of phenotypic reversion that explains the MMC-resistance of the lymphoblastoid cell line in one of our patients. Four of our five mosaic patients showed a gradual improvement of blood counts during a 3 to 6 year observation period, indicating the clinical relevance of revertant mosaicism.

5.3 Material and methods

5.3.1 Cell culture, cell cycle studies and complementation testing

The five patients we describe were referred to our laboratory in order to confirm or rule out the clinical suspicion of Fanconi anemia. Peripheral blood and skin

biopsy samples were obtained with informed consent. Mononuclear blood cells isolated by Ficoll separation were used for direct transfections, for 72 h PHA lymphocyte cultures, and for the establishment of EBV-transformed lymphoid cell lines (LCL). LCL were maintained in RPMI-media (Gibco) with 16% fetal bovine serum. Fibroblast cultures were established using standard cell culture procedures and MEM-media supplemented with glutamine and 16% fetal bovine serum. All cell cultures were kept in high humidity incubators equipped with CO_2 and O_2 sensors in an atmosphere of 5% (v/v) CO_2 and 5% (v/v) O_2 by replacing air through nitrogen. Chromosome breakage studies following exposure to various concentrations of mitomycin C were performed according to standard protocols (Schroeder-Kurth et al., 1989). Cell cycle studies employed bivariate BrdUrd/Hoechst flowcytometry as previously described (Poot et al., 1994; Seyschab et al., 1995). Assignment of patient cells to a given FA complementation group was achieved via complementation analysis using retroviral vectors containing inserts of the full-length cDNAs of FANCA or FANCC (Hanenberg et al., 2002) and (Lobitz et al, manuscript in preparation).

5.3.2 Mutation analysis

Genomic DNA extracted from 2nd to 4th passage primary fibroblast cultures was used for SSCP-prescreening. SSCP analysis was performed on native acrylamide gels (Amersham Pharmacia, Freiburg, Germany) at 14°C. Fragments showing an aberrant SSCP pattern were sequenced using the ABI 310 sequencer to identify the mutations. The FANCA-gene was amplified in 41 fragments containing all 43 exons and the FANCC-gene was amplified in 14 fragments containing all 14 exons of the gene, using primers flanking the exon/intron boundaries. All detected mutations were confirmed at the cDNA level. In patients where only a single mutation had been detected by SSCP screening we performed RT-PCR in order to rule out large heterozygous deletions. Aberrant bands in 1% agarose gels of cDNA-fragments were sequenced directly.

5.3.3 Site directed mutagenesis

In order to confirm the pathogenic status of novel amino acid exchanges they were analyzed by transducing the FANCA wildtype containing the mutations L324R and R951Q as well as the combination of R951Q and Q966A into the pSL1180/FANCA vector (Amersham Biosciences, Freiburg, Germany; modified by introducing wildtype FANCA). Sequences of the phosphorylated mutagenic primers were designed according to the manufacturer´s instructions (QuikChange Site-Directed Mutagenesis Kit, Stratagene, Amsterdam, Netherlands) and were as follows:
L324R/for 5´-TCAGTCATACCC**G**GACTCAGATACTC-3´,

L324R/rev 5´-GAGTATCTGAGTC**C**GGGTATGACTGA-3´,
R951Q/for 5´-CAGATACTGAAC**A**GCAGGACTTCCAC-3´,
R951Q/rev 5´-GTGGAAGTCCTGC**T**GTTCAGTATCTG-3´,
Q966A/for 5´-CTTTCTCCCTG**C**GTCCTCGGCTTC-3´ and
Q966A/rev 5´-GAAGCCGAGGAC**G**CAGGGAGAAAG-3´
(modified nucleotides are written in bold letters). The reaction was performed in 50 μl-reactions containing 20 ng of plasmid DNA, 125 ng of each mutagenesis primer, 1,5 mM $MgCl_2$, 1x reaction buffer, 200 nM dNTPs and 2,5 Units Pfu-Turbo DNA Polymerase (Stratagene, Amsterdam, Netherlands). An initial denaturation for 30 s at 95°C was followed by amplification for twelve cycles, each with denaturation for 30 s at 95°C, annealing for 60 s at 55°C and extension for 16 min at 68°C. After placing the reactions on ice for 2 min, 10 Units DpnI were added and the reaction was incubated at 37°C for 1 h to digest the parental supercoiled dsDNA. The FANCA coding sequences carrying the desired mutations were directionally cloned as 4.5 kb NotI/NotI DNA fragments into the LFAPEG expression vector and sequenced to show the integrity of the entire coding sequence except for the presence of the targeted mutations. The FA-A null cell lines PIR and SNE (both patients carrying large homozygous deletions in the FANCA gene) were stably transfected with the targeted cDNAs and tested for correction of MMC-hypersensitivity as described below.

5.3.4 Retroviral vectors and transfections

The LFAPEG and LFCPEG plasmids were used for complementation studies as previously described (Hanenberg et al., 2002). LFAPEG was used for site directed mutagenesis testing. This vector is a derivative from MSCV2.1 (Hawley et al., 1994) containing FANCA-WT and EGFP (enhanced green fluorescent protein), where the FANCA cDNA is expressed off the retroviral LTR and the EGFP cDNA is under control of the internal murine PGK promotor from MSCV2.1. In LEG (mock transfection), EGFP was directly expressed off the LTR. For site directed mutagenesis studies, the packing cell line, 293T-derived ecotropic Phoenix cells (Nolan, 1996, Phoenix cells, www-leland.stanford.edu/group/nolan), was transfected with 10-20 μg of the FANCA plasmid DNA using FuGene6 (Roche, Mannheim, Germany). After two days retrovirus containing supernatants (SNs) were harvested, filtered with 45 μm and used for transducing NIH/3T3-derived pg13 cells (Miller et al., 1991) three times in the presence of 7.5 $\mu g/ml$ protamin (Roche, Mannheim, Germany). GALV-pseudotyped retroviruses were harvested from pg13 cells as previously described (MacNeill et al., 1999). The cell lines of our patients as well as the PIR and SNE cell lines were cultured on the fibronectin fragment CH-296 as described previously (MacNeill et al., 1999; Pollok et al., 1998; Hanenberg et al., 1996; Hanenberg et al., 1997). Cells were grown for at least two days after the last exposure to virus-supernatants in order to

ensure stable expression of the cDNAs from the integrated proviruses. Treated cells were cultured in triplicates in the presence of increasing concentrations of MMC (Medac, Hamburg, Germany). Six days later, cells were harvested for flowcytometry.

5.3.5 Flowcytometry

Flowcytometry was carried out on a BD-LSR Instrument (Becton Dickinson, Heidelberg, Germany) equipped with Argon (emission at 488nm) and Helium-Cadmium (emission at 325nm) lasers. 30 min prior to cell harvest, the DNA-dye Hoechst 33342 was added to the cultures at a final concentration of 8 $\mu g/ml$. After harvest by trypsinisation, cells were stained for 10 min with propidium iodide (PI) (Sigma, Heidelberg, Germany) at a final concentration of 6 $\mu g/ml$. Using the PI versus Hoechst setting, live and dead cells were first separated, followed by the EGFP versus Hoechst setting, allowing discrimination between successfully transfected (green fluorescence) and non-transfected cells. DNA-content distributions showing G1 and G2 phase peaks were determined for EGFP positive cells.

5.4 Results

When 72 h cultures of peripheral blood mononuclear cells from healthy donors are exposed to increasing concentrations of MMC, there is only a minimal increase of metaphases that show more than three breakage events per cell (figure 5.1, left-most columns). In contrast, metaphases from FA-patients show more than ten breaks per cell after exposure to MMC (figure 5.1, rightmost columns). In our five patients, chromosome breakage studies of 72 h mononuclear blood cell cultures yielded various degrees of bimodal breakage distributions after exposure to 50 and 100 ng/ml MMC, indicating the simultaneous presence of MMC-sensitive and MMC-resistant cells (figure 5.1, middle columns, patient STT). Bivariate BrdUrd/Hoechst flowcytometry of 72 h peripheral blood mononuclear cell cultures of our five patients revealed cell cycle distributions and G2-phase accumulations intermediate (figure 5.2b) between controls (figure 5.2a) and previously confirmed FA-patients (figure 5.2c). At the time of their first diagnostic evaluation, the cell cycle test placed all five of our patient blood samples within the lower end of the Fanconi anemia cohort (figure 5.2d). However, during the course of a 3 to 6 year observation period, their cell cycle patterns gradually merged with those of the healthy controls (data not shown).

The bimodal distribution of chromosomal breakage rates and the results of the cell cycle analysis therefore suggested the presence of a mixture of MMC-sensitive

and MMC-resistant mononuclear blood cells and a mixture of cells with a disturbed and a normal cell cycle pattern. EBV-transformed lymphoid cell lines of the five patients were uniformly MMC-resistant. In contrast, the patients´ fibroblast cultures were uniformly MMC-sensitive as evidenced by elevated chromosome breakage (with unimodal breakage distributions), G2-phase blockage, and lack of cell growth following five day exposures to 3 *ng/ml* MMC (data not shown). Altogether, these findings indicated a FA-positive cellular phenotype in the patients´ fibroblasts, whereas the patients´ mononuclear blood cells consisted of variable mixtures of cells with FA and control cell phenotypes. In order to clarify the cellular genotype-phenotype correlations in our patients, complementation and mutation studies were performed with DNA isolated from their fibroblasts, and the results were compared to DNA isolated from their primary peripheral blood mononuclear cells and EBV-transformed lymphoid cell lines.

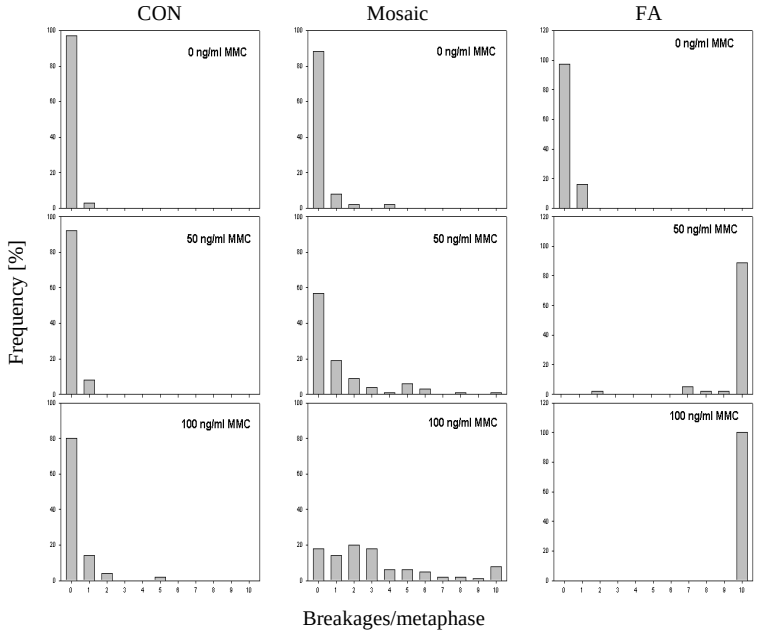


Figure 5.1: Chromosome breakage analysis of 72 h peripheral blood mononuclear cells in control (left panel), mosaic (center panel) and other FA blood (right panel) donors without and after exposure to 50 and 100 ng/ml MMC. The number of breaks per cell (horizontal axis) was analyzed in 100 metaphases each. Bars represent percentages of metaphases with zero to more than 10 breaks per cell. The center panel represents results of the breakage analysis in patient STT with evidence for a bimodal distribution of breakage rates after exposure to MMC.

Patient 1, **URD**, was assigned to complementation group A by showing elimination of G2-phase blockage after transfection of his fibroblasts with the FANCA vector (figure 5.3a). Mutation analysis (SSCP and sequencing) of the patient´s

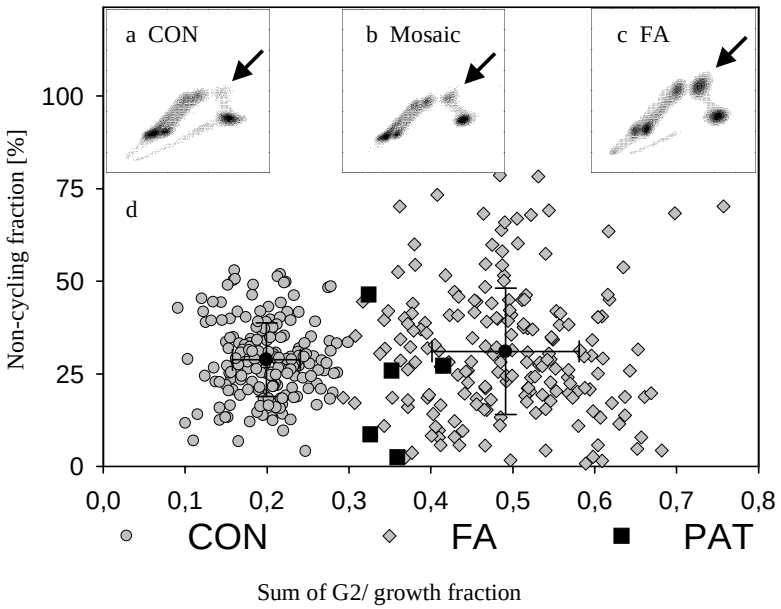


Figure 5.2: (a-c) 72 h cell cycle distributions of PHA-stimulated peripheral blood monunuclear cells analyzed via two parameter flowcytometry (BrdUrd/Hoechst technique, (Poot et al., 1994)). First cell cycle G2-phase compartments are denoted by arrows. Compared to controls (a), mosaic (b) and other FA-patients (c) show increasing accumulations of cells in G2 and decreasing cell cycle progression. (d) Sum of G2-phases over growth fraction (horizontal axis) versus percentage of G0/G1 phase cells (vertical axis) were assessed via flowcytometry and plotted for controls (solid circles), the 5 mosaic patients under investigation (solid squares) and other FA-patients (solid diamonds). Our 5 mosaic patients display positions within, but on the leftmost margin of the FA-patient cluster. Mosaicism has not been excluded for other FA-patients with similar low X-axis positions. Assuming all patients with X-axis positions between 0.29 and 0.41 would be mosaics, this would amount to 38% (44 of 169 FA-patients).

fibroblast cultures revealed compound heterozygosity for two FANCA nonsense mutations (856C>T, Q286X in exon 10 and 3976C>T, Q1326X in exon 40; table 5.1). Sequencing of the corresponding exons in DNA prepared from the patient´s MMC-resistant lymphoblastoid cell line showed that the exon 10 nonsense mutation had reverted to wildtype (figure 5.3b). The same reversion was found in DNA extracted from the patient´s peripheral blood mononuclear cells. As a consequence of the reversion, the patient´s blood cells have acquired a heterozygous state which normalizes their chromosome breakage rates and cell cycle patterns, and which explains the MMC-resistance of the patient´s LCL. These *in vitro* changes were accompanied *in vivo* (beginning at age 11) by improvements of the patient´s haematological parameters (figure 5.4).

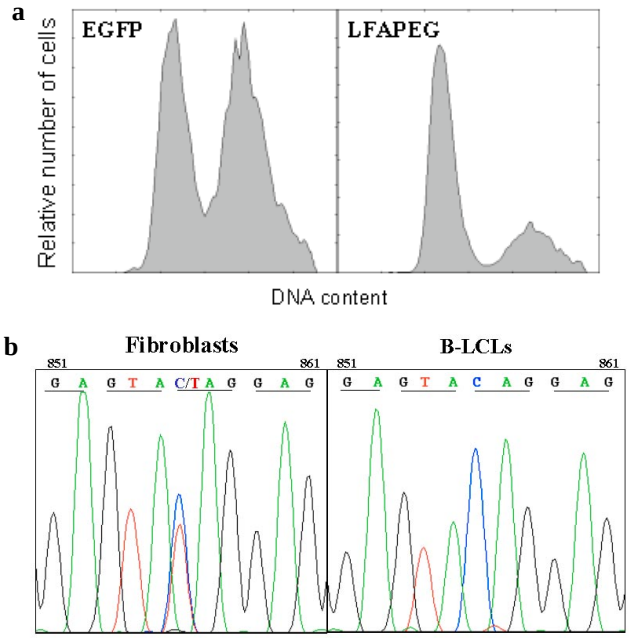


Figure 5.3: Complementation and mutation analysis in patient URD (a) Elimination of G2-phase blockage of virus-transfected fibroblasts after MMC treatment in patient URD shown by flowcytometry. Mock transfected fibroblasts (EGFP) display massive G2-phase accumulation, whereas the G2-phase peak of fibroblasts transfected with the vector LFAPEG containing the WT FANCA gene has returned to control levels. URD fibroblasts transfected with WT FANCC or WT FANCG containing viruses (LFCPEG and LFGPEG) behave like mock transfected fibroblasts (data not shown). (b) URD fibroblasts carry the heterozygous mutation 856C>T, Q256X, leading to a premature stop-codon, whereas the lymphoblastoid cell line derived from patient URD displays homozygosity for the WT codon CAG (Glutamine). The same reversion was found in the patient´s native peripheral blood mononuclear cells (data not shown).

Patient 2, **STT**, was assigned to complementation group A by retroviral transfection of his fibroblast culture. Mutation analysis (SSCP and sequencing) revealed a single nonsense mutation, 862G>T, E288X, in exon 10 of FANCA, which has been described before (Morgan et al., 1999). Sequencing of the complete genomic DNA failed to detect a second mutation. On the basis of cDNA analysis a defective promoter region is unlikely because of the presence of a second allele. Rather, the distribution of heterozygous polymorphisms suggests the presence of a large deletion as the second mutation. The extent and nature of the deletion could not be further clarified with the materials available. Sequencing of the patient´s MMC-resistant lymphoblastoid cell line and of his native peripheral blood

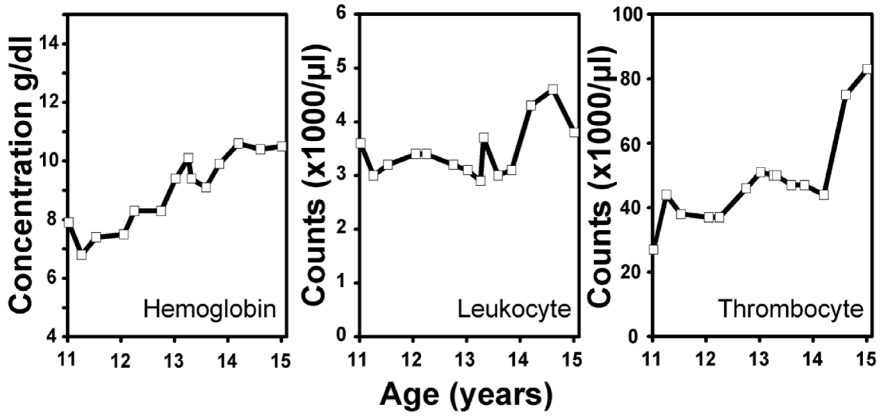


Figure 5.4: Time course of haematological parameters in patient URD.

