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From the Molecular Biology to the Gene Therapy of a DNA Repair Syndrome: Fanconi Anemia

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1. Introduction

Fanconi anemia (FA) was first described in 1927 by the Swiss pediatrician Guido Fanconi in a family with five children, three of which had various physical abnormalities and hematological defects, in a condition that resembled pernicious anemia [1]. In all the three children the disease was manifested between ages of five to seven years old and had fatal consequences. Studies in peripheral blood cells from these and other patients with the same symptoms made Guido Fanconi to realize that the disorder affected all the hematopoietic lineages, not only erythropoiesis, and that these alterations were usually the main cause of mortality (See review in [2]). From the beginning of his studies, he thought that this disease was too complex to be caused by mutations in one single gene, but neither he, nor the rest of the research community could imagine at that time that mutations in at least fourteen different genes, currently known as FA genes, could account for the same disease. The description of the chromosomal instability of FA cells as a hallmark of the disease in 1964[3] focused the etiology of the disease as a DNA repair failure, but it was almost forty years later when this idea was confirmed.

In this chapter we will make an overview of the implications of the FA pathway in DNA repair and cell survival, and discuss the advances, limitations and perspectives of the therapeutic approaches used for the treatment of the most severe problem that takes place in FA patients, the bone marrow failure (BMF).

2. Fanconi anemia proteins form a complex pathway involved in the repair of DNA inter-strand cross-links

Mutations in at least 14 genes have been associated with FA. Patients with biallelic mutations in any of these FA genes (except in *FANCB,* which is X-linked) are assigned to different complementation groups (Table 1). The identification of the first FA complementation group was conducted by the fusion of cell lines generated from different FA patients[4]. The first FA gene, *FANCC*, was then identified by the transfection of cells from a FA patient with a cDNA expression library, followed by their exposure to mitomycin

C (MMC), a DNA cross-linking drug that is extremely toxic and generates specific chromosomal instability in FA cells[5]. Only those cells complemented with *FANCC* grew after MMC exposure, allowing the identification of the defective gene in these patients. Similar approaches, together with positional cloning and linkage analysis, allowed the identification of other protein members of the so called "FA core complex", which included FANCA, FANCG, FANCF and FANCE[6-10]. Although the description of FANCD2[11] and its activation by monoubiquitination after DNA damage linked FA with DNA repair, the confirmation of the involvement of the FA pathway in DNA repair and its link to homologous recombination occurred in 2002, when *BRCA2* was identified as the *FANCD1* gene[12]. After the discovery of *BRCA2* as a FA gene, several other FA genes were described, initially *FANCL*, containing the ubitiquitin ligase activity of the complex [13]and later on, *FANCB*; the only FA gene linked to the X chromosome[14]. The description of *FANCJ* (*BRIP1*)[15-17], *PALB2*[18] and *RAD51C* (not formally assigned yet as a FA gene)[19, 20] together with the previously described *BRCA2,* definitively linked the FA/BRCA pathway with increased cancer susceptibility[19, 21-24]. In subsequent studies FANCI [25-27] was found to be the partner of FANCD2. Additionally, the finding that FANCM[28] can interact with DNA, and the observation that FANCP (SLX4) had endonuclease activity, is allowing to unravel the role of the FA/BRCA pathway in the repair of DNA interstrand cross-links (ICLs) during replication [29](Table 1).

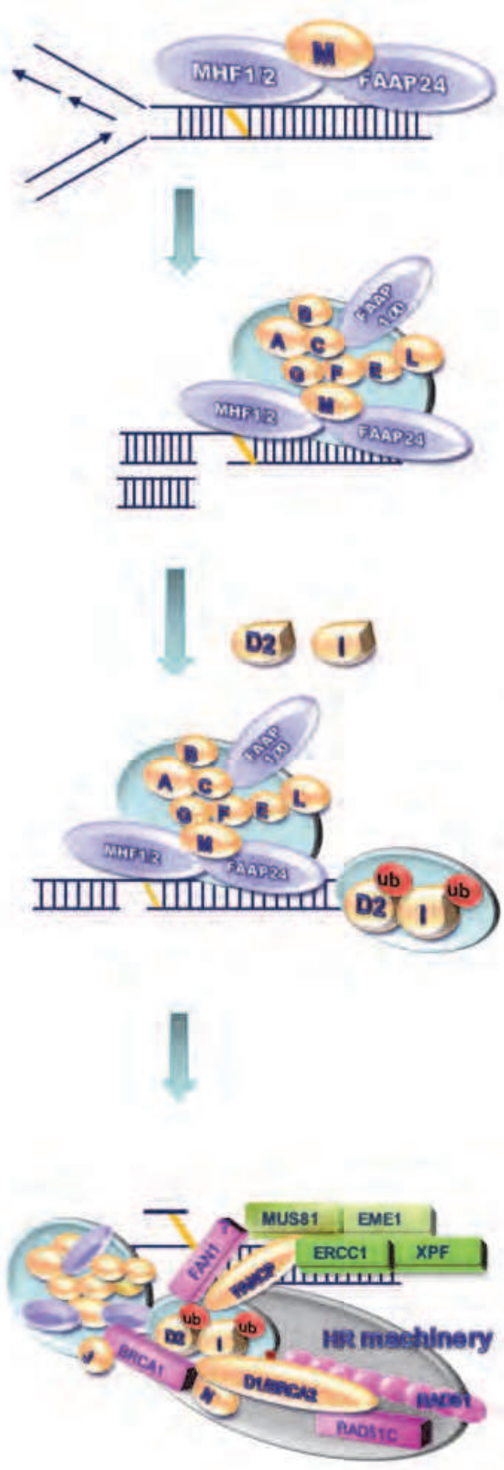
Interstrand cross-linking drugs covalently bind both strands of the DNA helix, blocking the DNA replication and transcription. As a consequence of stalled replication produced by ICLs or at S-phase entry, the FA pathway is activated (See review in [29]). Although the exact role of FA proteins in the repair of ICLs is not clear yet, it is known that they work together in a complex network, where the key event is the monoubiquitination of FANCD2[30] and FANCI [25-27] (D2-I complex). This monoubiquitination requires the presence of the FA core complex, currently known to be formed by FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL and FANCM[31], together with other FA associated proteins(FAAP; see Figure 1). The description of the last members of the FA/BRCA pathway, particularly FANCM, FAN1 (associated nuclease, with no mutations still found in FA patients)[32-35] and FANCP (SLX4)[36, 37] has added new critical elements in this intriguing pathway.

