Human Gene Therapy

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Advances in the Gene Therapy for Fanconi Anemia

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Summary

Fanconi anemia (FA) is a rare inherited disease that is associated with bone marrow failure (BMF) and a predisposition to cancer. Previous clinical trials emphasized the difficulties that accompany the use of gene therapy to treat BMF in FA patients. Nevertheless, the discovery of new drugs that can efficiently mobilize hematopoietic stem cells (HSC) and the development of optimized procedures for transducing HSCs using safe, integrative vectors markedly improved the efficiency by which the phenotype of hematopoietic repopulating cells from FA patients can be corrected. Additionally, these achievements allowed the demonstration of the *in vivo* proliferation advantage of gene-corrected FA repopulating cells in immunodeficient mice. Significantly, new gene therapy trials are currently ongoing to investigate the progressive restoration of hematopoiesis in FA patients by gene-corrected autologous HSCs. Further experimental studies are focused on the *ex vivo* transduction of unpurified FA HSCs using new pseudotyped vectors that have HSC tropism. Due to the resistance of some of these vectors to serum complement, new strategies for *in vivo* gene therapy for FA HSCs are in development. Finally, due to the rapid advancements in gene editing techniques, correction of $CD34^+$ cells isolated from FA patients is now feasible using gene targeting strategies. Taken together, these advances indicate that gene therapy can soon be used as an efficient and safe alternative for the hematopoietic treatment of FA patients.

1. Clinical features of Fanconi anemia (FA)

Fanconi anemia (FA) is a rare inherited disorder that occurs at a rate of 1-5 cases per million[1], and is associated with defects in the repair of inter-strand crosslinks (ICL) in DNA and the maintenance of genomic stability[2, 3]. FA is mainly characterized by congenital abnormalities, bone marrow failure (BMF) and a predisposition to cancer. Congenital abnormalities are present in 60-70% of FA patients, and generally include skeletal abnormalities (e.g., radial ray, hip, vertebral scoliosis, rib), skin hyperpigmentation (café au lait spots), microphthalmia and renal abnormalities. Bone marrow failure (BMF) is the main characteristic of FA and generally occurs when patients are between 5 and 10 years-old. Thrombocytopenia or leukopenia typically precedes anemia. Eighty percent of 15 year-old FA patients develop BMF, and the risk of BMF exceeds 90% in FA patients who are

40 and older[4, 5]. FA patients are also prone to develop cancer, principally, acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS). Older FA patients also have a high risk of developing solid tumors, mainly squamous cell carcinomas (SCC)[5-10].

2. Molecular biology of FA

FA is a complex disorder characterized by marked chromosomal instability that arises due to defects in the repair of ICLs. Although exogenous agents that could generate ICLs (i.e., different chemotherapeutic agents such as cis-platinum) are known, endogenous sources that promote formation of ICLs have remained elusive. Recent studies confirmed that aldehydes generated from the metabolism of various molecules, including alcohols or fats, constitute important endogenous agents that can generate ICLs[11]. ICLs generated by either external agents or endogenous sources can be extremely damaging for FA cells, including HSCs[12-15].

FA is caused by mutations in any of the 22 genes that are known as *FANC* genes (*FANCA, FANCB, FANCC, FANCD1/BRCA2, FANCD2, FANCE, FANCF, FANCG/XRCC9, FANCI, FANCJ/BRIP1, FANCL, FANCM, FANCN/PALB2, FANCO/RAD51C, FANCP/SLX4, FANCQ/ERCC4, FANCR/RAD51, FANCS/BRCA1, FANCT/UBE2T, FANCU/XRCC2, FANCV/REV7* and *FANCW/RFWD3*)[16-21]. In most cases the disease is autosomal recessive, with the exception of *FANCB*, which is X-linked, and mutations in the oligomerization domain of *RAD51/FANCR,* which are dominant negative. Mutations in *FANCA* occur in 60% of FA patients, followed in frequency by mutations in *FANCG* and *FANCC*[22, 23]. These FA proteins cooperate in the so-called FA/BRCA pathway, which mainly functions to sense lesions and coordinate ICL repair[18, 24].

At a molecular level, FANCM is the first FA protein that, together with the FA associated protein (FAAP24), and the DNA binding co-factors MHF1 and MHF2, recognizes ICLs[25, 26]. This recognition facilitates recruitment of other members of the FA core complex, including FANCA, FANCB, FANCC, FANCE, FANCG, FANCL, FANCT, FAAP100, MHF1, MHF2, FAAP20 and FAAP24. This complex can monoubiquitinate two different proteins, FANCD2 and FANCI[27, 28], through FANCL (ubiquitin ligase subunit)[29] and FANCT (UBE2T; E2 ubiquitin-conjugating enzyme)[17, 19, 20]. After monoubiquitination, the FANCD2-FANCI complex moves

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to chromatin where recruitment of SLX4 to act as a scaffold activates MUS81-EME1, a heterodimer of SLX1 and XPF(FANCQ)-ERCC1 that facilitates the unhooking of the ICL lesion[30, 31]. Then, translation synthesis polymerases bypass the lesion through insertion and extension of the opposite strand facilitated by REV1 and REV3-REV7[21] to generate a DNA duplex that can be used as a template for the homologous recombination (HR) of the double strand break (DSB) in cooperation with the other FA proteins and canonical HR proteins[16]. When the ICL is resolved, the USP1/UAF1 complex deubiquitinates FANCD2-FANCI, allowing its re-activation when a new ICL is generated[32, 33].