Patient code	YoB[a]	Compl. group[b]	Mutations	Reference	Mechanism of reversion
URD	1987	FA-A	856C>T, Q256X	(Wijker et al., 1999)	Back mutation to WT
			3976C>T, Q1326X	present paper	
STT	1992	FA-A	862G>T, E288X	(Morgan et al., 1999)	Back mutation to WT
			not detected		
MRB	1994	FA-A	971T>G, L324R	present paper	Back mutation to WT
			IVS9-1G>T	present paper	
RNT	1991	FA-C	67delG	(Strathdee et al., 1992)	Intragenic
			IVS11-2A>G	present paper	crossover
EUFA-173	1974	FA-A	Del. exons 17-31	(Joenje et al., 2000)	
			2852G>A, R951Q	(Joenje et al., 2000)	Back mutation to WT
			2897A>C, E966A	present paper	Compensatory missense mutation

[a] Year of Birth [b] Complementation group

Table 5.1: Patient codes, birthdates and constitutional mutations as determined from fibroblast DNA.

mononuclear cells showed that the nonsense mutation in exon 10 had reverted to wildtype, both in the patient´s T- and B-cells (data not shown).

Clinically, the patient suffered from anemia and thrombocytopenia right after birth. His erythrocyte and leukocyte counts improved gradually during his first year of life, whereas his thrombocyte counts started to improve between ages 3 and 6. Currently, at age 12, thrombocytes are 112 Gpt/l, hemoglobin is 11.1 $mmol/l$, erythrocytes are 3.5 Gpt/l and leukocytes 7.4 Gpt/l.

Patient 3, **MRB**, was assigned to complementation group A by normalization of his cell cycle pattern after transfection of his fibroblasts with the FANCA vector (figure 5.5a). Mutation analysis of the patient´s fibroblast DNA revealed two not previously reported mutations, one splice-site and one missense mutation. The paternal splice-site mutation IVS9-1G>T destroys the conserved acceptor splice-site in front of exon 10. An alternative AG dinucleotide dowstream is used, which is located in exon 10 and results in a 32 nt deletion at the mRNA level, leading to a frameshift and a truncated protein nine amino acids downstream. The maternal missense mutation (971T>G, L324R) affects a conserved residue in the murine Fanca sequence, suggesting its functional significance. In order to provide definitive proof of its pathogenicity, the substitution was introduced into pSL1180/FANCA by site directed mutagenesis and the altered FANCA coding sequence was directionally cloned as a 4.5 kb NotI/NotI DNA fragment into the LFAPEG expression vector. After packaging this plasmid into the retroviral vector, the FANCA$^{-/-}$ cell lines SNE and PIR were stably transfected with the targeted FANCA cDNA and tested for correction of their MMC-hypersensitivity. As shown in figure 5.5b, transfection with the targeted FANCA vector failed to abrogate the G2-phase cell cycle blockage of FANCA null cells, just like mock transfection with the EGFP-only vector (LEG) or transfection with the FANCC containing vector (LFCPEG). In contrast, transfection of null cells with the LFAPEG vector carrying the wildtype FANCA gene restored a normal cell cycle pattern. Altogether, these experiments prove that the amino acid change L324R is a pathogenic mutation. Sequencing of the patient´s MMC-resistant EBV-transformed cell line and of his native peripheral blood mononuclear cells revealed that the mutation 971G>T had reverted to wildtype (figure 5.5c), explaining the reversion of the patient´s blood cells to functional heterozygosity.

Within an observation period of four years, the patient´s thrombocyte counts increased from 38.000 to 139.000 Gpt/ml, total leukocytes from 4.1 to 5.6 Gpt/l, Granulocytes from 1.2 to 3.5 Gpt/l and hemoglobin from 11.9 to 13.1 $mmol/l$. This suggests that the reversion event took place in a very early progenitor of all these cell lineages.

Patient 4, **RNT**, was assigned to complementation group C by transfection of his cultured fibroblasts with the FANCC vector. His genomic DNA (from fibroblasts) had the known founder mutation 67delG (Strathdee et al., 1992) in addition to a not previously reported splice-site-mutation IVS11-2A>G, which destroys the conserved acceptor splice-site in front of exon 12 and leads to skipping of exon 12. In contrast to the previous patients, both mutations were still present in

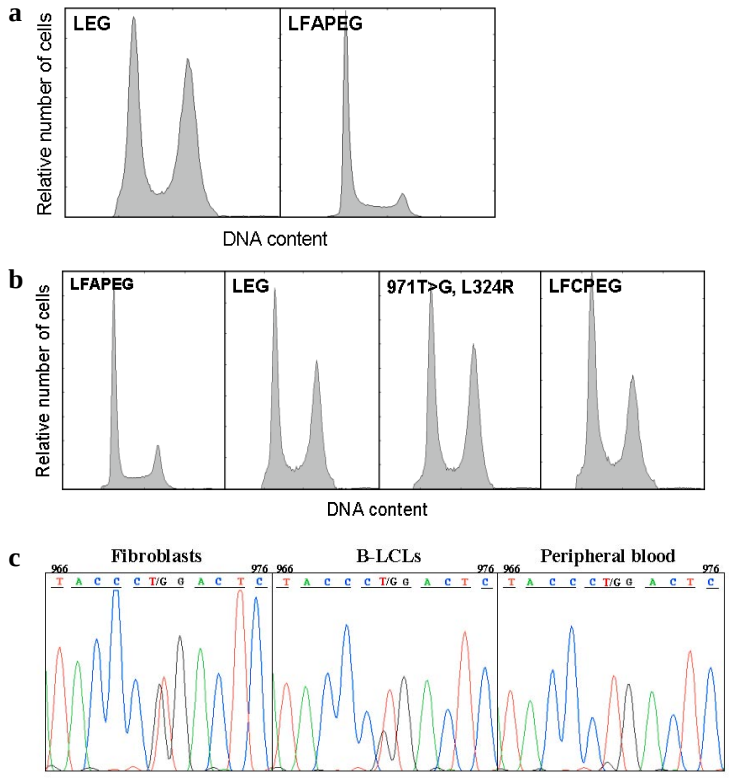


Figure 5.5: Complementation and mutation analysis in patient MRB. (a) Mock transfected fibroblasts (LEG) show the typical FA G2-phase arrest, whereas G2-phase accumulations of fibroblasts with the complementing vector containing the WT FANCA gene (LFAPEG) have returned to WT levels. MRB-fibroblasts transfected with WT FANCC or WT FANCG containing viruses (LFCPEG and LFGPEG) behave like mock transfected fibroblasts (data not shown). (b) Testing for pathogenicity of the maternal missense mutation 971T>G, L324R, of patient MRB, using the FANCA$^{-/-}$ lymphoblastoid cell line SNE. The respective cell cycle distributions (from left to right) show complementation of SNE after transfection with the WT FANCA vector (LFAPEG), lack of complementation in mock transfected cells (LEG) after transfection with LFAPEG vector containing the 971T>G-mutation (which proves the pathogenicity of the mutation) as well as lack of complementation with the WT FANCC (LFCPEG) containing vector. (c) Sequencing analysis of the reversion of patient MRB. His fibroblasts carry the heterozygous missense mutation 971T>G, L324R, whereas the mutated maternal allele decreases in the lymphoblastoid cell line and in native peripheral blood cells, confirming the patient´s incomplete mosaic status. In the patient´s fibroblasts, the proportion of WT (T) and mutated nucleotides (G) amounts to 1:1. This ratio increases to 2:1 in the lymphoblastoid cell line which was established in the year 1998, but two years later the patient´s native peripheral blood cells exhibit a WT to mutant ratio of 10:1, indicating a selective growth advantage of the reverted cell population.

the patient´s MMC-resistant EBV-transformed cell line indicating a mechanism other than back-mutation for the self-correction of his blood cells. There were no detectable sequence variations in the vicinity of the two mutations, but their location at opposite ends of the gene suggested the possibility of self-correction via intragenic crossover. In order to separate the two different alleles from patient RNT, his full-length FANCC-cDNA was amplified using Pfu-Turbo DNA Polymerase (Stratagene, Amsterdam, Netherlands) and primers containing a tail with a NotI-binding site. The full length fragment was cloned as a NotI/NotI DNA fragment into the pSL1180 vector and transfected into TOP TEN electrocompetent cells (Gibco). Exons 1 and 12 of the resulting 26 colonies were sequenced in order to prove intragenic crossover by the detection of both wildtype alleles and alleles carrying both mutations. Eight colonies carried a single mutation, 15 colonies showed wildtype sequences, whereas three colonies included the 67delG-mutation as well as a lack of exon 12, confirming intragenic crossover as the mechanism of reversion in patient RNT. The presence of colonies with only one of the two mutations suggests that mosaicism had been incomplete at the time when the cell line was established. Due to the patient´s sudden death from cerebral hemorrhage, no primary blood cells were available for further studies. Since there was no improvement of his haematological parameters during the observation period of 3 years prior to his death, the somatic recombination event giving rise to mosaicism may have been limited to a common precursor of his T- and B-cell lineages.

The last patient in this series is patient **EUFA173**, who had previously been re-assigned from complementation group H to group A by the Amsterdam group (Joenje et al., 2000). We confirmed this re-assignement by showing normalization of the G2-phase cell cycle blockage after transfection of his primary fibroblasts with the wildtype FANCA vector. Two mutations in the FANCA gene, a large deletion spanning exons 17 to 31 and the missense mutation 2852G>A, R951Q, had been previously described by the group of H. Joenje (Joenje et al., 2000). Even though the maternal missense mutation affects a position that is conserved in the murine gene, its pathogenic status had yet to be proven. The strategy used was the same as described for the L324R substitution in patient MRB. As shown in figure 5.6a, transfection of PIR and SNE with LFAPEG/2852 failed to reduce the G2-phase accumulations of the null mutant cells, confirming the pathogenicity of the R951Q substitution. Sequencing of the patient´s MMC-resistant lymphoblastoid cell line revealed the presence of both mutations. However, a second amino acid substitution located 15 amino acids downstream of the first one (2897A>C, E966A) was also present. In order to test the possiblity whether this second mutation (not at the conserved residue in the mouse genome) might have arisen *in vitro* and might act as a compensatory mutation, both substitutions were introduced into pSL1180/FANCA and transfected into our null cell lines PIR and SNE. As shown in figure 5.6b, transfection with the double mu-

tant decreased G2-phase cell cycle blockage in the same way as transfection with the wildtype LFAPEG vector. The restoration of normal cell cycle progression by the presence of both amino acid changes is suggestive of restoration of normal protein function by the second mutation. Much to our surprise, sequencing of the patient´s peripheral blood mononuclear cells did not show the second amino acid substitution that had been found in his lymphoblastoid cell line, but rather showed a reversion of the maternal mutation 2852G>A to wildtype. Likewise, a newly established lymphoblastoid cell line from the patient contained only a reversion of 2852G>A. This proves that the second amino acid change found in the patient´s original LCL must have arisen *in vitro* during long-term cultivation. The substitution E966A therefore is responsible for the MMC-resistance of this cell line, since it obviously compensates the defective function caused by the constitutional mutation. The G to A change at position 2852 is obviously methylation-mediated, the CG-dinucleotide located on the antisense strand being changed to TG, resulting in a G>A miscorrection on the coding strand. With regard to the reversion event, the G>A mutation creates a new direct repeat, CAGCAG, that could trigger back mutation via a slippage mechanism. There are a number of Alu-repeats in the region of exons 27 to 33 of FANCA that are known to increase the likelihood of replication errors (Morgan et al., 1999).

From a clinical point of view it is worth noting that, between the ages 15 and 21, the patient´s thrombocyte counts increased from 54.000 to 142.000 Gpt/l, his total leukocytes rose from 2.5 to 4.8, his granulocytes from 0.8 to 2.5 Gpt/l, and his hemoglobin from 12.1 to 15.8 $mmol/l$.

5.5 Discussion

Somatic cell mosaicism due to forward somatic mutation is increasingly recognized as explanation for deviations from predicted genotype-phenotype correlations (Gottlieb et al., 2001). In contrast, somatic cell mosaicism due to reverse somatic mutation (revertant mosaicism) is less well-known, but should in principle contribute in the same way to variable expressivity of genetic disorders (Wahn et al., 1998; Jonkman, 1999). In recessive disesases, somatic reversion of one of the two inherited mutations restores heterozygosity in the descendants of the reverted cell. Depending on the mechanism of somatic reversion, the function of the affected cell lineage may be partly or completely restored. Complete restoration of a cellular phenotype to wildtype usually results from mechanisms such as intragenic recombination, back-mutation (reverse point mutation), or gene conversion. Partial restoration of protein function has been observed with so-called compensating or second site mutations. Such second site somatic mutations in cis leave the constitutional mutation unchanged but alter the downstream DNA sequence via insertion, deletion or point mutation. As a consequence of the com-

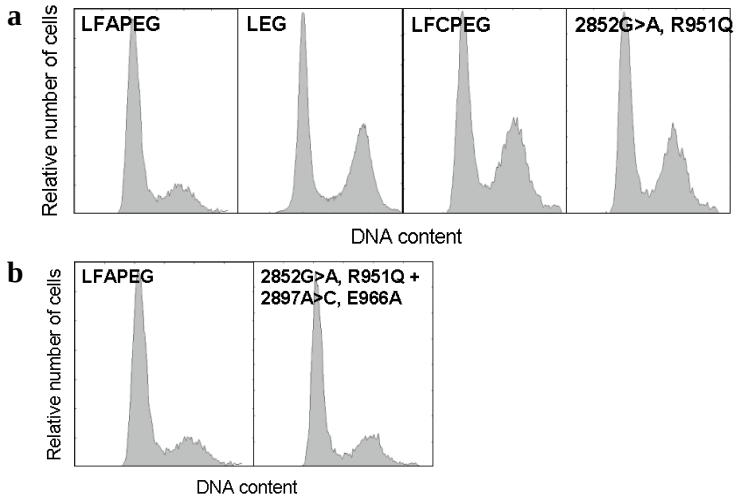


Figure 5.6: Complementation assays in patient EUFA173. (a) Cell cycle distributions (from left to right) indicating successful complementation of EUFA173 fibroblasts by the WT FANCA vector (LFAPEG), lack of complementation in mock transfected fibroblasts (LEG), lack of complementation after transfection with the WT FANCC vector (LFCPEG) and lack of complementation after transfection with the FANCA vector containing the maternal missense 2852G>A mutation (which proves its pathogenicity). (b) Cell cycle distributions (from left to right) showing successful complementation of the PIR cell line ($FANCA^{-/-}$ cells) after transfection with the WT FANCA vector (LFAPEG) and after transfection with the FANCA vector containing both the 2852G>A, R951Q, and the 2897A>C, E966A, mutations. The latter result indicates the compensatory nature of the in vitro 2897A>C mutation.

pensating mutation a protein with at least partial function is produced (Waisfisz et al., 1999b).

Revertant mosaicism has been described as a rare cause of phenotypic modulation in single instances of X-linked and autosomal recessive diseases (for review see (Jonkman, 1999; Wada et al., 2001)), but revertant mosaicism appears to be a rather frequent phenomenon in the autosomal recessive genetic instability syndromes. For example, in Bloom syndrome, compound heterozygosity combined with genetic instability sets the stage for somatic reversion of the high SCE-phenotype via intragenic recombination (Ellis et al., 1995). In occasional homozygous Bloom syndrome individuals back mutation has been reported as well (Ellis et al., 2001). In FA, intragenic recombination, gene conversion and compensatory second site mutations have been reported in lymphoblastoid cell lines of four FANCC-patients, and cytogenetic evidence for mosaicism in additional five unclassified patients (Lo Ten Foe et al., 1997; Waisfisz et al., 1999b).

A single compound heterozygous FANCA-patient has been reported to date who developped mosaicism as a consequence of a putative back mutation (Gregory et al., 2001), and two FANCA-patients with homozyogous mutations were shown to harbor compensatory mutations in their peripheral blood mononuclear cells (Waisfisz et al., 1999b). The case history of patient EUFAR173 demonstrates that such a compensatory change can also arise during the *in vitro* cultivation of lymphoblastoid cells, explaining the conversion of these cells from MMC-sensitivity to MMC-resistance.

Intragenic crossover could be proven as the mechanism of self-correction in our FANCC-patient. Intragenic crossover represents a reversion mechanism that requires compound heterozygosity. It has been described as the predominant mechanism of reversion in Bloom syndrome (Ellis et al., 1995) and in the lymphoblastoid cell line of a single FANCC-patient (Lo Ten Foe et al., 1997), but there are no reports to date of self-correction via intragenic recombination for any of the other known FA-genes. The previously published FANCC-patient had a single base deletion in exon 1 (67delG) together with a single base insertion in exon 14 (1806insA). Our FANCC-patient has the same single base deletion in exon 1 but a different second mutation located in exon 12. In both patients, the intragenic recombination event seems to have been facilitated by the mutually distant locations of the paternal and the maternal mutations.