After the generation of an ICL in the DNA, the progression of the DNA replication fork is stalled, and this activates the FA core complex and ATR. The first protein from FA pathway that seems to initiate DNA repair is FANCM. This protein, thanks to its translocase activity [28], together with FAAP24 and MHF1/2[29], moves along the DNA to sense the stalled replication fork[38], facilitating the translocation and anchoring the rest of the FA core complex to damaged DNA. In this way, the FA core complex can monoubiquitinate FANCD2/I, inducing the replication fork pause and the incision/unhooking of the ICL, facilitating the translesion synthesis and finally the HR machinery required for the repair of the DNA damage [39, 40]. Once the D2-I complex is monoubiquitinated, another FA protein - FAN1 - is also recruited to sites of DNA damage, where all these proteins form large nuclear foci. The endonuclease activity of FAN1 suggests that this protein could also pair with MUS81-EME1 to unhook the ICL[32, 35, 40] necessary for DNA repair. In this step, the endonuclease XPF-ERCC1, whose activity is coordinated by the last described member of the FA family SLX4 (FANCP[36, 37]), probably also plays an important role in the repair of the DNA damage (See Figure 1).

C. group	FA genes	Prevalence	Chromosomal location	Protein size (kDa)	Protein characteristics	Required for Ub-FANCD2/I	RAD51 foci
FA-A	*FANCA*	66%	16q24.3	163	Member of the core complex. Phosphorylated by ATR kinase. Two NLS	Yes	Normal
FA-B	*FANCB*	~2%	Xp22.31	95	Member of the core complex. Contains a NLS	Yes	Normal
FA-C	*FANCC*	10%	9q22.3	63	Localized in the nucleus and cytoplasm. Member of the core complex.	Yes	Normal
FA-D1	*BRCA2*	~2%	13q12-13	380	Essential in HR by controlling RAD51. Interacts with FANCG, FANCD2 and PALB2. Cancer susceptibility gene.	No	Reduced
FA-D2	*FANCD2*	~2%	3q25.3	155,162	Monoubiquitinated by FA core complex. Phosphorylated by ATR and probably ATM after DNA damage.	Yes	Normal
FA-E	*FANCE*	~2%	6p21-22	60	Member of the core complex. Directly binds FANCD2. Contains two NLS.	Yes	Normal
FA-F	*FANCF*	~2%	11p15	42	Required for the assembly of FA core complex.	Yes	Normal
FA-G	*FANCG/ XRCC9*	9%	9p13	68	Member of the core complex. Interacts with FANCD1, FANCD2 and XRCC3.	Yes	Normal
FA-I	*FANCI*	~2%	15q25-26	140,147	Monoubiquitinated by FA complex in a FANCD2 dependent manner. It is also phosphorylated after DNA damage.	Yes	Normal
FA-J	*FANCJ/ BRIP1*	~0.2%	17q22-24	140	DNA dependent ATPase and a 5'-3' DNA helicase. Binds the BCRT domain of BRCA1. Cancer susceptibility gene.	No	Normal
FA-L	*FANCL (PHF9)*	~0.2%	2p16.1	43	Core complex member with Ubiquitin ligase activity.	Yes	Normal
FA-M	*FANCM/HEF*	~0.2%	14q21.3	250	Member of the core complex with translocase activity and endonuclease domain. DNA damage sensor.	Yes/ Partially in mice	Normal
FA-N	*FANCN/ PALB2*	~2%	16p12.1	140	Partner and localizer of BRCA2. It links BRCA2 and BRCA1 proteins. Cancer susceptibility gene.	No	Reduced
FA-O	*RAD51C**	~0.2%	17q22	43	Participates in several protein complexes involved in HR. Cancer susceptibility gene.	No	Reduced
FA-P	*SLX4*	~0.2%	16p13.3	200	Scaffold protein for endonucleases MUS81-EME1 and XPF-ERCC1. Involved in resolution of HR intermediates such as Holliday junctions.	No	Normal

* *FANCO/RAD51C* is not formally defined as a FA gene. NLS; nuclear localization signals. HR; Homologous recombination

Table 1. Main characteristics of the Fanconi anemia genes and their proteins.