In the remaining four of our five patients the uniformity of the type of reversion is impressive. Each of these four patients carries compound mutations in the FANCA gene, and in each of them one of the two constitutional point mutations has reverted back to the original wildtype sequence. In each of these four cases, reversion is complete in the patient´s B-cell derived lymphoblastoid cell lines, whereas the findings in peripheral blood mononuclear cells (mostly T-cells) indicate variable degrees of co-existence of mutated and reverted cells. Three of the four reversions took place in the region of exons 10 to 11 of the FANCA gene which is known as a highly mutable region due to the abundance of repetitive elements (Morgan et al., 1999; Wijker et al., 1999). To illustrate this point, table 5.2 summarizes the types of DNA sequence motifs surrounding the respective mutation/reversion events, including motifs found within +/- 20 bp that are known to play an important role in the breakage and rejoining of DNA (Cooper and Krawcak, 1993; Huff et al., 1995; Levran et al., 1997; Ianzano et al., 1997).

How can the apparent non-randomness of the reversion events, leading in each instance to restoration of precisely the wildtype sequence, be reconciled with the stochastic nature of mutations? One obvious explanation concerns the constraints imposed by selection, which create a proliferative advantage for cells with complete rather than partial restoration of protein function. In the case of our patients, random mutation would overwhelmingly result in non-conservative amino acid exchanges such that protein function might be impaired. In addition, there might be constraints imposed by DNA-structure which would favor the

Patient code	Exon	Mutation	Reversion	Mutation motifs[a] (+/-20 bp around the mutation)	Direct repeats (+/-20 bp)
STT	10	AGGAG**T**AGTCCTCC	A[GGAG**G**AGTCCTCC]	2x CTT, TGGA, CAGG(CCTG)	AGGAGG, AGTAGT, TCCTCC
URD	10	**T**A[GGAGGAGTCCTCC]	**C**A[GGAGGAGTCCTCC]	CTT, 2x TGGA, CAGG	AGGAGG, TCCTCC
MRB (= UPN127)	11	CCC**G**GA	CC**T**GA	2x CTT	CCC, TTCTTC, ACTCACTC
EUFA173 (Blood cells)	29	CA**G**CAGG	C**G**GCAGG	CAGG, 2x CTT, TGGA	CAGCAG
EUFA173 (Lymphoblastoid cell line)[b]	30	CA**G**CAGG	TTTCTCCCTG**C**GTCC	2x CTT, 2x CAGG, TGGA	TTTCTCCC, GGGGG

Table 5.2: Sequence environments and DNA-motifs surrounding the mutation/reversion sites in FANCA (Mutations and reversions are shown in bold letters; motifs directly found in the mutation locus are underlined; the sequence in parentheses [] represents a palindrome).

[a] CTT = Topoisomerase I cleavage site consensus sequence (Bullock et al., 1975); TGGA = Deletion hot spot consensus sequence, which resembles the putative arrest site for DNA polymerase α (Krawczak and Cooper, 1991); CAGG/CCTG = Mutation hot spot sequence (Huff et al., 1995; Levran et al., 1997) [b] In the lymphoblastoid cell line of patient EUFA173, the reversion is located 45 nucleotides downstream of the mutation.

restoration of the original sequence. For example, in our patients STT and URD the respective mutations/reversions are located at the 5´ end of a palindromic sequence whose hairpin-structure might be important for protein binding. Back mutation combined with selection therefore might explain the surprisingly uniform pattern of reversion in our FANCA-patients. Another possibility would be gene conversion. Gene conversion requires some sort of somatic pairing between homologous chromosomes. Since FANCA is located on human chromsome 16 that harbors a large block of heterochromatin known to promote somatic pairing (Haaf et al., 1986), and since all of our patients are compound heterozygotes, gene conversion cannot be ruled out as an alternative explanation for the fidelity with which restoration of the wildtype sequence has occurred.

The clinical course of FA is highly variable and may be determined in part by complementation group and mutation type (Gillio et al., 1997; Faivre et al., 2000). Whether revertant mosaicism leads to clinical improvement depends on when and where the reversion occurred during evolution of the various bone marrow cell lineages (Lo Ten Foe et al., 1997; Gregory et al., 2001). From a hematological and functional point of view, both T- and B-lymphocytic cell types were reverted in all of our patients. The fact that we observed bimodality of chromosome breakage and intermediate cell cycle patterns at the initial evaluation of these patients probably reflects the relative longevity of subpopulations of peripheral blood lymphocytes in which constitutionally defective cells persist long after somatically self-corrected cells have emerged. In contrast to the patient´s normal lymphocyte situation, their erythroid, leukocytic and megakaryocytic cell lineages showed various degrees of impairment. However, the status of these affected cell lineages improved over time in at least three of our five patients. In only a single patient (RNT) the hematological situation did not improve during a three year observation period, and he died from cerebral haemorrhage at six years of age after his thrombocyte counts had continuously decreased to 11 Gpt/l and his hemoglobin from 11.2 to 5.9 $mmol/l$. These data indicate that reversion in our patients must have taken place at least in a precursor cell of both lymphocytic cell lineages. Moreover, in three of the patients self-correction must have taken place in a much earlier precursor cell, possibly a hematopoietic stem cell. With respect to the prospects of gene therapy, the obvious *in vivo* selective advantage of such spontaneously self-corrected cells is encouraging.

5.6 Acknowledgements

The authors gratefully acknowledge the reported Fanconi anemia patients, their families and the "Deutsche Fanconi-Anaemie Hilfe e.V." for their cooperation. We also thank Richard Friedl, Gitta Emmert and Ariane Klobe for expert technical assistance.

Chapter 6

Spontaneous hematological cure in Fanconi anemia due to revertant alleles in somatic stem cells

*Michaela Gross[1] *, Detlev Schindler[1] *, Lydia Vieten[2], Sonja Gräsl[1], Stephan Lobitz[2], Alexandra Sobeck[1], Sabine Herterich[1] and Helmut Hanenberg[2]*

**These authors contributed equally to this work.*

[1] Department of Human Genetics, University of Wuerzburg, Biozentrum, Am Hubland, D-97074 Wuerzburg, Germany

[2] Department of Pediatric Hematology and Oncology, Heinrich Heine University Medical Center, Moorenstrasse 5, D-40225 Duesseldorf, Germany.

to be submitted to *Nature Genetics*

6.1 Introduction

Fanconi anemia (FA) is an autosomal recessive DNA instability disorder with progressive bone marrow failure. Here we demonstrate spontaneous reversion of one mutant FANCA allele in a single hematopoietic stem cell in two FA-patients. Corrected progeny of this cell over time repopulated the hematopoietic system and completely abrogated the hematological disease manifestations.

6.2 Results

6.2.1 Patient EUFA173

The EUFA173 patient manifested with severe pancytopenia at 15 years of age and was reported to have inherited a maternal point mutation 2852G>A in exon 29 of FANCA with substitution of R951Q and a large genomic deletion of exons 17-31 from his father (Joenje et al., 2000). In contrast to the natural course of FA (Auerbach et al., 1998; Butturini et al., 1994; Grompe and D´Andrea, 2001), he improved in all peripheral blood counts over the following eleven years to normal values without any treatment (figure 6.1). Sequencing analysis from whole blood at the age of 26 revealed that in contrast to DNA from skin fibroblasts, DNA from total white blood cells (WBC) had only minor amounts of the 2852G>A substitution, while most of the missense mutation had reverted to the WT sequence. For lineage-specific analysis, granulocytes and T-cells were separated to a purity >96% and a new B-lymphoblast cell line was established. All three cell types revealed the reversion of 2852A>G at different degrees with the short-lived granulocytes harboring predominantly a reverted normal FANCA allele and the long-lived T-cells still demonstrating large proportions of uncorrected cells. Moreover, the course of red blood cells (RBC) and platelet counts over the past eleven years strongly suggested that the reversion also involved the erythroid and megakaryocytic lineages.

6.2.2 Patient UPN127

The UPN127 patient manifested at 2.5 years with thrombocytopenia and 32 months later was started on androgens due to severe anemia and thrombocytopenia (figure 6.2). Retroviral complementation group analysis (Hanenberg et al., 2002) followed by sequencing demonstrated two mutant FANCA alleles, a maternal point mutation 971T>G and a paternal splice mutation IVS9-1G>T. The dramatic and stable improvements of the peripheral blood (PB) values however allowed to taper the androgen dose as early as eight months later with a continuous reduction to <0.5 mg/kg over the next 20 month. This clinical course

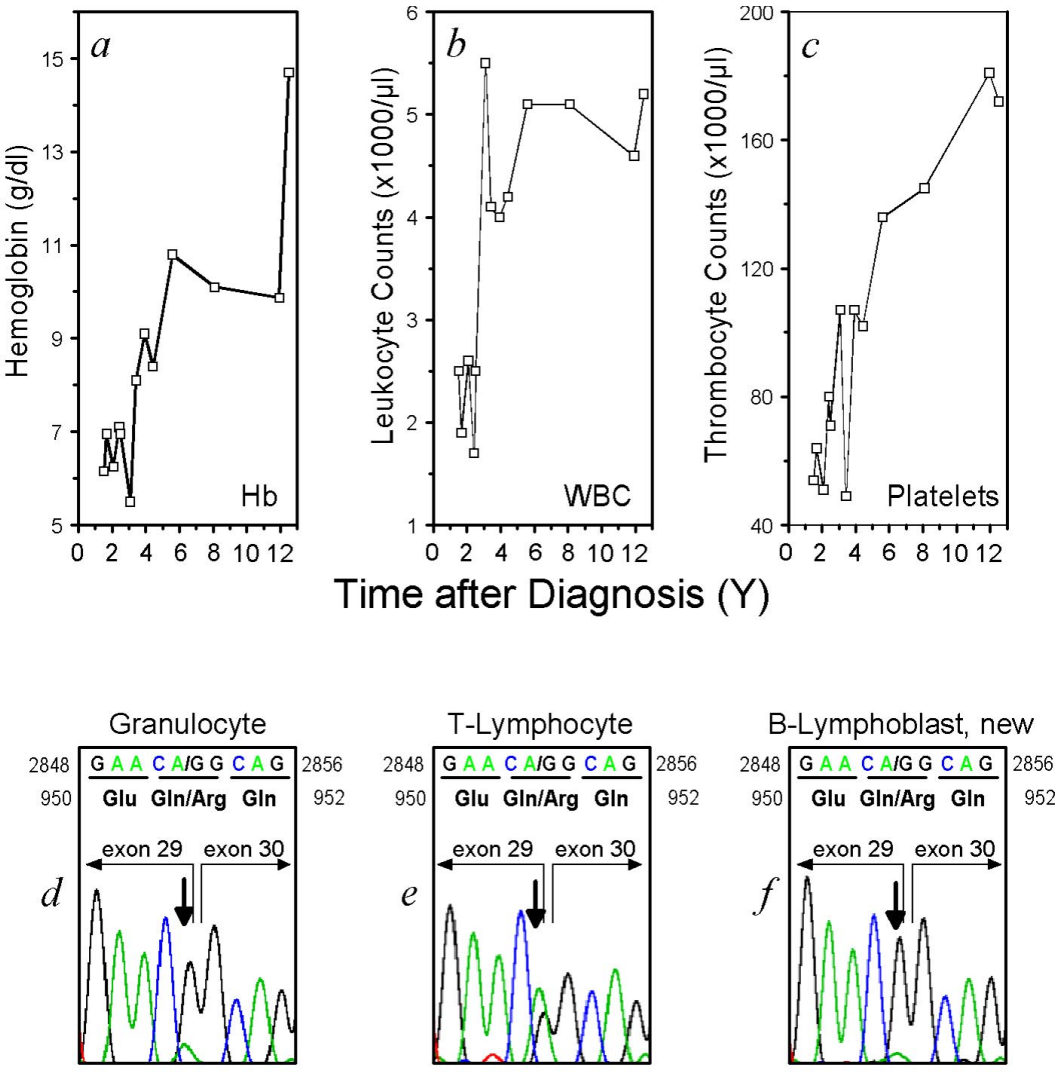


Figure 6.1: Patient EUFA0173. (a-c) Hemoglobin concentrations, leukocyte and platelet counts of PB normalized over 11 years after clinical manifestation of the pancytopenia. (d-f) Highly purified granulocytes, T-cells and EBV-transformed B-lymphoblasts show variable degrees of the reversion 2852G>A back to wildtyp.

prompted us to sequence whole blood cDNA 4.5 years after diagnosis revealing that the maternal point mutation had almost disappeared in PB while it was still present in fibroblasts. To investigate whether the reversion to WT sequence was present in the different hematopoietic lineages, leukocytes were highly purified into monocytes, T- and B-lymphocytes and individual white (CFU-GM) and red (BFU-E) colonies plucked from a standard marrow progenitor assay. DNA sequencing revealed the presence of the reversion in all lineages.

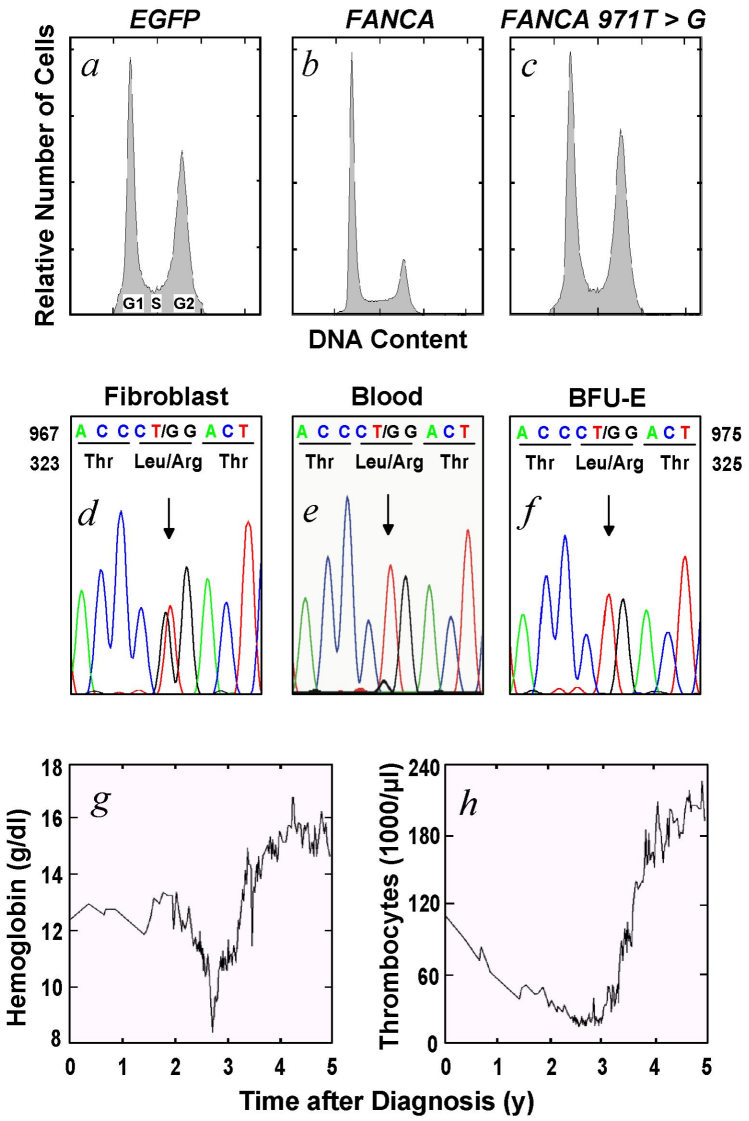


Figure 6.2: Patient UPN127. (a-c) Cell cycle analysis of FANCA-/- lymphoblasts transduced with retroviral vectors expressing the EGFP, the WT FANCA or the FANCA with 971T>G cDNAs demonstrates that the inherited point mutation abrogates the function of the FANCA protein. (d-f) Sequence analysis of patient derived cells reveals the reversion to be present in different hematological lineages, but not in skin fibroblasts. (g-h) Hemoglobin and platelet counts in the PB began to rise after starting oxymethalon almost three years after diagnosis and remained stable despite gradual reduction of oxymethalon to <0.5 mg/kg.

6.3 Discussion

In both patients, the reversions in the inherited FANCA alleles were only present in hematopoietic cells thereby demonstrating that these mutations occurred in so-

matic cells. Considering the common ontogenetic descent of granulocytes, monocytes, T- and B-lymphocytes, erythrocytes and platelets, but not skin fibroblasts, from a putative hematopoietic stem cell and considering the likelyhood of such somatic mosaicism to occur in more than one stem cell at a time, we postulate that a single normal hematopoietic stem cell spontaneously appeared after mitosis. This single corrected stem cell then was able to undergo self-renewal and also to create corrected progeny. Due to the strong selective growth advantages *in vivo* and the increased apoptosis of the mutant hematopoietic cells, corrected progeny from this single cells over time repopulated all hematopoietic lineages and cured the pancytopenia in both patients. These two fortunate experiments of nature indicate that a natural gene therapy occurs in FA, which is highly encouraging for ongoing and future gene therapy trials in this devastating disease. However, these two cases also suggest that any gene therapy approach should start early as it takes ample time for the corrected cells to impinge on the natural course of the disease.

6.4 Acknowledgements

We thank the reported patients and their parents for their participation in the present study facilitated by R. Dietrich of the German FA-familiy association. We are indebted to H. Joenje for kindly providing us with the lymphoblast line EUFA173 and the pREP4 plasmid containing the FANCA cDNA. We also thank J. Enczmann and P. Wernet for HLA typing, and U. Goebel and H. Hoehn for helpful discussions. Financial support was from the 'Deutsche Fanconi-Anaemie-Hilfe', the 'Schroeder-Kurth-Fond', the 'Bundesministerium fuer Bildung und Forschung' (Forschungsverbund Somatische Gentherapie, Koordinator Prof. Dr. Ulrich Goebel, BEO 311061), the Kinderkrebsklinik e. V. and the Fanconi Anemia Research Fund.

Chapter 7

General discussion

7.1 Mutation analysis

Mutation analysis of the past few years revealed different, but typical mutation spectra for the different FA genes. The different spectra for the most prevalent three complementation groups (FA-A, FA-C and FA-G) published until the year 2000 as well as those mutations detected in our patients until then are summarized in chapter 2. In the FANCA gene, our new mutations consist of four large deletions, five splice-site mutations, three microdeletions/-insertions, three nonsense mutations as well as one missense mutation, the pathogenicity of which has been confirmed by sequencing of 100 control alleles and by site directed mutagenesis followed by retroviral transfection (not reported in this thesis). In addition to those alterations, the new mutations detected after the year 2000 in our patients were as follows (data not shown in this thesis, Lobitz et al., manuscript in preparation): three large deletions, one splice-site mutation, one microdeletion, one nonsense and one confirmed missense mutation. The frequencies of the different types of mutation (table 7.1) as well as the mutation spectrum fit to those recently published and summarized in figure 2.1 in chapter 2.

The mutations noted in table 7.1 are dispersed throughout the FANCA gene and only two mutations could be detected twice in unrelated patients, confirming the high proportion of so-called "private mutations". The differences in the percentages of the diverse mutation types might arise from the different methods used for mutation analysis in the respective publications. For example, the percentage of large intragenic deletions in one study (Morgan et al., 1999) was 40% and thus much higher than the mean percentage in the literature (23%). This discrepancy is likely to be due to a screening method developed to detect heterozygous large deletions. There are two further missense substitutions at our laboratory still waiting to be confirmed as pathogenic mutations, which would abolish the differences in the proportion of missense mutations.

Mutation type	Mutation spectrum in literature [%]	New mutations at Würzburg [%]	Resulting new mutation spectrum [%]
Large deletions	**23%** (28/122)	30,5% (7/23)	**24%** (35/145)
Microdeletions/ -insertions	**33%** (40/122)	17,5% (4/23)	**30%** (44/145)
Splice-site mutations	**16,5%** (20/122)	26% (6/23)	**18%** (26/145)
Nonsense mutations	**12%** (15/122)	17,5% (4/23)	**13%** (19/145)
Missense mutations	**15,5%** (19/122)	8,5% (2/23)	**15%** (21/145)

Table 7.1: Comparison of all FANCA mutations found in literature with those identified at Würzburg.(This table only reflects the mutation spectrum, that means it does not consider founder mutations and frequencies.)

For FANCG, there are only two publications dealing with mutations, one of which contains the results of mutation analysis at Würzburg as well as those from other groups (chapter 4 = (Demuth et al., 2000)). It can be stated that to date no large deletions have been found in that gene, and that 94% of the mutations identified result in a truncated protein compared to 81% in FANCA and 75% in FANCC.

Interestingly, the types of mutation identified in the FANCC gene at Würzburg are quite different from those described in the literature. Pathogenic alterations published to date are clustered in both the amino- and the carboxy-terminal region as well as around exons 5-6 suggesting mutation hot spot regions (Strathdee et al., 1992; Gibson et al., 1993b; Gibson et al., 1996; Whitney et al., 1993; Verlander et al., 1994; Lo Ten Foe et al., 1996c; Lo Ten Foe et al., 1996a; Lo Ten Foe et al., 1998). We found new mutations in exons 7, 12 and 14 as well as in introns 9 and 11, rendering a clustered mutation distribution questionable. In contrast to the literature, we were unable to detect nonsense mutations in our FANCC patients, but found the first large deletion in the FANCC gene (table 7.2).

Compared to the literature the percentage of large deletions increases from 0 to 6% and demonstrates that intragenic deletions should be considered in mutation analysis of FANCC when a second mutation is not found by conventional SSCP screening. The newly detected pathogenic missense mutations (L423P and T529P) are likely to disrupt secondary structure in this region since they are found next to L554P and L496R in the 3´-region of FANCC suggesting the impairment of a functional domain. Since these mutations are all unable to abolish MMC-hypersensitivity and to complement FA-C cells after retroviral transfection (chapter 3), their pathogenicity is firmly established.

Mutation type	Mutation spectrum in literature [%]	New mutations at Würzburg [%]	Resulting new mutation spectrum [%]
Large deletions	**0%** (0/10)	14,5% (1/7)	**6%** (1/16)
Microdeletions/ -insertions	**20%** (2/10)	28,5% (2/7)	**25%** (4/16)
Splice-site mutations	**10%** (1/10)	28,5% (2/7)	**19%** (3/16)
Nonsense mutations	**50%** (5/10)	0% (0/7)	**31%** (5/16)
Missense mutations	**20%** (2/10)	28,5% (2/7)	**25%** (4/16)

Table 7.2: Comparison of all FANCC mutations found in literature with those identified at Würzburg. (This table only reflects the mutation spectrum, that means it does not consider founder mutations and frequencies.)

7.2 Identification of pathogenic missense mutations by retroviral transfection

Within the framework of this thesis, mutation analysis was carried out in the FANCA, FANCC and FANCG gene and mainly based on SSCP analysis ("**S**ingle **S**trand **C**onformational **P**olymorphism"). Therefore, the spectrum of identified mutations contains many missense mutations, the pathogenicity of which has always to be tested. Generally, there are four different proceedings used for pathogenicity confirmation:

1. Detection of further, conclusively pathogenic mutations
2. Screening of at least 100 ethnically matched control alleles
3. Cosegregation with clinical phenotype
4. Complementation analysis

In coorporation with the group of Dr. H. Hanenberg (University of Düsseldorf) we used a protocol for complementation analysis of missense mutations, which is a modification of that used for determination of the complementation group of FA-patients. The protocol is as reliable as the determination of one´s complementation group and depends on the same requirements described for complementation analysis in section 1.7. The method is based on "Site directed mutagenesis" and a retrovirus-mediated transfer of the mutated cDNA into a FA cell line belonging to the same complementation group. Final testing for MMC-hypersensitivity or MMC-resistance proves the pathogenic or polymorphic character of the alteration. More detailed information about the method is given in section 3.3. Retroviral complementation analysis is more conclusive than those

methods listed under points one to three because it represents a test of the functionality of the FA protein that cannot be disturbed by nondetected mutations. The confirmation tests one to three should only be used in combination with each other, because many pathogenic mutations, such as heterozygous large deletions, remain undetected, if using the wrong detection method. Pathogenicity cannot be considered as confirmed if there is no further mutation found. 100 control alleles often are not enough to distinguish between rare polymorphisms and pathogenic mutations, especially in small ethnic groups. Cosegregation with the clinical phenotype does not always work, because the number of siblings (FA as well as non-FA) available is usually small.

Our functional test has been applied for several amino acid substitutions detected in our patients. Some of them are described in chapter 3, chapter 5 and chapter 6, others are not mentioned in this thesis, because they have not been published to date. Amongst our FA-C-patients, there were two homozygous amino acid changes, L423P and T529P, the first in a patient from Uzbekistan and the latter in a Spanish patient. There was no data available concerning consanguinity. After sequencing of the entire gene without detecting any pathogenic variants, retroviral complementation analysis revealed pathogenicity of both mutations. Amongst our mosaic FA-A-patients, two missense mutations, L324R and R951Q as well as a combination of the two alterations R951Q and E966A, were examined. L324R as well as R951Q were confirmed to be pathogenic. R951Q in combination with the second amino acid substitution E966A (a situation found in the patient´s lymphoid cell line, but not *in vivo*) caused MMC-resistance of the transfected FA-A cell lines PIR and SNE.

In order to demonstrate the sensitivity of our retroviral pathogenicity assay, some known polymorphisms were also tested (figure 7.1). The left panel of figure 7.1 shows transfection of the SNE null line with the WT vector (with and without addition of MMC) as well as transfection with a vector carrying the mutation T529P. The right panel shows the result of mock transfections and transfection with two previously documented polymorphisms, S26F and N170S. G2 phase blockage still persists after transfection with the mock vector and the T529P-containing vector, demonstrating that both are not capable of complementing MMC-hypersensitivity of the SNE cell line; the latter because of a non-functional protein and the mock virus because of its lack of FA-protein. However, transfection with the two vectors carrying the known polymorphisms and transfections with the WT vector obviously decrease G2 phase blockage to normal levels and normalize the cell cycle pattern. These examples show that the retroviral pathogenicity assay provides a definitive distinction between mutations that are pathogenic and those that are mere polymorphic variants.

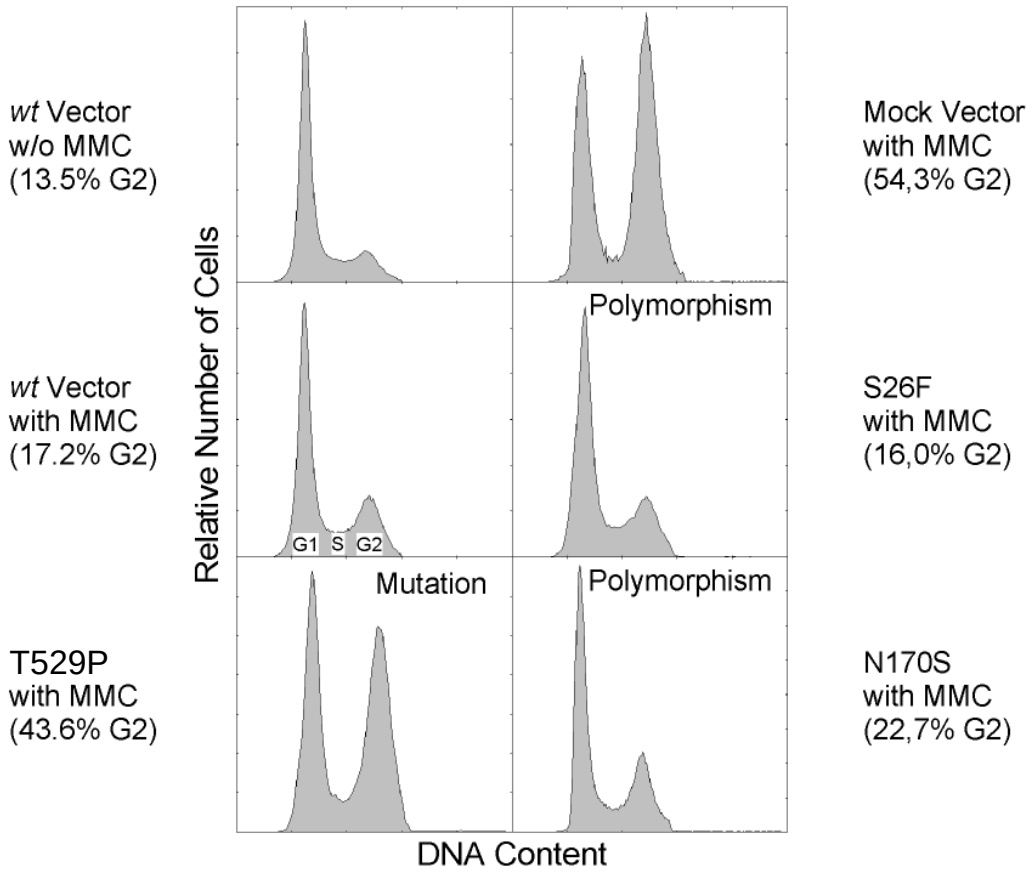


Figure 7.1: Site directed mutagenesis in FANCC vectors.

7.3 Mosaicism

Somatic mosaicism (loss or compensation of a constitutional recessive mutation) in genetic diseases might arise in replicating cells at any time of life. In order to influence the clinical course of the disease, a long-lived cell, which is capable of numerous cell divisions, must be involved. The resulting correction must provide a growth or selective advantage *in vivo*, and enough time must be allowed for the corrected cell to give rise to sufficient corrected progeny.

Somatic mosaicism has been shown to provide a selective growth advantage of the revertant hematological cells in genetic hematological disorders, such as severe combined immunodeficiency (SCID) and FA (Hirschhorn et al., 1996; Lo Ten Foe et al., 1997; Gregory et al., 2001). If the reversion occurs in a pluripotent stem cell, that situation represents an example of "spontaneous" natural gene therapy and can serve as a model for the potential success of gene therapy. Mechanisms that cause functional correction in FA lymphoblast lines in culture include somatic recombination (Lo Ten Foe et al., 1997), gene conversion (Gregory et al.,

2001; Lo Ten Foe et al., 1997), compensatory mutation ((Waisfisz et al., 1999b) and chapter 5) and back mutation (chapter 5). In each case the process results in one allele becoming functionally repaired with respect to the specific FA mutation. Peripheral blood cells or cultured lymphocytes, where one of such events has occured, have been shown to lose their cellular FA phenotype. In the case of FA, revertant mosaicism in hematopoietic stem cells has to date only be suspected, but not been proven. In order to define, where the reversion of our two FA mosaic patients, EUFAR173 and UPN127, had occured, we sorted their blood cells in order to perform lineage specific analysis (chapter 6). In EUFAR173, granulocytes and T-cells were separated and a new B-lymphoblast cell line was established; in UPN127, leukocytes were highly purified into monocytes, T- and B-lymphocytes. DNA sequencing revealed the presence of the reversion in all lineages from both patients, but not in their skin fibroblasts. Moreover, the red blood cell and platelet counts increased over the past few years. Considering the common ontogenetic descent of granulocytes, T- and B-lymphocytes, monocytes, erythrocytes and thrombocytes, but not skin fibroblasts, the reversion of the constitutional mutation must have taken place in a single hematopoietic stem cell. This self-corrected cell then was able to produce healthy progeny and repopulate all hematological lineages, thereby "curing" both patients from pancytopenia. These results seem to be encouraging for ongoing and future gene therapy trials, although to date there is no evidence for long-term correction of FA cells after somatic stem cell gene therapy with retroviral vectors expressing WT FA cDNA. One reason for the lack of success might be that mosaicism occurs in the patient´s bone marrow, which inplies that the corrected cell is located in its physiological environment. In human transplantation studies, the stem cells are harvested, extensively purified and returned intravenously. They can settle down in their physiological environment only after such extensive manipulations. It is conceivable that the bone marrow stroma cells, which still carry their FA mutations may influence repopulation of the bone marrow.

Although the above mentioned patients, as well as patients URD and STT (chapter 5), have obviously been cured from their pancytopenia after development of mosaicism, they clearly remain FA-patients. One of our mosaic patients (chapter 5) died at the age of 6 years as a consequence of cerebral haemorrhage and infections. Looking at the blood cell counts of the past years, there had been no increase, suggesting that mosaicism had not taken place in a hematopietic stem cell. It is more likely that mosaicism had been restricted to a precursor cell of the lymphoid lineage, such that it could be found in the patient´s isolated lymphoblasts by flocytometry and sequencing of the lymphoblastoid cell line. The limitation of self-correction to a single cell lineage might also explain the lack of improvement in the patient´s hematological symptoms. As pointed out in chapter 5, four of the mosaic patients showed a clear improvement of their hematological parameters during a 3 to 6 year observation period. The figure 7.2

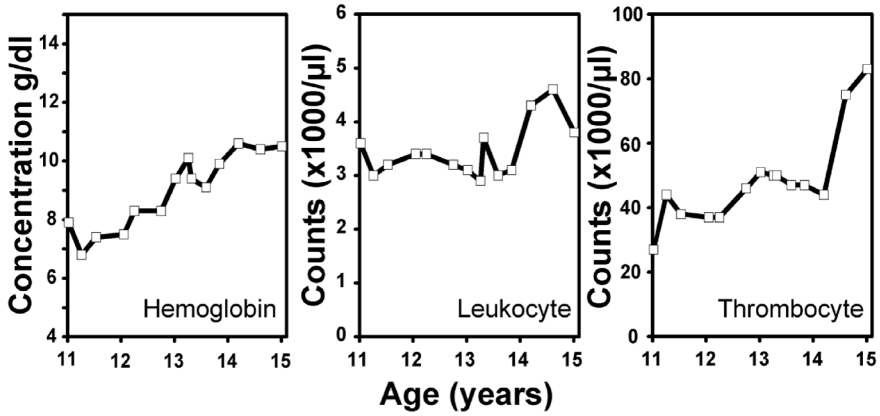


Figure 7.2: Time course of haematological parameters in patient URD.

illustrates the time course of the recovery of the hematopoietic system in one of these mosaic patients. It is quite obvious that the repopulation of the patient´s bone marrow with progeny of a (presumably single) self-corrected cell is a slow process that can take several years. However, in the end a situation arises that dramatically improves the patient´s prognosis.

Chapter 8

Summary/ Zusammenfassung

8.1 Summary

Fanconi anemia (FA) is a genetically and phenotypically heterogenous autosomal recessive disease associated with chromosomal instability, progressive bone marrow failure, typical birth defects and predisposition to neoplasia. The clinical phenotype is similar in all known complementation groups (FA-A, FA-B, FA-C, FA-D_1, FA-D_2, FA-E, FA-F and FA-G). The cellular phenotype is characterized by hypersensitivity to DNA crosslinking agents, such as MMC or DEB, which is exploited as a diagnostic tool. Alltogether, the FA proteins constitute a multiprotein pathway whose precise biochemical function(s) remain unknown. FANCA, FANCC, FANCE, FANCF and FANCG interact in a nuclear complex upstream of $FANCD_2$. Complementation group FA-D_1 was recently shown to be due to biallelic mutations in the human breast cancer gene 2 (BRCA2). After DNA damage, the nuclear complex regulates monoubiquitylation of $FANCD_2$, resulting in targeting of this protein into nuclear foci together with BRCA1 and other DNA damage response proteins. The close connection resp. identity of the FA genes and known players of the DSB repair pathways (BRCA1, BRCA2, Rad51) firmly establishs an important role of the FA gene family in the maintenance of genome integrity.

The chapter 1 provides a general introduction to the thesis describing the current knowledge and unsolved problems of Fanconi anemia. The following chapters represent papers submitted or published in scientific literature. These five chapters are succeeded by a short general discussion (chapter 7).

Mutation analysis in the Fanconi anemia genes revealed gene specific mutation spectra as well as different distributions throughout the genes. These results are described in chapter 1 and chapter 2 with main attention to the first genes identified, namely FANCC, FANCA and FANCG. In chapter 2 we provide general background on mutation analysis and we report all mutations published for

FANCA, FANCC and FANCG as well as our own unpublished mutations until the year 2000.