Step I:

•FANCM-FAAP24-MHF complex move across the DNA

Step II:

•Incision in the DNA and FANCM-FAAP24-MHF complex recruitment of the FA core complex

Step III:

•Monoubiquitination of FANCD2-I complex and accumulation of FANCD2-I in the chromatin

Step IV:

•Recruitment of downstream porteins involved in HR.

•Unhook of ICL by MUS81-EME1 and XPF-ERCC1 and possibly FAN1.

•Repair of the DSB by HR machinery.

Fig. 1. Description of the four essential steps in the repair of DNA interstrand cross-links of the FA/BRCA pathway (Adapted from Niedernhofer [39] , Kee et al.[29], Valeri *et al*. [41] and Cybulski and Howlett[42]).

3. Clinical features of Fanconi anemia

Fanconi anemia is a rare autosomal recessive disease (except for FA-B which is X-linked), with an estimated frequency of 1-5 per 100,000 births. Although the presentation of the disease is very heterogeneous, around 70% of FA patients show physical anomalies, such as skeletal abnormalities, including radial hypoplasia, short stature and microphtalmia. Most FA patients present hypo or hyperpigmentation of the skin, showing the characteristic *café au lait* spots, and with a lower frequency, cardiac, renal, gastrointestinal and other organ malformations [43]. The most common feature of the disease is the bone marrow failure (BMF), which is manifested at a median age of 8 years, being the primary cause of morbidity and mortality in FA patients[44]. Clinical data from the International Fanconi anemia Registry (IFAR) and the German Fanconi anemia Registry (GEFA) have shown that virtually all FA patients would develop BMF by the age of 40 years[45, 46]. At birth, the blood counts are usually normal, macrocytosis usually being the first symptom detected in these patients, followed by thrombocytopenia and neutropenia. Pancytopenia is generally presented between 5 and 10 years of age. The German and American studies showed that the use of a defined abnormality score, based on the analysis of five different congenital abnormalities, can significantly predict the development of the BMF in FA patients[46, 47].

In addition to the BMF, the incidence of cancer in FA patients is also markedly increased compared to the normal population, the incidence of myelodisplasia (MDS) or acute myeloid leukemia (AML) being 33% at the age of 40 years (more than eight hundred fold increased incidence compared to a healthy population). Reports from the IFAR and GEFA have also shown that FA patients are at extraordinary risk of developing specific solid tumors, such as head and neck squamous cell carcinomas (HN-SCCs; with several hundred fold increased frequency), esophagus SCCs (several thousand fold increase) and vulvar cancer in women (also several thousand fold increase)[46, 48, 49].GEFA also observed an increase in breast and brain tumors, while the North American study observed an increased incidence of cervical,ostesarcoma and liver tumors [46].

Myelodisplasia is often presented as a refractory cytopenia with multilineage dysplasia, with or without excess of blasts[49]. Acute myeloid leukemia can be diagnosed primarily or after a MDS phase, with an increasing fraction of blast cells in the BM[50]. The high selective pressure during their teens or early adulthood is probably involved in the development of clonal MDS and AML. The most common abnormalities found in FA patients with MDS/AML are gains of chromosome 1q, monosomy 7, gains of 3q (where *EVI1* is included) [44, 49-51], and abnormalities in *RUNX1/AML1* gene at chromosome 21q[50]. Results by Quentin *et al* also suggest a model of multi-step oncogenesis progression in the BM of FA patients, in which 1q+ (which can be found in the aplastic anemia form of the disease) would possibly constitute the initiating event, while the 3q+, -7/7q and *RUNX1* abnormalities would lead to high grade MDS or AML. In this model 1q+ might clonally rescue the BMF of FA patients, but would not protect against progression towards MDS and leukemia[50].

A remarkable aspect concerning the clinical symptoms of the disease is the observation that some of FA patients can undergo somatic mosaicism by means of a spontaneous reverse mutation or mitotic recombination in one of the FA pathogenic mutations in particular somatic cells[52-54]. If such a reversion occurs in a hematopoietic progenitor or stem cell (HSC), it may confer a proliferation advantage to the reverted cell, leading to the recovery of the BMF in the patient[52-55].