In chapter 3 we report a shift of the mutation spectrum previously reported for FANCC after examining ten FA-patients belonging to complementation group C. Seven of those ten patients carried at least one previously unknown mutation, whereas the other three patients carried five alleles with the Dutch founder mutation 65delG and one allele with the Ashkenazi founder mutation IVS4+4A>T, albeit without any known Ashkenazi ancestry. We also describe the first large deletion in FANCC. The newly detected alterations include two missense mutations (L423P and T529P) in the 3´-area of the FANCC gene. Since the only previously described missense mutation L554P is also located in this area, a case can be made for the existence of functional domain(s) in that region of the gene.

In chapter 4 we report the spectrum of mutations found in the FANCG gene compiled by several laboratories working on FA. As with other FA genes, most mutations have been found only once, however, the truncating mutation, E105X, was identified as a German founder mutation after haplotype analysis. Direct comparison of the murine and the human protein sequences revealed two leucine zipper motifs. In one of these the only identified missense mutation was located at a conserved residue, suggesting the leucine zipper providing an essential protein-protein interaction required for FANCG function. With regard to genotype-phenotype correlations, two patients carrying a homozygous E105X mutation were seen to have an early onset of the hematological disorder, whereas the missense mutation seems to lead to a disease with later onset and milder clinical course.

In chapter 5 we explore the phenomenon of revertant mosaicism which emerges quite frequently in peripheral blood cells of patients suffering from FA. We describe the types of reversion found in five mosaic FA-patients belonging to complementation groups FA-A and FA-C. For our single FA-C-patient intragenic crossover could be proven as the mechanism of self-correction. In the remaining four patients (all of them being compound heterozygous in FANCA), either the paternal or maternal allele has reverted back to WT sequence. We also describe a first example of *in vitro* phenotypic reversion via the emergence of a compensating missense mutation 15 amino acids downstream of the constitutional mutation explaining the MMC-resistance of the lymphoblastoid cell line of this patient.

In chapter 6 we report two FA-A mosaic patients where it could be shown that the spontaneous reversion had taken place in a single hematopoietic stem cell. This has been done by separating blood cells from both patients and searching for the reverted mutation in their granulocytes, monocytes, T- and B-lymphocytes as well as in skin fibroblasts. In both patients, all hematopoietic lineages, but not the fibroblasts, carried the reversion, and comparison to their increase in erythrocyte and platelet counts over time demonstrated that reversion must have taken place in a single hematopoietic stem cell. This corrected stem cell then has been able to undergo self-renewal and also to create a corrected progeny,

which over time repopulated all hematopoietic lineages. The pancytopenia of these patients has been cured due to the strong selective growth advantage of the corrected cells *in vivo* and the increased apoptosis of the mutant hematopoietic cells.

8.2 Zusammenfassung

Fanconi Anämie (FA) stellt eine sowohl genetisch als auch phänotypisch heterogene, autosomal rezessive Erkrankung dar. Charakteristische Merkmale dieser Erkrankung sind die chromosomale Instabilität, ein fortschreitendes Knochenmarksversagen, multiple kongenitale Abnormalitäten und eine Prädisposition zu diversen Neoplasien. Dieser klinische Phänotyp ist bei allen bisher bekannten Komplementationsgruppen (FA-A, FA-B, FA-C, FA-D_1, FA-D_2, FA-E, FA-F and FA-G) ähnlich, ebenso wie der zelluläre Phänotyp, der durch Hypersensitivität zu DNA-quervernetzenden Substanzen, wie MMC und DEB, gekennzeichnet ist. Diese Hypersensitivität wird dementsprechend in der FA-Diagnostik verwandt. Alle FA-Proteine arbeiten in einem "Multiprotein-Pathway" zusammen, dessen exakte biochemische Funktion noch nicht geklärt ist. FANCA, FANCC, FANCE, FANCF und FANCG interagieren in einem nukleären Komplex, der nach DNA-Schädigung die Monoubiquitylierung von $FANCD_2$ reguliert, woraufhin man $FANCD_2$ zusammen mit BRCA1 und anderen DNA-Reparaturproteinen in nukleären Foci detektieren kann. Die Komplementationsgruppe FA-D_1 wurde kürzlich biallelischen Mutationen im menschlichen Brustkrebsgen BRCA2 zugeordnet. Die enge Verbindung zwischen den FA-Genen und den Doppelstrangbruch(DSB)-Reparaturgenen (BRCA1, BRCA2, Rad51) deutet auf eine wichtige Rolle der FA-Genfamilie in der Erhaltung der genomischen Stabilität hin.

Kapitel 1 gibt eine allgemeine Einleitung dieser Promotionsarbeit. Es liefert Hintergrundinformationen zu Fanconi Anämie basierend auf Publikationen bis einschließlich Mai 2002. In den darauffogenden Kapiteln 2-6 sind eigene Veröffentlichungen zur Fanconi Anämie wiedergegeben, die entweder schon publiziert oder zur Veröffentlichung eingereicht worden sind. Zusätzlich zu den Diskussions-Abschitten in den einzelnen Veröffentlichungen werden diese fünf Arbeiten in Kapitel 7 kurz gemeinsam diskutiert.

Die Mutationsanalyse in den diversen FA-Genen lieferte genspezifische Mutationsspektren sowie genspezifische Mutations-Verteilungen. Diese werden in Kapitel 1 und 2 beschrieben, wobei Kapitel 2 nur auf die zuerst entdeckten FA-Gene, FANCC, FANCA und FANCG, eingeht. In Kapitel 2 werden allgemeine Hintergrundinformationen zur Mutationsanalyse geliefert und alle bis zum Jahr 2000 für FANCA, FANCC und FANCG publizierten Mutationen sowie unsere eigenen bis dato unveröffentlichten Veränderungen dargestellt.

In Kapitel 3 berichten wir über eine bemerkenswerte Verschiebung des bisher beschriebenen FANCC-Mutationsspektrums. Von den zehn von uns untersuchten FA-C-Patienten trugen acht zumindest eine neue Mutation, wohingegen die drei restlichen Patienten fünf 65delG-Allele und ein IVS4+4A>T-Allel besaßen. Interessanterweise fanden wir auch erstmals große Deletionen im FANCC-Gen, deren Existenz bisher nur für FANCA beschrieben war. Weiterhin werden zwei bisher nicht bekannte Missense Mutationen (L423P und T529P) im 3´-Bereich des Gens beschrieben. In dieser Region findet sich auch der bisher einzige pathogene Aminosäureaustausch, L554P, was auf die Existenz einer funktionellen Domäne in dieser Genregion hindeutet. Außerdem scheinen unsere neu detektierten Mutationen vielmehr verstreut im Gen vorzuliegen als dies bisher angenommen worden war. Denn die bisher beschriebenen Veränderungen betreffen vor allem den Exonbereich 5-6 sowie das amino- und carboxyterminale Ende von FANCC.

Kapitel 4 beschreibt das Mutationsspektrum für FANCG, zusammengetragen von verschiedenen FA-Arbeitsgruppen. Wie in den anderen FA-Genen traten die meisten Mutationen auch hier nur einmal auf. Allerdings konnte die trunkierende Mutation, E105X, nach einer Haplotyp-Analyse als deutsche Gründermutation beschrieben werden. Ein direkter Vergleich der Proteinsequenzen von Mensch und Maus ergab Hinweise auf konservierte Genabschnitte sowie auf zwei Leuzin-Zipper-Motive. Die einzige beschriebene Missense Mutation befindet sich in einem konservierten Bereich eines dieser beiden Leuzin-Zipper, was auf eine wichtige Rolle dieses Motivs für FANCG in Bezug auf Protein-Protein-Interaktionen schließen läßt. Obwohl die Anzahl der Patienten mit vergleichbaren Mutationen zu gering für statistisch signifikante Aussagen war, so fiel doch auf, daß bei den beiden Patienten mit einer homozygoten E105X-Mutation wesentlich früher hämatologische Probleme auftraten als bei dem Patienten mit der heterozygoten Missense Mutation, für den ein milder klinischer Verlauf sowie ein späteres Einsetzen hämatologischer Probleme berichtet wurde.

Kapitel 5 und 6 behandeln das Phänomen des reversen Mosaizismus, der sehr häufig im Blut von FA-Patienten zu diagnostizieren ist. In Kapitel 5 beschreiben wir die Reversionsmechanismen von fünf Patienten, von denen einer der Komplementationsgruppe C und die anderen vier der Komplementationsgruppe A angehören. Der Mechanismus, welcher der Selbstkorrektur des FA-C-Patienten zugrunde lag, konnte als intragene Rekombination definiert werden. Bei den verbleibenden vier compound heterozygoten FA-A-Patienten war jeweils eine Rückmutation zum Wildtyp auf dem mütterlichen bzw. väterlichen Allel ursächlich für die phänotypische Gesundung der Blutzellen. Des weiteren beschreiben wir eine *in vitro* Reversion in der lymphoblastoiden Linie eines unserer Patienten erstmals den Mechanismus einer sekundären Missense Mutation 15 Aminosäuren nach der konstitutionellen Mutation. Diese "kompensatorische" Mutation ist für die MMC-Resistenz der Zellinie verantwortlich. 4 von 5 der untersuchten Mosaik-Patienten zeigten eine eindeutige Verbesserung ihrer Blutwerte. Die Diagnose "Mosaizis-

mus" verbessert offenbar die Prognose des Krankheitsbildes vor allem dann, wenn die Reversion eines Allels in einem frühen Stadium der Hämatopoiese auftritt.

In Kapitel 6 berichten wir von zwei Mosaik-Patienten, bei denen untersucht wurde, wann in der Hämatopoiese die Reversion stattgefunden haben muß. Es konnte gezeigt werden, daß die Reversion in einer einzelnen hämatopoietischen Stammzelle erfolgte. Der Nachweis wurde durch die Isolierung einzelner Blutzelltypen, wie Granulozyten, Monozyten, T- und B-Zellen, aus dem peripheren Blut unserer Patienten sowie durch das Vorhandensein bzw. Nichtvorhandensein der Reversion in diesen Zellen geführt. Zum Vergleich wurden Hautfibroblasten herangezogen, da diese bei Mosaizismus im Blut nicht revertiert sind. In beiden Patienten trugen alle isolierten Blutzellen, nicht jedoch die Hautzellen, die Reversion. Dies und ein zusätzlicher Vergleich mit den zu diesem Zeitpunkt angestiegenen Erythrozyten- und Thrombozytenzahlen zeigten, daß die Reversion in einer einzigen hämatopoietischen Stammzelle stattgefunden haben muß. Dieser revertierten Stammzelle sind alle jeweils phänotypisch korrigierten Blutzellen zuzuschreiben, die dann die gesamte Hämatopoiese übernahmen und aufgrund eines *in vivo* Wachstumsvorteils sowie der erhöhten Apoptoserate der mutierten Zellen die Panzytopenie beider Patienten im Sinne einer "natürlichen" oder "spontanen" Gentherapie zur Ausheilung brachten.

Bibliography

Alter, B. (1995). Hematologic abnormalities in Fanconi anemia. *Blood*, 85:1148–1149.

Alter, B. and Tenner, M. (1994). Brain tumors in patients with Fanconi´s anemia. *Arch.Pediatr.Adolesc.Med.*, 148:661–663.

Alter, B. P. (1996). Fanconi´s anemia and malignancies. *Am J Hematol*, 53:99–110.

Auerbach, A. (1993). Fanconi anemia diagnosis and the diepoxybutane (DEB) test. *Exp.Hematol.*, 21:731–733.

Auerbach, A. and Allen, R. (1991). Leukemia and preleukemia in Fanconi anemia patients: a review of the literature and report of the International Fanconi Anemia Registry. *Cancer Genet.Cytogenet.*, 51:1–12.

Auerbach, A., Buchwald, M., and Joenje, H. (1997). Fanconi anemia. In Vogelstein, B. and Kinzler, K., editors, *The genetic basis of human cancer*, pages 317–332. McGraw-Hill, New York.

Auerbach, A., Buchwald, M., and Joenje, H. (1998). Fanconi anemia. In Vogelstein, B. and Kinzler, K., editors, *The genetic bases of human cancer*, volume 2. McGraw-Hill, New York.

Auerbach, A., Buchwald, M., and Joenje, H. (2001). Fanconi anemia. In Scriver, C., Beaudet, A., Sly, W., and Valle, D., editors, *The metabolic and molecular bases of inherited disease*, volume 1. McGraw-Hill, New York.

Auerbach, A. and Wolman, S. (1976). Susceptibility of Fanconi´s anemia fibroblasts to chromosome damage by carcinogens. *Nature*, 261:494–496.

Bagby, G., Segal, G., Auerbach, A., Onega, T., Keeble, W., and Heinrich, M. (1993). Constitutive and induced expression of hematopoietic growth factor genes by fibroblasts from children with Fanconi anemia. *Exp.Hematol.*, 21:1419–1426.

Bagnara, G., Bonsi, L., Strippoli, P., Ramenghi, U., Timeus, F., Bonifazi, F., Bonafe, M., Tonelli, R., Bubola, G., and Brizzi, M. (1993). Production of interleukin 6, leukemia inhibitory factor and granulocyte-macrophage colony stimulating factor by peripheral blood mononuclear cells in Fanconi´s anemia. *StemCells*, 11:137–143.

Baum, C., Hegewisch-Becker, S., Eckert, H., Stocking, C., and Ostertag, W. (1995). Novel retroviral vectors for efficient expression of the multidrug resistance (mdr-1) gene in early hematopoietic cells. *J.Virol.*, 69:7541.

Boland, C. (2001). Hereditary Nonpolyposis Colorectal Cancer (HNPCC). In Scriver, C., Beaudet, A., Sly, W., and Valle, D., editors, *The metabolic and molecular bases of inherited disease*, volume 1, pages 769–783. McGraw-Hill, New York.

Bootsma, D., Kraemer, K., Cleaver, J., and Hoeijmakers, J. (2001). Nucleotide excision repair syndromes: Xeroderma pigmentosum, Cockayne syndrome and Trichothiodystrophy. In Scriver, C., Beaudet, A., Sly, W., and Valle, D., editors, *The metabolic and molecular bases of inherited disease*, volume 1, pages 677–703. McGraw-Hill, New York.

Bullock, P., Champoux, J., and Botchan, M. (1975). Association of crossover points with topoisomerase I cleavage sites: a model for nonhomologous recombination. *Science*, 230:954–958.

Busch, D., Zdzienicka, M., Natarajan, A., Jones, N., Overkamp, W., Collins, A., Mitchell, D., Stefanini, M., Botta, E., Albert, R., Liu, N., White, D., van Gool, A., and Thompson, L. (1996). A CHO mutant, UV40, that is sensitive to diverse mutagens and represents a new complementation group of mitomycin C sensitivity. *Mutat.Res.*, 363:209–221.

Butturini, A., Gale, R., Verlander, P., Adler-Brecher, B., Gillio, A., and Auerbach, A. (1994). Hematologic abnormalities in Fanconi anemia: an international Fanconi anemia registry study. *Blood*, 84:1650–1655.

Cahill, D., Kinzler, K., Vogelstein, B., and Lengauer, C. (1999). Genetic instability and darwinian selection in tumours. *TrendsCell.Biol.*, 12:57–60.

Carreau, M., Gan, O., Liu, L., Doedens, M. McKerlie, C., Dick, J., and Buchwald, M. (1998). Bone marrow failure in the Fanconi anemia group C mouse model after DNA damage. *Blood*, 91:2737–2744.

Cassinat, B., Guardiola, P., Chevret, S., Schlageter, M., Toubert, M., Rain, J., and Gluckman, E. (2000). Constitutive elevation of serum α-fetoprotein in Fanconi anemia. *Blood*, 96:859–863.

Centra, M., Memeo, E., D´Apolito, M., Savino, M., Ianzano, L., Notarangelo, A., Liu, J., Doggett, N., Zelante, L., and Savoia, A. (1998). Fine exon-intron structure of the Fanconi anemia group A (FAA) gene and characterization of two genomic deletions. *Genomics*, 51:463–467.

Centurion, S., Kuo, H., and Lambert, W. (2000). Damage-resistant DNA synthesis in Fanconi anemia cells treated with a DNA cross-linking agent. *Exp.CellRes.*, 260:216–221.

Chen, M., Tomkins, D., Auerbach, W., McKerlie, C., Youssoufian, H., Liu, L., Gan, O., Carreau, M., Auerbach, A., Groves, T., Guidos, C., Freedman, M., Cross, J., Percy, D., Dick, J., Joyner, A., and Buchwald, M. (1996). Inactivation of Fac in mice produces inducible chromosomal instability and reduced fertility reminiscent of Fanconi anemia. *Nat.Genet.*, 12:448–451.

Cheng, N., van de Vrugt, H., van der Valk, M., Oostra, A., Krimpenfort, P., de Vries, Y., Joenje, H., Berns, A., and Arwert, F. (2000). Mice with a targeted disruption of the Fanconi anemia homolog Fanca. *Hum.Mol.Genet.*, 9:1805–1811.

Clarke, A., Philpott, N., Gordon-Smith, E., and Rutherford, T. (1997). The sensitivity of Fanconi anemia group C cells to apoptosis induced by mitomycin C is due to oxygen radical generation, not DNA crosslinking. *Br.J.Hematol.*, 96:240–247.

Cone, R. and Mulligan, R. (1984). High efficiency gene transfer into mammalian cells: generation of helper-free retroviruses with broad mammalian host range. *PNAS*, 81:6349–6353.

Cooper, D. and Krawcak, M. (1993). *Human Gene Mutation*. BIOS Scientific Publishers, Oxford.

Couch, F. and Weber, B. (2001). Breast cancer. In Scriver, C., Beaudet, A., Sly, W., and Valle, D., editors, *The metabolic and molecular bases of inherited disease*, volume 1, pages 999–1031. McGraw-Hill, New York.

Cox, P., Gibson, R., Morgen, N., and Brueton, L. (1997). VACTERL with hydrocephalus in twins due to Fanconi anemia (FA): mutation in the FAC gene. *Am.J.Med.Genet.*, 68:86–90.

Cumming, R., Lightfoot, J., Beard, K., Youssoufian, H., O´Brien, P., and Buchwald, M. (2001). Fanconi anemia group C protein prevents apoptosis in hematopoietic cells through redoxregulation of GSTP1. *Nat.Med.*, 7:814–820.

D´Andrea, A. (1996). Fanconi anemia forges a novel pathway. *Nature Genet.*, 14:240–242.

Daniel, D. (2002). Highlight: BRCA1 and BRCA2 proteins in breast cancer. *Microsc.Res.Tech.*, 59:68–83.

de Boer, J., Andressoo, J., de Wit, J., Huijmans, J., Beems, R., van Steeg, H., Weeda, G., van der Horst, G., van Leeuwen, W., Themmen, A., Meradji, M., and Hoeijmakers, J. (2001). Premature aging in mice deficient in DNA repair and transcription. *Science*, 296:1276–1279.

De Cremoux, P., Gluckman, E., Podgorniak, M., Menier, C., Thierry, D., Calvo, F., and Socie, G. (1996). Decreased IL-1 beta and TNF alpha secretion in long-term bone marrow culture supernatant from Fanconi´s anemia patients. *Eur.J.Hematol.*, 57:202–207.

De silva, I., McHugh, P., Clingen, P., and Hartley, J. (2000). Defining the roles of nucleotide excision repair and recombination in the repair of DNA interstrand cross-links in mammalian cells. *Mol.CellBiol.*, 20:7980–7990.

De Winter, J., Leveille, F., van Berkel, C., Rooimans, M., van der Weel, L., Steltenpool, J., Demuth, I., Morgan, N., Alon, N., Bosnoyan-Collins, L., Lightfoot, J., Leegwater, P., Waisfisz, Q., Komatsu, K., Arwert, F., Pronk, J., Mathew, C., Digweed, M., Buchwald, M., and Joenje, H. (2000a). Isolation of a cDNA representing the Fanconi anemia complementation group E gene. *Am.J.Hum.Genet.*, 67:1306–1308.

De Winter, J., Rooimans, M., van der Weel, L., van Berkel, C., Alon, N., Bosnoyan-Collins, L., de Groot, J., Zhi, Y., Waisfisz, Q., Pronk, J., Arwert, F., Mathew, C., Scheper, R., Hoatlin, M., Buchwald, M., and Joenje, H. (2000b). The Fanconi anemia gene FANCF encodes a novel protein with homology to ROM. *Nat.Genet.*, 24:15–16.

de Winter, J., van der Weel, L., de Groot, J., Stone, S., Waisfisz, Q., Arwert, F., Scheper, R., Kruyt, F., Hoatlin, M., and Joenje, H. (2000). The Fanconi anemia protein FANCF forms a nuclear complex with FANCA, FANCC and FANCG. *Hum.Mol.Genet.*, 9:2665–2674.

De Winter, J., Waisfisz, Q., Rooimans, M., van Berkel, C., Bosnoyan-Collins, L., Alon, N., Carreau, M., Bender, O., Demuth, I., Schindler, D., Pronk, J., Arwert, F., Hoehn, H., Digweed, M., and Buchwald, M. Joenje, H. (1998). The Fanconi anemia group G gene is identical with XRCC9. *Nat.Genet.*, 20:281–283.

Demuth, I., Wlodarski, M., Tipping, A., Morgan, N., de Winter, J., Thiel, M., Gräsl, S., Schindler, D., D´Andrea, A., Altay, C. Hülya, K., Zatterale, A.,

Kunze, J., Ebell, W., Mathew, C., Joenje, H., Sperling, K., and Digweed, M. (2000). Spectrum of mutations in the Fanconi anemia group G gene, FANCG/XRCC9. *Europ.J.Hum.Genet.*, 8:861–868.

Digweed, M. and Sperling, K. (1996). Molecular analysis of Fanconi anemia. *BioEssays*, 18:579–585.

Digweed, M., Zakrzewski-Lüdcke, S., and Sperling, K. (1988). Fanconi´s anemia: correlation of genetic complementation group with psoralen/UVA response. *Hum.Genet.*, 78:51–54.

Dronkert, M. and Kanaar, R. (2001). Repair of DNA interstrandcross-links. *Mut.Res.*, 486:217–247.

Duckworth-Rysiecki, G., Cornish, K., Clarke, C., and Buchwald, M. (1985). Identification of two complementation groups in Fanconi anemia. *Som.CellMol.Genet.*, 11:35–41.

Ellis, N., Ciocci, S., and German, J. (2001). Back mutation can produce phenotype reversion in Bloom syndrome somatic cells. *Hum.Genet.*, 108:167–173.

Ellis, N., Lennon, D., Proytcheva, M., Alhadeff, B., Henderson, E., and German, J. (1995). Somatic intragenic recombination within the mutated locus BLM can correct the high sister-chromatide exchange phenotype of Bloom syndrome cells. *Am.J.Hum.Genet.*, 57:1019–1027.

Ellis, N., Lennon, D., Proytcheva, M., Alhadeff, B., Henderson, E., and German, J. (1996). Somatic intragenic recombination within the mutated locus BLM can correct the high sister-chromatide exchange phenotype of Bloom syndrome cells. *Am.J.Hum.Genet.*, 58:254.

Faivre, L., Guardiola, P., Lewis, C., Dokal, I., Ebell, W., Zatterale, A., Altay, C., Poole, J., Stones, D., Kwee, M., van Weel-Sipman, M., Havenga, C., Morgan, N., de Winter, J., Digweed, M., Savoia, A., Pronk, J., de Ravel, T., Jansen, S., Joenje, H., Gluckman, E., and Mathew, C. (2000). Association of complementation group and mutation type with clinical outcome in Fanconi anemia. European Fanconi Anemia Research Group. *Blood*, 96:4064–4070.

Fanconi, G. (1927). Familiäre infantile perniziosartige Anämie (perniziöses Blutbild und Konstitution). *Jahrb.Kinderh.*, 117:257.

Fanconi, G. (1967). Familial constitutional panmyelocytopathy, Fanconi´s anemia (F.A.) I. Clinical aspects. *Semin.Hematol.*, 4:233–240.

Flowers, M., Zanis, J., Pasquini, R., Deeg, H., Ribeiro, R., Longton, G., Medeiros, C., Doney, K., Sanders, J., and Bryant, J. e. a. (1996). Marrow transplantation for Fanconi anemia: conditioning with reduced doses of cyclophosphamide without radiation. *Br.J.Hematol.*, 92:699–706.

Freie, B., Dutt, P., and Clapp, D. (1996). Correction of Fanconi anemia type C phenotypic abnormalities using a clinically suitable retroviral vector infection protocol. *CellTransplant*, 5:385–393.

Fu, K., Lo Ten Foe, J., Joenje, H., Rao, K., Liu, J., and Walsh, C. (1997). Functional correction of Fanconi anemia group A hematopoietic cells by retroviral gene transfer. *Blood*, 90:3296–3303.

Garcia-Higuera, I., Kuang, Y., Denham, J., and D´Andrea, A. (2000). The Fanconi anemia proteins FANCA and FANCG stabilize each other and promote the nuclear accumulation of the Fanconi anemia complex. *Blood*, 96:3224–3230.

Garcia-Higuera, I., Kuang, Y., Näf, D., Wasik, J., and D´Andrea, A. (1999). Fanconi anemia proteins FANCA, FANCC and FANCG/XRCC9 interact in a functional nuclear complex. *Mol.Cell.Biol.*, 19:4866–4873.

Garcia-Higuera, I., Taniguchi, T., Ganesan, S., Meyn, M., Timmers, C., Hejna, J., Grompe, M., and D´Andrea, A. (2001). Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. *Mol.Cell*, 7:249–262.

Gatti, R. (1998). Ataxia telangiectasia. In Vogelstein, B. and Kinzler, K., editors, *The genetic basis of human cancer*, pages 275–300. McGraw-Hill, New York.

Gatti, R. (2001). The inherited basis of human radiosensitivity. *Acta Oncol*, 40:702–711.

Gavish, H., Dos Santos, C., and Buchwald, M. (1993). Leu554Pro substitution completely abolishes complementing activity of the Fanconi anemia (FAC) protein. *Hum.Mol.Genet.*, 2:123–126.

German, J. and Ellis, N. (2001). Bloom syndrome. In Scriver, C., Beaudet, A., Sly, W., and Valle, D., editors, *The metabolic and molecular bases of inherited disease*, volume 1, pages 733–751. McGraw-Hill, New York.

Giampietro, P., Adler-Brecher, B., Verlander, P., Pavlakis, S., Davis, J., and Auerbach, A. (1993). The need for more accurate and timely diagnosis in Fanconi anemia: a report from the International Fanconi Anemia Registry. *Pediatrics*, 91:1116–1120.

Giampietro, P., Verlander, P., Davis, J., and Auerbach, A. (1997). Diagnosis of Fanconi anemiain patients without congenital malformations: an international Fanconi anemia registry study. *Am.J.Med.Genet.*, 68:58–61.

Gibson, R., Buchwald, M., Roberts, R., and Mathew, C. (1993a). Characterization of the exon structure of the Fanconi anemia group C gene by vectorette PCR. *Hum.Mol.Genet.*, 2:35–38.

Gibson, R., Hajianpour, A., Murer-Orlando, M., Buchwald, M., and Mathew, C. (1993b). A nonsense mutation and exon skipping in the Fanconi anemia group C gene. *Hum.Mol.Genet.*, 2:797–799.

Gibson, R., Morgan, N., Goldstein, L., Pearson, I., Kesterton, I., Foot, N., Jansen, S., Havenga, C., Pearson, T., de Ravel, T., Cohn, R., Marques, I., Dokal, I., Roberts, I., Marsh, J., Ball, S., Milner, R., Llerena, J., Samochatova, E., Mohan, S., Vasudevan, P., Birjandi, F., Hajianpour, A., Murer-Orlando, M., and Mathew, C. (1996). Novel mutations and polymorphisms in the Fanconi anemia group C gene. *Hum.Mutat.*, 8:140–148.

Gillio, A., Verlander, P., Batish, S., Giampietro, P., and Auerbach, A. (1997). Phenotypic consequences of mutations in the Fanconi anemia FAC gene: an International Fanconi Anemia Registry study. *Blood*, 90:105–110.

Glanz, A. and Fraser, F. (1982). Spectrum of anomalies in Fanconi anemia. *J.Med.Genet.*, 19:412–416.

Gottlieb, B., Beitel, L., and Trifiro, M. (2001). Somatic mosaicism and variable expressivity. *TrendsGenet.*, 17:79–82.

Gregory, J., Wagner, J., Verlander, P., Levran, O., Batish, S., Eide, C., Steffenhagen, A., Hirsch, B., and Auerbach, A. (2001). Somatic mosaicism in Fanconi anemia: evidence of genotypic reversion in lymphohematopoietic stem cells. *PNAS*, 98:2532–2537.

Grompe, M. and D´Andrea, A. (2001). Fanconi anemia and DNA repair. *Hum.Mol.Genet.*, 10:2253–2259.

Gruenert, D. and Cleaver, J. (1985). Repair of proralen-induced cross-links and monoadducts in normal and repair-deficient human fibroblasts. *CancerRes.*, 45:5399–5404.

Guardiola, P., Socie, G., Pasquini, R., Dokal, I., Ortega, J., van Weel-Sipman, M., Marsh, J., Locatelli, F. Souillet, G., Cahn, J., Ljungman, P., Miniero, R., Shaw, J., Vermylen, C., Archimbaud, E., Bekassy, A., Krivan, G., Di Bartolomeo, P., Bacigalupo, A., and Gluckman, G. (1998). Allogenic stem cell transplantation for Fanconi anemia. severe Aplastic Anemia Working Party

of the EBMT and EUFAR. european group for blood and marrow transplantation. *Bone Marrow Transplant*, 21:24–27.

Gush, K., Fu, K., Grompe, M., and Walsh, C. (1999). Phenotypic correction of Fanconi anemia group C knockout mice. *Blood*, 95:700–704.

Haaf, T., Steinlein, K., and Schmid, M. (1986). Preferential somatic pairing between homologous heterochromatic regions of human chromosomes. *Am.J.Hum.Genet.*, 38:319–329.

Haneline, L., Gobbett, T., Ramani, R., Carreau, M., Buchwald, M., Mervin, C., and Clapp, D. (1999). Loss of FancC function results in decreased hematopoietic stem cell repopulation ability. *Blood*, 94:1–8.

Hanenberg, H., Batish, S., Pollok, K., Vieten, L., Verlander, P., Leurs, C., Cooper, R., Göttsche, K., Haneline, L., Clapp, D., Lobitz, S., Williams, D., and Auerbach, A. (2002). Phenotypic correction of primary Fanconi anemia T cells with retroviral vectors as a diagnostic tool. *Exp.Hematol.*, 30:1–11.

Hanenberg, H., Hashino, K., Konishi, H., Hock, R., Kato, I., and Williams, D. (1997). Optimization of fibronectin-assisted retroviral gene transfer into human CD34+ hematopoietic cells. *Hum.GeneTher.*, 8:2193–2206.

Hanenberg, H., Xiao, X., Dilloo, D., Hashino, K., Kato, I., and Williams, D. (1996). Colocalization of retrovirus and target cells on specific fibronectin fragments increases genetic transduction of mammalian cells. *Nat.Med.*, 2:876–882.

Hawley, R., Lieu, F., Fong, A., and Hawley, T. (1994). Versatile retroviral vectors for potential use in gene therapy. *Gene Therapy*, 1:136–138.

Heinrich, M., Silvey, K., Stone, S., Zigler, A., Griffith, D., Montalto, M., Chai, L., Zhi, Y., and Hoatlin, M. (2000). Posttranscriptional cell cycle-dependent regulation of human FANCC expression. *Blood*, 95:3970–3977.

Hildinger, M., Eckert, H., Schilz, A., John, J., Ostertag, W., and Baum, C. (1998). FMEV vectors: both retroviral long terminal repeat and leader are important for high expression in transduced hematopoietic cells. *GeneTher*, 5:1575.

Hirschhorn, R., Yang, D., Puck, J., Huie, M., Jiang, C.-K., and Kurlandsky, E. (1996). Spontaneous in vivo reversion to normal of an inherited mutation in a patient adenosine deaminase deficiency. *Nat.Genet.*, 13:290–295.

Hirschhorn, R., Young, D., Israni, A., Huie, M., and Ownby, D. (1994). Somatic mosaicism for a newly identified splice-site mutation in a patient with adenosine deaminase-deficient immunodeficiency and spontaneous clinical recovery. *Am.J.Hum.Genet.*, 55:59–68.

Hoatlin, M., Christianson, T., Keeble, W., Hammond, A., Zhi, Y., Heinrich, M., Tower, P., and Bagby, G. (1998). The Fanconi anemia group C gene product is located in both the nucleus and cytoplasm of human cells. *Blood*, 91:1418–1425.

Hoshino, T., Wang, J., Devetten, M., Iwata, N., Kajigaya, S., Wise, R., Liu, J., and Youssoufian, H. (1998). Molecular chaperone GRP94 binds to the Fanconi anemia group C protein and regulates ist intracellular expression. *Blood*, 91:4379–4386.

Howlett, N., Taniguchi, T., Olson, S., Cox, B., Waisfisz, Q., de Die-Smulders, C., Persky, N., Grompe, M., Joenje, H., Pals, G., Ikeda, H., Fox, E., and D´Andrea, A. (2002). Biallelic inactivation of BRCA2 in Fanconi anemia. *Science*, 297:606–609.

Huber, P., Medhurst, A., Youssoufian, H., and Mathew, C. (2000). Investigation of Fanconi anemia protein interactions by yeast two-hybrid analysis. *Biochem.Biophys.Res.Commun.*, 268:73–77.

Huff, V., Jaffe, N., Saunders, G., Strong, L., Villalba, F., and Ruteshouser, E. (1995). WT1 exon 1 deletion/insertion mutations in Wilms tumor patients, associated with di- and trinucleotide repeats and deletion hotspot consensus sequences. *Am.J.Hum.Genet.*, 56:84–90.

Ianzano, L., D´Apolito, M., Centra, M., Savino, M., Levran, O., Auerbach, A., Cleton-Jansen, A., Doggett, N., Pronk, J., Tipping, A., Gibson, R., Mathew, C., Whitmore, S., Apostolou, S., Callen, D., Zelante, L., and Savoia, A. (1997). The genomic organization of the Fanconi anemia group A (FAA) gene. *Genomics*, 41:309–314.

Ishida, R. and Buchwald, M. (1982). Susceptibility of Fanconi´s anemia lymphoblasts to DNA cross-linking and alkylating agents. *Cancer Res.*, 42:4000–4006.

Jakobs, P., Fiddler-Odell, E., Reifsteck, C., Olson, S., Moses, R., and Grompe, M. (1997). Complementation grous assignments in Fanconi anemia fibroblast cell lines from North America. *Somat.Cell/Mol.Genet.*, 23:1–7.

Joenje, H. (1996). Fanconi anemia complementation groups in Germany and the Netherlands. European Fanconi Anemia Research Group. *Hum.Genet.*, 97:280–282.

Joenje, H., Arwert, F., Eriksson, A., de Koning, H., and Oostra, A. (1981). Oxygen-dependence of chromosomal aberrations in Fanconi´s anemia. *Nature*, 290:142–143.

Joenje, H., Arwert, F., Kwee, M., Madan, K., and Hoehn, H. (1998). Confounding factors in the diagnosis of Fanconi anemia. *Am.J.Med.Genet.*, 79:403–405.

Joenje, H., Levitus, M., Waizfisz, Q., D´Andrea, A., Garcia-Higuera, I., Pearson, T., van Berkel, C., Rooimans, M., Morgan, N., and Mathew, C. (2000). Complementation analysis in Fanconi anemia: assignment of the reference FA-H patient to group A. *Am.J.Hum.Genet.*, 67:759–762.

Joenje, H., Lo Ten Foe, J., Oostra, A., van Berkel, C., Rooimans, M., Schroeder-Kurth, T., Wegner, R., Gille, J., Buchwald, M., and Arwert, F. (1995a). Classification of Fanconi anemia patients by complementation analysis: evidence for a fifth genetic subtype. *Blood*, 86:2156–2160.