A recent paper described for the first time a characteristic phenotype in a number of FA patients who showed two of the FA hallmark features (high number of chromosomal aberrations after DNA damage and absence of FANCD2 monoubiquitination), compatible with normalized hematological counts[56]. Remarkably, blood cells from these patients did not show the FA characteristic G2/M cell cycle arrest after exposure to DNA cross-linkers[56]. Moreover, in contrast to mosaic FA patients, no reversion in the pathogenic mutations occurred in this new group of FA patients. Based on the above observations, a phenomenon defined as attenuation of the phenotype was described in FA[56]. Attenuation was associated with almost normal blood cell counts, and in some cases with development of MDS or AML. This process was accompanied by clonal hematopoiesis, implying that all peripheral blood cells derive from a single progenitor cell, in which a molecular event reducing CHK1 expression took place, resulting in the attenuated G2 arrest. The conclusions of this work have important implications in the management of FA patients because patients with this phenotype, although having essentially normal blood cell counts, should be followed closely to prevent the development of MDS or AML. Additionally, this study offers new explanations that could account for differences in the severity of the disease between FA siblings with the same mutation. Another important study has recently shown that FA proteins also play a role in mitosis, since FANCD2, FANCI and FANCM are localized to the extremities of ultrafine DNA bridges (UFBs), which link sister chromatids during cell division. FA cells show increase number of UFBs that may inhibit cytokinesis, leading to binucleated or multinucleated cells, a phenomenon that could account for the increased apoptosis and thus, also contribute to BMF [57, 58].

4. Main phenotypic characteristics of Fanconi anemia cells

FA cells are hypersensitive to ICL agents such as mitomycin C (MMC) and diepoxibutane (DEB). Additionally, these drugs specifically induce a high number of chromosomal aberrations in FA cells[59, 60], thus constituting a hallmark for the diagnosis of FA patients[60, 61]. Although normal cells exposed to DNA cross-linkers develop a transient accumulation in the G2/M phase of the cell cycle, the defective ability of FA cells to repair the DNA damage leads to a very significant accumulation of these cells in G2/M after exposure to ICLs[62]. This property has been also used for the diagnosis of FA patients, particularly in skin fibroblasts for the characterization of FA mosaic patients in which no obvious phenotypic markers may be apparent in their peripheral blood cells[63, 64].

FA cells are also characterized by their hypersensitivity to ambient oxygen conditions [65-67], manifested by a poor *ex vivo* growth and clonogenic capacity. This impaired growth properties of FA cells can be significantly restored when the incubation atmosphere is changed to hypoxic conditions (<5% of oxygen)[67], mimicking the low oxygen concentrations present in most tissues, including the BM niche[68, 69].

FA cells also show an increased apoptotic predisposition, something that may account for the BMF of these patients and for the development of malformations during embryonic development. One of the biochemical pathways involved in the increased apoptosis of FA cells is related to the over-production of cytokines such as TNFα and interferon-γ [70, 71]; an observation that has been confirmed in BM from FA patients[72].

FA patient hematopoietic progenitor cells have also shown a defective adhesion and homing activities, associated with an aberrant regulation of Cdc42 activity[73].

5. Identification of complementation groups in Fanconi anemia patients

As described before, fourteen different FA genes have been described so far, whose mutations account for the different FA complementation groups already identified (Table 1). However, there is still a number of patients with clinical symptoms of FA without mutations in any of the 14 FA genes, suggesting that more FA genes will be added to this long list. The identification of the FA complementation group (FA subtyping) in a patient has several advantages in the management of the disease. In this respect FA subtyping 1) confirms the FA diagnosis; 2) facilitates the identification of pathogenic mutations in FA genes, 3) allows to investigate relationships between the phenotype and the genotype in the patients, and 4) it is required for a potential future treatment of the patient by gene therapy.

Once a patient is diagnosed with FA, several alternatives have been used for FA subtyping. Because of the technical difficulties of the cell fusion approaches initially developed by Buschwald[4, 5], Hanenberg *et al.* in 2002 developed a new strategy based on genetic complementation strategies with retroviral vectors harboring the different FA genes[74]. This strategy, combined with Western blot analyses, principally of the monoubiquitinated and non ubiquitinated forms of FANCD2 (to detect if the mutation is upstream or downstream FANCD2 monoubiquitination), and also with the analysis of foci of nuclear proteins, has allowed the assignment of FA patients to the different complementation groups[75]. Sequencing has also been used [76]however, the large number and complexity of some FA genes and their mutations, together with the necessity of verifying the pathogenicity of each new mutation, implies that subtyping of patients with FA by mutational analysis is often time consuming and laborious [75]. Nevertheless, sequencing combined with genetic complementation strategies can be useful to deeply characterize FA patients.

6. Current treatments of the bone marrow failure in Fanconi anemia patients

As discussed above, the BMF is the main cause of mortality in FA patients. Many of the treatments of the BMF are palliative and directed to maintain acceptable numbers of peripheral blood cells. The use of androgens, in some cases combined with corticoids, constitute one of the most common treatments of FA patients in early stages of the disease, when a residual endogenous hematopoiesis remains[43, 77]. However, not all the patients respond to this treatment and, in most cases the response is slow, transient and normally limited to the red blood cells. Although a longer survival has been reported in patients who have been treated with androgens, in comparison with those who have not (20 years *vs* 14 years) [77], it has been reported that their use might constitute an adverse predictor when hematopoietic cell transplantation (HCT) is required [78]. Additionally, side effects such as liver tumors[48, 79] and masculinization may occur when androgens are used[80].