Joenje, H. and Oostra, A. (1986). Oxygen-induced cytogenetic instability in normal human lymphocytes. *Hum.Genet.*, 74:438–440.

Joenje, H., Oostra, A., Wijker, M., di Summa, F., van Berkel, C., Rooimans, M., Ebell, W., van Weel, M., Pronk, J., and Buchwald, M. (1997). Evidence for at least eight Fanconi anemia genes. *Am.J.Hum.Genet.*, 61:940–944.

Joenje, H. and Patel, K. (2001). The emerging genetic and molecular basis of Fanconi anemia. *Nat.Rev.Genet.*, 2:446–457.

Joenje, H., Youssoufian, H., Kruyt, F., Dos Santos, C., Wevrick, R., and Buchwald, M. (1995b). Expression of the Fanconi anemia gene FAC in human cell lines: lack of effect of oxygen tension. *BloodCellsMol.Dis.*, 21:182–191.

Jonkman, M. (1999). Revertant mosaicism in human genetic disorders. *Am.J.Med.Genet.*, 85:361–364.

Jonkman, M., Scheffer, H., Stulp, R., Pas, H., Nijenhuis, M., Heeres, K., Owaribe, K., Pulkkinen, L., and Uitto, J. (1997). Revertant mosaicism in epidermolysis bullosa caused by mitotic gene conversion. *Cell*, 88:543–551.

Kennedy, A. and Hart, W. (1982). Multiple squamous cell carcinoma in Fanoni´s anemia. *Cancer*, 50:811–814.

Kinzler, K. and Vogelstein, B. (1998). Familial cancer syndromes: the role of caretakers and gatekeepers. In Vogelstein, B. and Kinzler, K., editors, *The genetic basis of human cancer*, pages 241–242. McGraw-Hill, New York.

Klocker, H., Burtscher, H., Auer, B., Hirsch-Kaufmann, M., and Schweiger, M. (1985). Fibroblasts from patients with Fanconi´s anemia are not deficient in excision of thymine dimer. *Europ.J.Cell.Biol.*, 37:240–242.

Koh, P., Hughes, P., Faulkner, G., Keeble, W., and Bagby, G. (1999). 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.*, 27:1–8.

Krasnoshtein, F. and Buchwald, M. (1996). Developmental expression of the Fac gene correlates with congenital defects in Fanconi anemia patients. *Hum.Mol.Genet.*, 5:85–93.

Krawczak, M. and Cooper, D. (1991). Gene deletions causing human genetic disease: mechanisms of mutagenesis and the role of the local DNA sequence. *Hum.Genet.*, 86:425–441.

Kruyt, F., Abou-Zahr, F., Mok, H., and Youssoufian, H. (1998a). Resistance to mitomycin C requires direct interaction between the Fanconi anemia proteins FANCA and FANCG in the nucleus through an arginine-rich domain. *Europ.J.Hum.Genet.*, 6:501–508.

Kruyt, F., Dijkmans, L., Arwert, F., and Joenje, H. (1997). Involvement of the Fanconi anemia protein FAC in a pathway that signals to the cyclin B/cdc2 kinase. *CancerRes.*, 57:2244–2251.

Kruyt, F., Hoshino, T., Liu, J., Joseph, P., Jaiswal, A., and Youssoufian, H. (1998b). Abnormal microsomal detoxification implicated in Fanconi anemia group C by interaction of the FAC protein with NADPH cytochrome P450 reductase. *Blood*, 92:3050–3056.

Kruyt, F. and Youssoufian, H. (1998). The Fanconi anemia proteins FAA and FAC function in different cellular compartments to protect against cross-linking agent cytotoxicity. *Blood*, 92:2229–2236.

Kubbies, M., Schindler, D., Hoehn, H., Schinzel, A., and Rabinovitch, P. (1985). Endogeneous 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.*, 37:1022–1030.

Kunze, J. (1980). Estren-Dameshek. Anämie mit myelomonozytärer Leukämie (Subtyp der Fanconi-Anämie?). In Tolksdorf, M. and Spranger, J., editors, *Klinische Genetik in der Pädiatrie*, pages 213–214. Springer, Stuttgart.

Kupfer, G., Näf, D., Suliman, A., Pulsipher, M., and D´Andrea, A. (1997a). The Fanconi anemia proteins, FAA and FAC, interact to form a nuclear complex. *Nat.Genet.*, 17:487–490.

Kupfer, G., Yamashita, T., Näf, D., Suliman, A., Asano, S., and D´Andrea, A. (1997b). The Fanconi anemia polypeptide, FAC, binds to the cyclin-dependent kinase, cdc2. *Blood*, 90:1047–1054.

Kutler, D., Sing, B., Satagopan, J., Batish, S., Berwick, M., Giampetro, P., Hanenberg, H., and Auerbach, A. (2002). A 20 year perspective of the international Fanconi anemia registry (IFAR). *Blood*.

Kvittingen, E., Rootwelt, H., Berger, R., and Brandtzaeg, P. (1994). Self-induced correction of the genetic defect in tyrosinemia type I. *J.Clin.Invest.*, 94:1657–1661.

Levran, O., Doggett, N., and Auerbach, A. (1998). Identification of Alu-mediateddeletions in the Fanconi anemia gene FAA. *Hum.Mutat.*, 12:145–152.

Levran, O., Erlich, T., Magdalena, N., Gregory, J., Batish, S., Verlander, P., and Auerbach, A. (1997). Sequence variation in the Fanconi anemia gene FAA. *PNAS*, 94:13051–13056.

Liu, N., Lamerdin, J., Tucker, J., Zhou, Z., Walter, C., Albala, J., Busch, D., and Thompson, L. (1997). The human XRCC9 gene corrects chromosomal instability and mutagen sensitivities in CHO UV40 cells. *PNAS*, 94:9232–9237.

Lo Ten Foe, J., Barel, M., Thuss, P., Digweed, M., Arwert, F., and Joenje, H. (1996a). Sequence variations in the Fanconi anemia gene, FAC: pathogenicity of 1806insA and R548X and recognition of D195V as a polymorphic variant. *Hum.Genet.*, 98:522–523.

Lo Ten Foe, J., Kruyt, F., Zweekhorst, M., Pals, G., Gibson, R., Mathew, C., Joenje, H., and Arwert, F. (1998). Exon 6 skipping in the Fanconi anemia C gene associated with a nonsense/missense mutation (775C>T) in exon 5: the first example of a nonsense mutation in one exon causing skipping of another downstream. *Hum.Mutat.*, 1:25–27.

Lo Ten Foe, J., Kwee, M., Rooimans, M., Oostra, A., Veerman, A., van Weel, M., Pauli, R., Shahidi, N., Dokal, I., Roberts, I., Altay, C., Gluckman, E., Gibson, R., Mathew, C., Arwert, F., and Joenje, H. (1997). Somatic mosaicism in Fanconi anemia: molecular basis and clinical significance. *Eur.J.Hum.Genet.*, 5:137–148.

Lo Ten Foe, J., Rooimans, M., Bosnoyan-Collins, L., Alon, N., Wijker, M., Parker, L., Lightfoot, J., Carreau, M., Callen, D., and Savoia, A. (1996b). Expression cloning of a cDNA for the major Fanconi anemia gene, FAA. *Nat.Genet.*, 14:320–323.

Lo Ten Foe, J., Rooimans, M., Joenje, H., and Arwert, F. (1996c). Novel frameshift mutation (1806insA) in exon 14 of the Fanconi anemia C gene, FAC. *Hum.Mutat.*, 7:264–265.

MacNeill, E., Hanenberg, H., Pollok, K., van der Loo, J., Bierhuizen, M., Wagemaker, G., and Williams, D. (1999). Simultaneous infection with retroviruses pseudotyped with different envelope proteins bypasses viral receptor interference associated with colocalization of gp70 and target cells on fibronectin CH-296. *J.Virol.*, 73:3960–3967.

Maniatis, T., Fritsch, E., and Sambrook, J. (1982). *Molecular cloning (A Laboratory Manual)*. Cold Spring Harbour Laboratory, New York.

Mann, R., Mulligan, R., and Baltimore, D. (1983). Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell*, 33:153–158.

Medhurst, A., Huber, P., Waisfisz, Q., de Winter, J., and Mathew, C. (2001). Direct interactions of the five known Fanconi anemia proteins suggest a common functional pathway. *Hum.Mol.Genet.*, 10:423–429.

Miglierina, R., Le Coniat, M., and Berger, R. (1991). A simple diagnostic test for Fanconi anemia by flowcytometry. *Anal.CellPath.*, 3:111–114.

Miller, A., Garcia, J., von Suhr, N., Lynch, C., Wilson, C., and Eiden, M. (1991). Construction and properties of retrovirus packaging cells based on gibbon ape leukemia virus. *J.Virol.*, 65:2220–2224.

Miller, A. and Rosman, G. (1989). Improved retroviral vectors for gene transfer and expression. *BioTechniques*, 7:980–990.

Morgan, N., Tipping, A., Joenje, H., and Mathew, C. (1999). High frequency of large intragenic deletions in the Fanconi anemia group A gene. *Am.J.Hum.Genet.*, 65:1330–1341.

Näf, D., Kupfer, G., Suliman, A., Lambert, K., and D´Andrea, A. (1998). Functional activity of the Fanconi anemia protein FAA requires FAC binding and nuclear localization. *Mol.Cell.Biol.*, 18:5952–5960.

Noll, M., Bateman, R., D´Andrea, A., and Grompe, M. (2001). Preclinical protocol for in vivo selection of hematopoietic stem cells corrected by gene therapy in Fanconi anemia group C. *Mol.Ther.*, 3:14–23.

Pang, Q., Fagerlie, S., Christianson, T., Faulkner, G., Diaz, J., Rathbun, R., and Bagby, G. (2000). The Fanconi anemia protein FANCC binds to and facilitates the activation of STAT1 by gamma interferon and hematopoietic growth factors. *Mol.CellBiol.*, 20:4724–4735.

Paull, T., Rogakou, E., Yamazaki, V., Kirchgessner, C., Gellert, M., and Bonner, W. (2000). A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. *Curr.Biol.*, 10:886–895.

Paulovich, A., Toczyski, D., and Hartwell, L. (1997). When checkpoints fail. *Cell*, 7:315–321.

Pollok, K., Hanenberg, H., Noblitt, T., Schroeder, W., Kato, I., Emanuel, D., and Williams, D. (1998). High-efficiency gene transfer into normal and adenosine deaminase-deficient T lymphocytes is mediated by transduction on recombinant fibronectin fragments. *J.Virol.*, 72:4882–4892.

Poot, M., Hoehn, H., Kubbies, M., Grossmann, A., Chen, Y., and Rabinovitch, P. (1994). Cell-cycle analysis using continuous bromodeoxyuridine labeling and Hoechst 33358-ethidium bromide bivariate flow cytometry. *Methods-Cell.Biol.*, 41:327–340.

Potter, N., Sarmousakis, C., and Li, F. (1983). Cancer in relatives of patients with aplastic anemia. *Cancer Genet.Cytogenet.*, 9:61–65.

Pronk, J., Gibson, R., Savoia, A., Wijker, M., Morgan, N., Melchionda, S., Ford, D., Temtamy, S., Ortega, J., Jansen, S., Havenga, C., Cohn, R., de Ravel, T., Roberts, I., Westerveld, A., Easton, D., Joenje, H., Mathew, C., and Arwert, F. (1995). Localization of the Fanconi anemia complementation group A gene to chromosome 16q24.3. *Nat.Genet.*, 11:338–340.

Rathbun, R., Faulkner, G., Ostroski, M., Christianson, T., Hughes, G., Jones, G., Cahn, R., Maziarz, R., Royle, G., Keeble, W., Heinrich, M., Grompe, M., Tower, P., and Bagby, G. (1997). Inactivation of the Fanconi anemia group C gene augments interferon-gamma-induced apoptotic responses in hematopoietic cells. *Blood*, 90:974–985.

Reuter, T., Herterich, S., Bernhard, O., Hoehn, H., and Gross, H. (2000). Strong FANCA/FANCG, but weak FANCA/FANCC interaction in the yeast 2-hybrid system. *Blood*, 95:719–720.

Rieger, R., Michaelis, A., and Green, M. (1976). *Glossary of genetics and cytogenetics: Classical and molecular*. Springer, Berlin, Heidelberg, New York, fourth edition.

Rischewski, J., Clausen, H., Leber, V., Niemeyer, C., Ritter, J., Schindler, D., and Schneppenheim, R. (2000). A heterozygous frameshift mutation in the Fanconi anemia C gene in familial T-ALL and secondary malignancy. *Klin.Padiatr.*, 212:174–176.

Roest, P., Roberts, R., Sugino, S., van Ommen, G., and Dunnen, J. (1993). Protein truncation test (PTT) for rapid detection of translation-terminating mutations. *Hum.Mol.Genet.*, 2:1719–1721.

Rosselli, F., Sanceau, J., Gluckman, E., Wietzerbin, J., and Moustacchi, E. (1994). 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*, 83:1216–1225.

Rosselli, F., Sanceau, J., Wietzerbin, J., and Moustacchi, E. (1992). Abnormal lymphokine production: a novel feature of the genetic disease Fanconi anemia. I. involvement of interleukin-6. *Hum.Genet.*, 89:42–48.

Ruppitsch, W., Meisslitzer, C., Hirsch-Kauffmann, M., and Schweiger, M. (1998). Overexpression of thioredoxin in Fanconi anemia fibroblasts prevents the cytotoxic and DNA damaging effect of mitomycin C and diepoxybutane. *FEBS Lett*, 422:99–102.

Saar, K., Schindler, D., Wegner, R., Reis, A., Wienker, T., Hoehn, H., Joenje, H., Sperling, K., and Digweed, M. (1998). Localization of a Fanconi anemia gene to chromosome 9p. *Europ.J.Hum.Genet.*, 6:501–508.

Saiki, R., Gelfand, D., Stoffel, S., Scharf, S., Higuchi, R., Horn, G., Mullis, K., and Ehrlich, H. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, 239:487–491.

Sala-Trepat, M., Rouillard, D., Escarceller, M., Laquerbe, A., Moustacchi, E., and Papadopoulo, D. (2000). Arrest of S-phase preogression is impaired in Fanconi anemia cells. *Exp.CellRes.*, 260:208–215.

Sasaki, M. and Tonomura, A. (1973). A high susceptibility of Fanconi´s anemia to chromosome breakage by DNA cross-linking agents. *Cancer Res.*, 33:1829–1836.

Savino, M., Ianzano, L., Strippoli, P., Ramenghi, U., Arslanian, A., Bagnara, G., Joenje, H., Zelante, L., and Savoia, A. (1997). Mutations of the Fanconi anemia group A gene (FAA) in Italian patients. *Am.J.Hum.Genet.*, 61:1246–1253.

Savoia, A., Centra, M., Ianzano, L., de Cillis, G., Zelante, L., and Buchwald, M. (1995). Characterization of the 5´region of the Fanconi anemia group C (FACC) gene. *Hum.Mol.Genet.*, 4:1321–1326.

Savoia, A., Garcia-Higuera, I., and D´Andrea, A. (1999). Nuclear localization of the Fanconi anemia protein FANCC is required for functional activity. *Blood*, 93:4025–4026.

Schellenberg, G., Miki, T., Yu, C.-E., and Nakura, J. (2001). Werner syndrome. In Scriver, C., Beaudet, A., Sly, W., and Valle, D., editors, *The metabolic and molecular bases of inherited disease*, volume 1, pages 785–797. McGraw-Hill, New York.

Schindler, D. and Hoehn, H. (1988). Fanconi anemia mutation causes cellular susceptability to ambient oxygen. *Am.J.Hum.Genet.*, 43:429–435.

Schmid, W. and Fanconi, G. (1978). Fragility and spralization anomalies of the chromosomes in three cases, including fraternal twins, with Fanconi´s anemia, type Estren-Dameshek. *Cytogenet.Cell.Genet.*, 20:141–149.

Schroeder, T., Anschütz, F., and Knopp, A. (1964). Spontane Chromosomenaberrationen bei familiärer Panmyelopathie. *Humangenetik*, 1:194–196. Abstract.

Schroeder, T., Tilgen, D., Kruger, J., and Vogel, F. (1976). Formal genetics of Fanconi´s anemia. *Hum.Genet.*, 32:257–288.

Schroeder-Kurth, T., Auerbach, A., and Obe, G. (1989). *Fanconi anemia. Clinical, cytogenetic and experimental aspects*. Springer-Verlag, Heidelberg New York.

Schultz, J. and Shahidi, N. (1993). Tumor necrosis factor-alpha overproduction in Fanconi´s anemia. *Am.J.Hematol.*, 42:196–201.

Scully, R., Chen, J., Ochs, R., Keegan, K., Hoekstra, M., Feunteun, J., and Livingston, D. (1997). Dynamic changes of BRCA1 subnuclear location and phosphorylation state are initiated by DNA damage. *Cell*, 90:425–435.

Seyschab, H., Friedl, R., Sun, Y., Schindler, D., Hoehn, H., Hentze, S., and Schroeder-Kurth, T. (1995). Comparative evaluation of diepoxybutane sensitivity and cell cycle blockage in the diagnosis of Fanconi anemia. *Blood*, 85:2233–2237.

Siddique, M., Nakanishi, K., Taniguchi, T., Grompe, M., and D´Andrea, A. (2001). Function of the Fanconi anemia pathway in Fanconi anemia complementation group F and D1 cells. *Exp.Hematol.*, 29:1448–1455.

Socie, G., Gluckman, E., Raynal, B., Petit, T., Landman, J., Devergie, A., and Brison, O. (1993). Bone marrow transplantation for Fanconi anemia using low-dose cyclophosphamide/thoracoabdominal irradiation as conditioning regimen: chimerism study by the polymerase chain reaction. *Blood*, 82:2249–2256.