The use of growth factors to activate specific hematopoietic lineages has been also used in FA patients[81, 82], although generally with a limited success due to the transient benefit [82] and risks of leukemia due to the activation of potential pre-leukemic clones already present in the patient[83].

So far, the only curative treatment capable of restoring the hematopoiesis of FA patients in the long-term is allogenic HCT. Many of the obstacles initially found for the HCT of FA patients have been overcome nowadays. The hypersensitivity of FA patients to conditioning regimens was a limiting factor for the success of the first HCTs in FA patients. However,

conditioning is necessary to eliminate the endogenous hematopoiesis and allow the engraftment of donor cells. In 1984, Gluckman *et al*. [84] developed the first successful conditioning regimen for FA patients consisting of a low dose of cyclophosphamide (CY) and a single dose of total body irradiation (TBI). Since then, many different protocols have been developed aiming to limit the radiation exposure in HCT preparative regimens[85], and thus to minimize risks of malignancies in the long-term[77]. As a result of these improvements, current HCTs from HLA(Human Leukocyte Antigen) identical siblings do not generally include irradiation in the conditioning. The use of mild conditioning regimens and the inclusion of fludarabine (FLU) (an antimetabolite with profound immunosuppressive effects) has, therefore, markedly improved the outcome of HCTs with HLA-matched grafts from related donors.

Although the outcome of transplants from alternative donors in FA has also markedly improved in the last decade, the morbidity and mortality associated to these transplants is still significant[86]. Problems like graft failure, acute and chronic graft versus host disease (GVHD) and opportunistic infections are the major obstacles to address. Again, the inclusion of FLU and the use of T cell depletion (TCD) have significantly improved the efficacy of the transplant of FA patients from unrelated donors[87]. Recent clinical trials conducted in the University of Minnesota have shown the relevance of thymic shielding during irradiation with reduced doses of TBI to limit opportunistic infections, and thus to increase overall survivals after unrelated HCTs in FA patients[88]. Ideally, transplantation should be done previously to the development of a myelodysplasic syndrome or leukemia[88]. The success of HCTs in patients that already have developed any of these pathologies is limited in comparison to those who have not. The main risk associated with the HCT of these patients is that the low doses of radio/chemotherapy that must be used in FA patients might not be enough to destroy the endogenous leukemic cells, thus increasing the risk of future relapses [88].

Traditionally the preferential source for the HCT for FA patients was BM or mobilized progenitors from peripheral blood (mPB). However, umbilical cord blood is nowadays also a good alternative for the transplantation of FA patients. This is not only the case for the HCT from HLA-identical siblings - in some instances derived from *in vitro* fertilization and preimplantation genetic diagnosis - but also from unrelated donors[89].

7. Gene therapy as a new strategy for the treatment of Fanconi anemia

Hematopoietic gene therapy, defined as the HCT of genetically corrected autologous HSCs, is considered a good alternative to allogenic HCT in FA. This strategy would avoid GVHD and limit, at least partially, the side effects associated to severe chemo/radiotherapy and immunosupression (See review in [90]).

The previous observation that a number of mosaic FA patients (those who have reverted a pathogenic mutation in a HSC) could progressively improve their hematological status[52-55] opened the possibility of rescuing the BMF of FA patients after the infusion of gene-corrected HSCs, even in the absence of conditioning. As it is the case with HSCs that have reverted a pathogenic mutation, it is expected that *ex vivo* corrected FA HSCs may also develop a proliferation advantage over uncorrected cells, thus restoring progressively hematopoietic system of the patient.

To allow the stable integration of the transgene in the HSCs genome, gamma-retroviral vectors (RVs) have been the most frequently used vectors in clinical gene therapy protocols

[90, 91]. The principle of most of these protocols was based on the purification of $CD34^+$ cells, either from BM or mPB, followed by the transduction with the therapeutic vector, and the re-infusion of the transduced cells in the patient, either pre-conditioned or not[91-94].
Two different gene therapy trials have been already conducted in FA. The first one was developed by Liu and colleagues in FA-C patients [95] and the second one by Kelly and colleagues in FA-A patients [96]. Both protocols used similar conditions to those previously used for the gene therapy of other monogenic diseases, such as X1-SCID. Essentially, in both protocols *in vitro* pre-stimulated $CD34^+$ cells were transduced with RVs for three or four days in culture, and thereafter infused into non conditioned patients. In contrast to the results observed in X1-SCID patients, none of these protocols improved the clinical status of FA patients, indicating the necessity of improving the therapeutic vector and/or the manipulation of the target cells.

8. Towards the development of improved protocols of Fanconi anemia gene therapy

8.1 Lessons from Fanconi anemia mouse models

Mouse models represent an invaluable tool for improving the understanding of the mechanisms responsible of different pathologies, and also for developing new therapies with improved efficacy and reduced side effects. In the case of FA, where intrinsic difficulties exist to engraft inmunodeficient mice with BM from FA patients, the relevance of mouse models is even higher.
Different FA mouse models with disruptions in FA genes such as *Fanca*[97, 98], *Fancc*[99, 100], *Fancd1(Brca2)*[101], *Fancd2*[102], *Fancg*[103], *Fancm*[104] and more recently *Fancp* (*Slx4*)[105] have been generated (See Table 2). Additionally, a FA mouse model based on the deletion of *Usp1* (the enzyme responsible for *FANCD2* deubiquitination) has been described[106]. This gene, however, is not currently considered a FA gene since no FA patients have been so far identified with *Usp1*mutations.
Although all FA mouse models are characterized by their hypersensitivity to DNA-cross-linking agents and in some instances to cytokines such as TNFα and IFN((See Table 2), the severe BMF that takes place in FA patients is far from being reproduced in these models. In fact, only the recently developed mouse model of FA-P ($Btbd12^{-/-}$ mice) is prone to develop marked blood cytopenias, reflected by a reduction in white blood cells (WBCs) and platelets in a significant number of animals[105] (Table 2).
Aiming to generate FA mouse models that resemble more closely the disease observed in FA patients, double knock-outs have been also generated. Among these mouse models, only the double knock-out $Fancc^{-/-}/Fancg^{-/-}$ mice [107] and the $Fancc^{-/-}/Sod1^{-/-}$ mice showed evidences of BMF [108].
Significantly, although some FA mouse models are prone to develop tumors (i.e. FA-D1, FA-D2, FA-M), AML is not spontaneously generated in the FA mouse models generated so far. Studies conducted in $Fancc^{-/-}$ mice have shown, however, that the *ex vivo* culture and/or incubation of $Fancc^{-/-}$ BM cells with TNF-α, whose expression is significantly increased in FA patients, induces leukemic clonal evolution after transplantation [109, 110], suggesting that leukemia development in FA patients could be at least partially related to the deregulated expression of this cytokine.
$Fanca^{-/-}$ as well as $Fancc^{-/-}$ mice, and also mice with a hypomorphic mutation in *Brca2/Fancd1* (FA-D1 mice) constitute the FA mouse models more frequently used both to understand the

role of FA genes in HSCs functionality, and also to evaluate the preclinical efficacy of new therapies in FA. Defects in the HSCs have been observed in all tested FA mouse models, not only in terms of clonogenic potential, but also regarding the engrafting ability and repopulating properties of these cells (see Table 2). Although no aplastic anemia was observed in FA-D1 mice, this mouse model showed a more severe hematopoietic phenotype compared to other models with mutations in FA genes upstream in the FA/BRCA pathway[111]. In this respect, results from our laboratory showed a defective function in the repopulating potential of endogenous FA-D1 HSCs in their own natural microenvironment. This was demonstrated by the observation that BM cells from WT animals could repopulate in the long-term the hematopoietic tissues of FA-D1 unconditioned recipients [111]. This contrasts with studies conducted in other FA mouse models where only after the treatment with IFNγ [112, 113] or DNA damaging drugs, wild-type BM cells could be engrafted in FA recipients (Table 2).

In the field of gene therapy, it was shown for the first time in *Fancc*$^{-/-}$ mice that the retroviral-mediated expression of *Fancc* corrects the defective repopulation ability of FA HSCs[114]. Similar conclusions were obtained in different FA mouse models using RVs, Lentiviral and also Foamyviral vectors (LVs and FVs; Table 1). Significantly, Li *et al*. showed that the *ex vivo* culture of *Fancc*$^{-/-}$ HSCs increases apoptosis and promotes the development of clonal aberrations[109]. Studies conducted with *Fanca*$^{-/-}$ and *Fancc*$^{-/-}$ mice showed, on the other hand, that rapid transductions with LVs or FVs markedly improved the repopulating properties of the HSCs (Table 2). Taken together, these studies suggest the convenience of using similar short-transduction protocols in human FA gene therapy.

Working with the FA-D1 mouse model, our group showed that the infusion of LV-transduced cells in mice pre-treated with a mild conditioning results in a progressive increase in the proportion of genetically-corrected cells, in the absence of any selection treatment. This is in contrast to data obtained in other FA mouse models, where exposures to cytokines or DNA damaging agents were required (Table 2). Moreover, our data showed that in the long term after transplantation, most of the hematopoietic cells of recipient FA-D1 mice were resistant to otherwise cytototoxic doses of MMC and became genetically stable [115], suggesting that a similar proliferation advantage of *ex vivo* corrected HSCs may occur in FA clinical trials.

8.2 Lessons from *in vitro* studies conducted with bone marrow samples from FA patients

Based on the current knowledge on the biology of FA cells, it is now clear that marked differences distinguish FA HSCs from HSCs successfully treated with gene therapy (i.e. X1-SCID HSCs). In this respect, it has been already shown that in the case of FA HSCs, *in vitro* incubation induces apoptosis and genomic instability [109, 116, 135]. Therefore, it is now considered that short transduction strategies would improve the possibilities of engrafting FA patients with genetically corrected cells, as it has been already shown in FA mouse models [119, 125].