Stephan, V., Wahn, V., le Deist, F., Dirksen, U., Broker, B., Muller-Fleckenstein, I., Horneff, G., Schroten, H., Fischer, A., and de Saint-Basile, G. (1996). Atypical X-linked severe combined immunodeficiency due to possible spontaneous reversion of the genetic defect in T cells. *N.Engl.J.Med.*, 335:1563–1567.

Stewart, G. and Elledge, S. (2002). The two faces of BRCA2, a FANCtastic discovery. *Mol. Cell*, 10:2–4.

Strathdee, C., Gavish, H., Shannon, W., and Buchwald, M. (1992). Cloning of cDNAs for Fanconi´s anemia by functional complementation. *Nature*, 356:763–767.

Sujanski, E. and Leonhard, B. (1983). VACTERL association with hydrocephalus - a new recessive syndrome? *Am.J.Hum.Genet.*, 35:119A. Abstract.

Tachibana, A., Kato, T., Ejima, Y., Yamada, T., Shimizu, T., Yang, L., Tsunematsu, Y., and Sasaki, M. (1999). The FANCA gene in Japanese Fanconi anemia: reports of eight novel mutations and analysis of sequence variability. *Hum.Mutat.*, 13:237–244.

Taniguchi, T., Garcia-Higuera, I., Xu, B., Andreassen, P., Gregory, R., Kim, S., Lane, W., Kastan, M., and D'Andrea, A. (2002). Convergence of the Fanconi anemia and Ataxia telangiectasia signaling pathways. *Cell*, 109:459–472.

Tavtigian, S., Simard, J., Rommens, J., Couch, F., Shattuck-Eidens, D., Neuhausen, S., Merajver, S., Thorlacius, S., Offit, K., Stoppa-Lyonnet, D., Belanger, C., Bell, R., Berry, S., Bogden, R., Chen, Q., Davis, T., Dumont, M., Frye, C., Hattier, T., Jammulapati, S., Janecki, T., Jiang, P., Kehrer, R., Leblanc, J., and Goldgar, D. (1996). The complete BRCA2 gene and mutations in chromosome 13q-linked kindreds. *Nat.Genet.*, 12:333–337.

The Fanconi anemia/Breast cancer consortium (1996). Positional cloning of the Fanconi anemia group A gene. *Nat.Genet.*, 14:324–328.

Timmers, C., Taniguchi, T., Hejna, J., Reifsteck, C., Lucas, L., Bruun, D., Thayer, M., Cox, B., Olson, S., and D´Andrea, A. (2001). Positional cloning of a novel Fanconi anemia gene, FANCD2. *Mol.Cell*, 7:241–248.

Tipping, A., Pearson, T., Morgan, N., Gibson, R., Kuyt, L., Havenga, C., Gluckman, E., Joenje, H., de Ravel, T., Jansen, S., and Mathew, C. (2001). Molecular and genealogical evidence for a founder effect in Fanconi anemia families of the Africaner population of South Africa. *PNAS*, 98:5734–5739.

Tower, P., Christianson, T., Peters, S., Ostroski, M., Hoatlin, M., Zigler, A., Heinrich, M., Rathbun, R., Keeble, W., and Faulkner, G.R. Bagby, G. (1998).

Expression of the Fanconi anemia group C gene in hematopoietic cells is not influenced by oxydative stress, cross-linking agents, radiation heat or mitoticinhibitory factors. *Exp.Hematol.*, 26:19–26.

Van de Vrugt, H., Cheng, N., de Vries, Y., Rooimans, M., de Groot, J., Scheper, R., Zhi, Y., Hoatlin, M., Joenje, H., and Arwert, F. (2000). Cloning and characterization of murine Fanconi anemia group A gene: Fanca protein is expressed in lymphoid tissues, testis and ovary. *Mamm.Genome*, 11:326–331.

Verlander, P., Kaporis, A., Liu, Q., Zhang, Q., Seligsohn, U., and Auerbach, A. (1995). Carrier frequency of the IVS4+4A>T mutation of the Fanconi anemia gene FAC in the Ashkenazi Jewish population. *Blood*, 86:4034–4038.

Verlander, P., Lin, J., Udono, M., Zhang, Q., Gibson, R., Mathew, C., and Auerbach, A. (1994). Mutation analysis of the Fanconi anemia gene FACC. *Am.J.Hum.Genet.*, 54:595–601.

Wada, T., Schurman, S., Otsu, M., Garabedian, E., Ochs, H., Nelson, D., and Candotti, F. (2001). Somatic mosaicism in Wiskott-Aldrich syndrome suggests in vivo reversion by a DNA slippage mechanism. *PNAS*, 98:8697–8702.

Wahn, V., Stephan, V., and Hirschhorn, R. (1998). Reverse mutations - spontaneous amelioration or cure of inherited disorders? *Eur.J.Pediatr.*, 157:613–617.

Waisfisz, Q., de Winter, J., Kruyt, F., de Groot, J., van der Weel, L., Dijkmans, L., Zhi, Y., Arwert, F., Scheper, R., Youssoufian, H., Hoatlin, M., and Joenje, H. (1999a). A physical complex of the Fanconi anemia proteins FANCG/XRCC9 and FANCA. *PNAS*, 96:10320–10325.

Waisfisz, Q., Morgan, N., Savino, M., de Winter, J., van Berkel, C., Hoatlin, M., Ianzano, L., Gibson, R., Arwert, F., Savoia, A., Mathew, C., Pronk, J., and Joenje, H. (1999b). Spontaneous functional correction of homozygous Fanconi anemia alleles reveals novel mechanistic basis for reverse mosaicism. *Nat.Genet.*, 22:379–383.

Waisfisz, Q., Saar, K., Morgan, N., Altay, C., Leegwater, P., de Winter, J., Komatsu, K., Evans, G., Wegner, R.-D., Reis, A., Joenje, H., Arwert, F., Mathew, C., Pronk, J., and Digweed, M. (1999c). The Fanconi anemia group E gene, FANCE, maps to chromosome 6p. *Am.J.Hum.Genet.*, 64:1400–1405.

Wang, J., Otsuki, T., Youssoufian, H., Foe, J., Kim, S., Devetten, M., Yu, J., Li, Y., Dunn, D., and Liu, J. (1998). Overexpression of the Fanconi anemia group C gene (FAC) protects hematopoietic progenitors from death induced by Fas-mediated apoptosis. *CancerRes.*, 58:3538–3541.

Wang, X., Peterson, C., Zheng, H., Nairn, R., Legerski, R., and Li, L. (2001). Involvement of nucleiotide excision repair in a recombination-independent and error-prone pathway of DNA interstrand cross-link repair. *Mol.CellBiol.*, 21:713–720.

Wevrick, R., Clarke, C., and Buchwald, M. (1993). Cloning and analysis of the murine Fanconi anemia group C cDNA. *Hum.Mol.Genet.*, 2:655–662.

Whitney, M., Jakobs, P., Kaback, M., Moses, R., and Grompe, M. (1994). The Ashkenazi Jewish Fanconi anemia mutation: incidence among patients and carrier frequency in the at-risk population. *Hum.Mutat.*, 3:339–341.

Whitney, M., Royle, G., Low, M., Kelly, M., Axthelm, M., Reifsteck, C., Olson, S., Braun, R., Heinrich, M., Rathbun, R., Bagby, G., and Grompe, M. (1996). Germ cell defects and hematopoietic hypersensitivity to gamma-interferon in mice with a targeted disruption of the Fanconi anemia C gene. *Blood*, 88:49–58.

Whitney, M., Saito, H., Jakobs, P., Gibson, R., Moses, R., and Grompe, M. (1993). A common mutation in the FACC gene causes Fanconi anemia in Ashkenazi Jews. *Nat.Genet.*, 4:202–205.

Whitney, M., Thayer, M., Reifsteck, C., Olson, S., Smith, L. Jakobs, P., Leach, R., Naylor, S., Joenje, H., and Grompe, M. (1995). Microcell mediated chromosome transfer maps the Fanconi anemia group D gene to chromosome 3p. *Nat.Genet.*, 11:341–343.

Wijker, M., Morgan, N., Herterich, S., van Berkel, C., Tipping, A., Gross, H., Gille, J., Pals, G., Savino, M., Altay, C., Mohan, S., Dokal, I., Cavenagh, J., Marsh, J., van Weel, M., Ortega, J., Schuler, D., Samachatova, E., Karwacki, M., Bekassy, A., Abecasis, M., Ebell, W., Kwee, M., de Ravel, T., Gibson, R., Gluckman, E., Arwert, F., Joenje, H., Savoia, A., Hoehn, H., Pronk, J., and Mathew, C. (1999). Heterogenous spectrum of mutations in the Fanconi anemia group A gene. *Europ.J.Hum.Genet.*, 7:52–59.

Witt, E. and Ashworth, A. (2002). D-Day for BRCA2. *Science*, 297:534.

Wooster, R., Bignell, G., Lancaster, J., Swift, S., Seal, S., Mangion, J., Collins, N., Gregory, S., Gumbs, C., and Micklem, G. (1995). Identification of the breast cancer susceptibility gene BRCA2. *Nature*, 378:789–792.

Xie, Y., de Winter, J., Waisfisz, Q., Nieuwint, A., Scheper, R., Arwert, F., Hoatlin, M., Ossenkoppele, G., Schuurhuis, G., and Joenje, H. (2000). Aberrant Fanconi anemia protein profiles in acute myeloid leukemia cells. *Br.J.Hematol.*, 111:1057–1064.

Yamada, T., Tachibana, A., Shimizu, T., Mugishima, H., Okubo, M., and Sasaki, M. (2000). Novel mutations of the FANCG gene causing alternative splicing in Japanese Fanconi anemia. *J.Hum.Genet.*, 45:159–166.

Yamashita, T., Barber, D., Zhu, Y., Wu, N., and D´Andrea, A. (1994). The Fanconi anemia polypeptide FACC is localized to the cytoplasm. *PNAS*, 91:6712–6716.

Yamashita, T., Kupfer, G., Näf, D., Suliman, A., Joenje, H., Asano, S., and D´Andrea, A. (1998). The Fanconi anemia pathway requires FAA phosphorylation and FAA/FAC nuclear accumulation. *PNAS*, 95:13085–13090.

Yang, Y., Kuang, Y., Montes De Oca, R., Hays, T., Moreau, L., Lu, N., Seed, B., and D´Andrea, A. (2001). Targeted disruption of the murine Fanconi anemia gene, Fancg/Xrcc9. *Blood*, 98:3435–3440.

Young, N. and Alter, B. (1994). Clinical features of Fanconi´s anemia; pathophysiology of Fanconi´s anemia. In Young, N. and Alter, B., editors, *Aplastic anemia acquired and inherited*, pages 275–324. Saunders, Philadelphia.

Youssoufian, H. (1994). Localization of Fanconi anemia C protein to the cytoplasm of mammalian cells. *PNAS*, 91:7975–7979.

Youssoufian, H. (1996a). Cytoplasmic localization of FAC is essential for the correction of a prerepair defect in Fanconi anemia group C cells. *J.Clin.Invest.*, 97:2003–2010.

Youssoufian, H. (1996b). Natural gene therapy and the Darwinian legacy. *Nat.Genet.*, 13:255–256.

Youssoufian, H., Auerbach, A., Verlander, P., Steimle, V., and Mach, B. (1995). Identification of cytosolic proteins that bind to the Fanconi anemia complementation group C polypeptide in vitro. evidence for a multimeric complex. *J.Biol.Chem.*, 270:9876–9882.

Youssoufian, H., Li, Y., Martin, M., and Buchwald, M. (1996). Induction of Fanconi anemia cellular phenotype in human 293 cells by overexpression of a mutant FAC allele. *J.Clin.Invest.*, 97:957–962.

Acknowledgements

Last not least, I want to thank all those who have been involved in this work for their manifold support, especially:

- Prof. Dr. Holger Hoehn for providing this very interesting subject and for technical as well as psychological ;-) assistance,
- PD Dr. Detlev Schindler for supporting this thesis,
- PD Dr. Helmut Hanenberg for the good cooperation, e.g. virus production and transfections,
- likewise Dr. Johannes Rischewski for good cooperation and interesting discussions on FA as well as other important topics,
- "Flow-virtuoso" Richard for his helping hand and for his stoical calmness,
- Alex, Reinhard, Gitta, Ariane, Gaby, Anette, Richard and "SPD" for their friendship and their encouraging words in times of experimental despair, for the beautiful biocenter barbecues, for technical assistance, etc.
- and all my friends and university colleagues (including the above-mentioned persons) for their helping hands during my pregnancy, so that this thesis could be finished so frictionless.

I am indebted to my family for their omnipresent support throughout all the years as well as to my husband Oliver and my little son Dominik who both make me happy day by day and who motivated me to finish this thesis as soon as possible in order to spend more time with them.

Lebenslauf

Persönliche Daten:

Name:	Groß, geb. Thiel
Vornamen:	Michaela Sandra
wohnhaft:	Falkensteiner Str. 11a, 65779 Kelkheim
Geburtsdatum:	26. Januar 1973
Geburtsort:	Coburg
Familienstand:	verheiratet

Schulausbildung:

1979 - 1983:	Volksschule Sonnefeld
1983 - 1992:	Gymnasium Alexandrinum, Coburg
Juni 1992:	Abitur

Studium:

1992 - 1998:	Studiengang Biologie/Diplom an der Julius-Maximilians-Universität Würzburg Diplomarbeit: *Molekulargenetische Untersuchungen am Fanconi-Anämie-Gen der Komplementationsgruppe A*
Juli 1998:	Biologie-Diplom

Berufliche Tätigkeit:

seit August 1998:	wissenschaftliche Mitarbeiterin am Institut für Humangenetik Julius-Maximilians-Universität, Würzburg

Würzburg, 14. November 2002 Michaela Groß

The results of the thesis were presented in two lectures, six poster presentations and seven publications or publications in preparation.

Lectures:

11/99: "Mutationsanalyse im Fanconi Anämie A Gen", FA-Jahrestreffen at Gersfeld

7/00: "Mutationsspektrum bei Fanconi Anämie", Fanconi Anämie Symposium des Schroeder-Kurth-Fonds at Würzburg

Poster presentations:

Michaela Thiel, Sabine Herterich, Gitta Emmert, Ralf Dietrich, Richard Friedl, Detlev Schindler and Holger Hoehn (1998) A high rate of mosaicism in adult FA patients. (abstract) *Med.Genet.* 1:P4A-40

Michaela Thiel, Sabine Herterich, Gitta Emmert, Matthias Wagner, Holger Hoehn and Detlev Schindler (1999) Mutation analysis in the Fanconi anemia group A (FANCA) gene. (abstract) *Med.Genet.* 1:P7-119

Michaela Thiel, Sonja Gräsl, Holger Hoehn, Detlev Schindler (2000) Mutation anlaysis in the fanconi anemia (FA) genes FANCA and FANCG (abstract) *Eur.J.Hum.Genet.* 8:P601

Detlev Schindler, Stephan Lobitz, Michaela Thiel, Sonja Gräsl, Sabine Herterich, Helmut Hanenberg (2000) Fanconi Anemia (FA) complementation group analysis using retroviraal vectors. (abstract) *Eur.J.Hum.Genet.* 8:P598

Detlev Schindler, Michaela Thiel, Richard Friedl, Gitta Emmert, Sabine Herterich, Matthias Wagner, Holger Hoehn, Stephan Lobitz Helmut Hanenberg (2000) Diagnosis and complementation group assignment of mosaic FA patients (abstract)

Publications:

Michaela Thiel (1999) Mutationsanalyse in den Fanconi-Anämie-Genen, *Fanconi-Handbuch*, *in preparation*

Ilja Demuth, Marcin Wlodarski, Alex J Tipping, Neil V Morgan, Johan P de Winter, Michaela Thiel, Sonja Gräsl, Detlev Schindler, Alan D D'Andrea, Cigdem Altay, Hülya Kayserili, Adriana Zatterale, Jürgen Kunze, Wolfram Ebell, Christopher G Mathew, Hans Joenje, Karl Sperling and Martin Digweed (2000) Spectrum of mutations in the Fanconi anemia group G gene, FANCG/XRCC9. *Eur.J.Hum.Genet.* 8,861-868

Michaela Gross*, Detlev Schindler*, Lydia Vieten, Sonja Gräsl, Stephan Lobitz, Alexandra Sobeck, Sabine Herterich and Helmut Hanenberg (2002) Spontaneous hematological cure in Fanconi anemia due to revertant alleles in somatic stem cells. *to be submitted to Nat. Genet.*

Michaela Gross, Helmut Hanenberg, Stephan Lobitz, Richard Friedl, Sabine Herterich, Ralf Dietrich, Holger Hoehn and Detlev Schindler (2002) Natural gene therapy: molecular self-correction in Fanconi anemia. *Hum.Mol.Genet. in review*

Michaela Gross (2002) Mutationsanalyse in den Fanconi Anämie Genen unter besonderer Berücksichtigung von FANCA. *medgenetik* edition 2, *in press*

Michaela Gross, Reinhard Kalb, Detlev Schindler, Helmut Hanenberg and Johannes Rischewski (2002) Novel mutations, including a large deletion, in the FANCC gene. *to be submitted to Hum. Mutat.*

Holger Hoehn, Michaela Thiel, Alexandra Sobeck, Matthias Wagner and Detlev Schindler (2001) Genetic instability in Fanconi anemia. in: Chromosomal instability and aging: Basic science and clinical implications (Hisama FM and Martin GM, eds) *in press*

Ehrenwörtliche Erklärung

gemäß §4 Absatz 3, Ziffern 3, 5 und 8
der Promotionsordnung der
Fakultät für Biologie
der Universität Würzburg

Hiermit erkläre ich ehrenwörtlich, daß ich die vorliegende Dissertation selbständig angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Die Dissertation wurde bisher weder vollständig noch teilweise einer anderen Hochschule mit dem Ziel, einen akademischen Grad zu erwerben, vorgelegt.

Von der Universität Würzburg wurde mir am 24. Juli 1998 der akademische Grad "Diplom-Biologin Univ." verliehen. Weitere akademische Grade habe ich weder erworben noch versucht zu erwerben.

Würzburg, 14. November 2002 Michaela Groß