Because of the limited number of hematopoietic progenitors and HSCs present in the BM of FA patients [136], we hypothesized that these precursor cells would be actively cycling in the patient and therefore, directly susceptible to transduction with RVs without further *in vitro* stimulation. Consistent with this hypothesis, our studies showed that hematopoietic progenitors from FA patients can be efficiently transduced by Gibbon Ape Leukemia Virus

(GALV)-packaged RVs in protocols that lasted only 12-24 h [136]. Whether or not the most primitive HSCs were also efficiently transduced in these protocols is, however, unknown due to current limitations to engraft immunodeficient animals with BM from FA patients.

DEFECTIVE GENE	HYPERSENSITIVITY		HEMATOPOIESIS		GENE THERAPY	
	MMC	CTKs	BMF	HSC Defects	*In vivo* efficacy	*In vivo* proliferation advantage
Fanca	[97, 116]	IFN-γ [113]	Mild hrombocyt [97,116]	CRA and HSC Mobilization[117]	LVs [118] LVs[119]	+ MMC: [118] + Chemo: [119]
Fancc	[99],[120]	TNFα: - In vitro[110, 121] - In vivo [113] IFN-γ: - In vitro[100, 109] - In vivo [112, 113] - Not sensitive [122]		CRA [123] HSC Growth[124] CRA [125] HSC Engraftment [117]	RVs: [114, 126] [127] [110] [128] LVs: [129] FVs: [125]	+MMC[126] +CPA / IR:[128] + MMC[129]
Fancd1/Brca2	[111]			CRA[111]	[115]	No Treatment [115]
Fancd2	[102],[106],[130]			CRA [130]		
Fancg	[103],[131]	IFN-γ [113]		Microenvironment defects [132]		
Fancm	[104]					
Fancp/Btbd12	[105]		Mild cytopenias [105]			
Usp1	[106]			CRA[130]		
Fancc/Fancc	[133]					
Fanca/Fancg	[107]		Mild cytopenias [107]	CRA [107]		
Fanca/SOD			Anemia and leukop. [108]	CFCs defects [108]		
Fancd2/Mlh1	[134]					

BMF: Bone marrow failure; CTK: Cytokines; CFC: colony forming cell; Chemo: chemotherapy; CPA: cyclophosfamide; CRA: Competitive repopulation ability; FV: foamyviral vector; HSC: hematopoietic stem cell; IR: Ionizing radiation; LV: lentiviral vector; MMC: Mitomycin C; RV: Retroviral vector

Table 2. Principal characteristics of the hematopoietic system of FA mouse models and results from *ex vivo* HSC gene therapy.

Experimental studies [137, 138] and more recently also human trials[139, 140] have demonstrated that LVs currently constitute the most efficient clinical vectors for stably transducing HSCs after very short transduction periods. Additional studies have shown that these vectors facilitate the stable expression of the transgene *in vivo*, while transgene inactivation has been frequently reported in RVs[137]. Finally, but not less importantly, LVs have shown improved safety properties, compared to the RVs already used in human gene therapy [141-145].

Concerning the level of expression that is required for inducing a therapeutic effect in FA cells, we have recently shown in FA-A and FA-D2 cells that a weak expression of FA genes, at least *FANCA* and *FANCD2*, is sufficient to revert the FA phenotype of hematopoietic cells ([146],[147] and unpublished data). Because FA-A is the most frequent FA complementation group [75, 148] our group [147], as well as Hans-Peter Kiem group[149] have proposed independently the same LV construct for the gene therapy of FA patients. In this vector *FANCA* expression is driven by the phosphoglycerate kinase (PGK) promoter and stabilized by a mutated version of the posttranscriptional regulatory element (WPRE) [147, 149](see Figure 2).

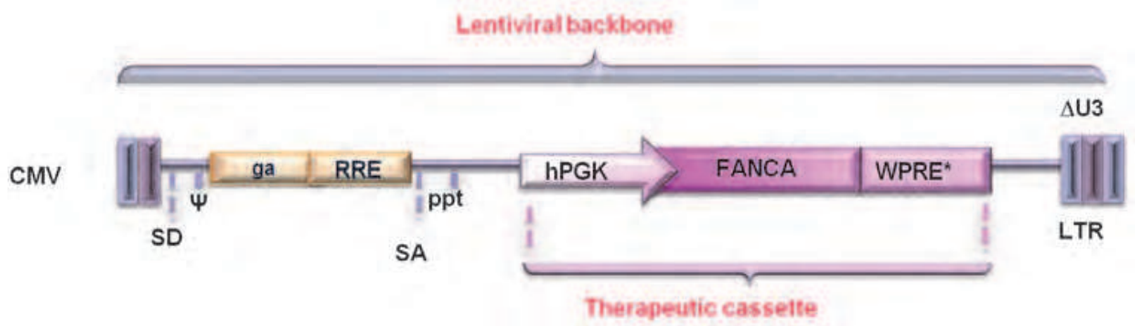


Fig. 2. Illustration of the FANCA-LV proposed independently by González-Murillo *et al* [147] and Becker *et al* [149] for the gene therapy of FA-A patients.

Studies conducted with BM samples from FA patients have also shown that FA progenitor cells are highly sensitive to cytokines such as TNF-α or IFN- γ and also to reactive oxygen species (ROS) [121, 150-154]. Thereafter it was shown that the use of *ex vivo* manipulation conditions that limit the oxidative damage (hypoxia or inclusion of N-acetyl-cysteine) improved the growth of FA progenitor cells[149, 155]. Similar observations were obtained when TNF-α was specifically inhibited with antibodies[72, 136]. Based on these observations and with the aim of improving the repopulation potential of genetically corrected HSCs, many of the experimental protocols aiming the genetic correction of FA HSCs use hypoxia and antibody-mediated inhibition of TNF-α[149, 155].

Concerning the ideal target population to be transduced in FA gene therapy trials, we reasoned that, if possible, the transduction of total BM would be the preferential option[155]. This suggestion derives from the fact that in FA every type of HSC, and also of accessory BM cell, would be directly affected by the genetic defect. Therefore, the genetic correction of each of these populations might be useful for the engraftment of the patient. Using GALV-TR (modified GALV envelope) packaged LVs carrying *FANCA* and/or *EGFP*, we demonstrated the possibility of efficiently transducing hematopoietic and mesenchymal progenitor cells in FA BM samples subjected to a very simple erythrocyte's depletion[155]. Although our data showed that, in contrast to LVs packaged with the G-protein from Vesicular Stomatitis Virus (VSV-G), GALV-TR packaged LVs can efficiently transduce FA BM samples at low multiplicities of infection (around 1-3 infective units/cell), there are still limitations in the production of GMP GALV-TR LVs at high titers. Because high MOIs (Multiplicity of infection) of VSV-G LVs produced in conditions approved for clinical use (GMP) are required to achieve efficient transductions, purified $CD34^+$ or $CD133^+$ cells currently constitute the preferential populations to be used in the next FA gene therapy protocols[156].

9. Perspectives for the future gene therapy of Fanconi anemia patients

The discovery by S. Yamanaka that the transfer of a few transcription factors can reprogram adult somatic cells and generate induced pluripotential cells (iPS cells) [157] has opened new perspectives for the cell and gene therapy of different diseases, particularly in FA. The collaborative study conducted between our group and J.C. Izpisua-Belmonte and J. Surralles groups demonstrated for the first time the possibility of generating disease-free hematopoietic progenitors from genetically corrected fibroblasts from patients with a monogenic disease,

specifically FA patients[158, 159]. Although the technology related to iPS cell generation should be further improved both in terms of efficiency and safety, these strategies have opened an unpredicted applicability in the management of genetic diseases like FA.

10. Concluding remarks

Since the description of the first FA patient by Guido Fanconi in 1927, an extraordinary advance in the understanding of the mechanisms accounting for the disease has occurred. Although more work is still required to elucidate the interactions between the FA/BRCA pathway with the different mechanisms of DNA repair, the relevance of this pathway in the repair of different insults to the DNA is now clear, accounting for the involvement of the FA/BRCA pathway in hereditary and also acquired cancer. Significant advances in the management of the hematopoietic syndromes that FA patients suffer have been produced within the last years, particularly in the field of hematopoietic transplantation. Thanks to these advantages, FA is nowadays not considered a restricted pediatric disease. New challenges have therefore emerged, particularly due to the necessity of developing improved therapies for syndromes that appear in adult FA patients, such as squamous cell carcinomas.

As happened in other diseases, gene therapy was soon considered a good option for the treatment of the BMF in FA patients. Although intrinsic difficulties in the manipulation of FA HSCs have limited the success of FA gene therapy, new vectors and improved FA HSC manipulations have emerged from studies conducted with FA mouse models and with samples from FA patients. All these technical advances have opened new hopes for the application of gene therapy in FA.

Finally, as it was the case with the clinical application of cord blood cells [160] or cells derived from siblings selected by pre-implantation genetic diagnosis[161, 162], FA is the first genetic disease where disease-free blood cells from non hematopoietic tissues, have been generated [158]. It is our hope that all these advances may have a translational clinical impact in our patients in the near future.

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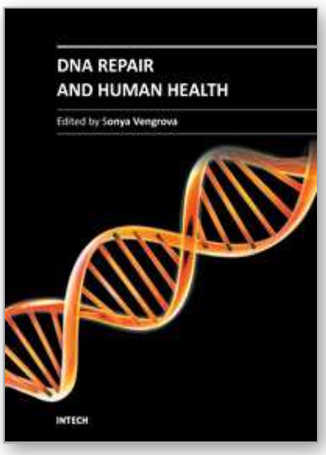

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Over the past decades, great advances have been made in understanding the cellular DNA repair pathways. At the same time, a wealth of descriptive knowledge of human diseases has been accumulated. Now, the basic research of the mechanisms of DNA repair is merging with clinical research, placing the action of the DNA repair pathways in the context of the whole organism. Such integrative approach enables understanding of the disease mechanisms and is invaluable in improving diagnostics and prevention, as well as designing better therapies. This book highlights the central role of DNA repair in human health and well-being. The reviews presented here, contain detailed descriptions of DNA repair pathways, as well as analysis of a large body of evidence addressing links between DNA damage repair and human health. They will be of interest to a broad audience, from molecular biologists working on DNA repair in any model system, to medical researchers.

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