3. Somatic mosaicism: a natural *in vivo* gene therapy in FA patients

Spontaneous reversions of FA gene mutations have been identified in around 20% of FA patients. Different mechanisms accounted for these genetic reversions including microdeletions, microinsertions, missense mutations, intragenic crossover, back mutations, or gene conversions[1, 34-38]. Although in most instances reversions occur in only one or a few specific cell types – mainly peripheral blood (PB) T cells – in a small proportion of FA patients the same reversion was observed in different hematopoietic lineages, indicating that they occurred in pluripotent HSCs[1,34-38]. The observation of progressive improvements in PB T cell counts in a number of mosaic FA patients evidenced the proliferative advantage of these corrected cells[36-38], suggesting that *ex vivo* gene therapy involving autologous FA HSCs could constitute a potential therapeutic approach for rescuing BMF in FA patients.

4. Hematopoietic transplantation in FA patients

Although androgen therapies can promote transient increases in the number of PB cells, allogeneic hematopoietic stem cell transplantation (HSCT) constitutes the preferred therapy for BMF in FA patients[39]. Due to the hypersensitivity of FA cells to DNA-damaging agents[40], reduced conditioning regimens are currently used for HSCT in these patients. Although outcomes for HSCT from HLA-identical siblings were generally good[41-43], those for transplants using cells from alternative donors were initially much poorer. The inclusion of fludarabine in conditioning regimens[44, 45] and T-cell depletion strategies in donor grafts resulted in marked improvements in the outcome of these HSCT modalities[43, 46-48]. Despite these advances, FA patients that underwent HSCT showed an increased incidence of solid

tumors - principally squamous cell carcinomas (SCC) - likely due to the conditioning regimen and the occurrence of graft versus host disease (GVHD)[6, 49, 50].

Based on the efficacy of hematopoietic gene therapy achieved for different monogenic diseases[51-55], and taking into account advances in preclinical and clinical gene therapy studies for FA[56, 57], this therapeutic approach is now considered to be an important alternative to HSCT for the restoration of hematopoietic function in FA patients.

5. Gamma-retroviral and lentiviral gene therapy studies in mouse models of FA

Soon after the discovery of the first FA gene, *FANCC,* by Manuel Buchwald's group in 1992[58], *in vitro* studies of gene therapy with gamma-retroviral vectors (RV) and adeno-associated vectors (AAV) were carried out both in lymphoblast cells lines (LCLs) and hematopoietic progenitors from FA-C patients[59, 60]. Once the murine *Fancc* gene was cloned by the same team[61] and the first mouse models of FA-C were generated[62, 63], gene therapy studies in FA mice were initiated. Evidence of therapeutic efficacy was demonstrated using RVs[64-68], lentiviral vectors (LVs)[69-74] or foamy viral vectors (FVs)[75]. Importantly, each of these vectors was tested in mouse models of FA, including *Fanca*$^{-/-}$ [66, 69, 70, 72-74], *Fancc*$^{-/-}$ [64, 65, 67-69, 76] and *Fancd1/Brca2*$^{-/-}$ [71]. Since HSC defects in *Fancd1*$^{-/-}$ mice closely resembled those observed in HSCs from FA patients[77], this model could be used to demonstrate that gene therapy confers a proliferative advantage of HSCs *in vivo* [71], thus mimicking the behavior of reverted HSCs in FA mosaic patients.

Difficulties in the collection of HSCs from FA patients were also reproduced in FA mouse models[78]. Thus, to improve HSC collection efficiency for gene therapy purposes, different drugs were combined with G-CSF to enhance its HSC mobilization potential. Significantly, the chemokine receptor antagonist AMD3100 was shown to synergize with G-CSF, resulting in a significant mobilization of HSCs in two FA mouse models[79]. Similarly, an interleukin-8 related chemoattractant protein also enhanced the efficiency of G-CSF-mediated mobilization of HSCs from *Fancg*$^{-/-}$ mice[80]. These studies, together with data obtained from patients who had a poor HSC reserve[81], and more recently in a FA clinical trial (see NCT02931071 clinical trial), suggested that the combined use of different

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mobilizing drugs would constitute efficient procedures for collecting significant numbers of HSCs from FA patients for gene therapy purposes.

To enhance the homing of corrected HSCs in transplanted patients, transduction protocols for FA HSCs have been significantly modified. In this respect, studies carried out in different FA mouse models showed improvements in the engraftment of HSCs that had been transduced for short periods of time[72, 75, 82]. In addition to improved engraftment, safety studies carried out in *Fanca*$^{-/-}$ mice transplanted with syngeneic HSCs transduced with the PGK-*FANCA*.Wpre* LV[83] confirmed the efficiency of this approach and showed the safety of LV-mediated gene therapy in a FA-A mouse model[73].

Myeloablative or submyeloablative conditioning constitutes a standard method to facilitate HSC engraftment in FA mouse models. Although conditioning may not be essential for FA patient gene therapy, the mild hematopoietic phenotype observed in most FA models requires the depletion of endogenous hematopoiesis to facilitate engraftment of transplanted HSCs. In most instances, ionizing radiation was used for conditioning, although cyclophosphamide has also been employed to develop gene therapy approaches that have greater clinical applicability [65, 84]. Because of the hypersensitivity of FA cells to cytokines such as tumor necrosis factor-alpha (TNF-α)[68] or interferon-γ[85, 86], these drugs have also been considered for use as conditioning agents[85, 86]. More recently, non-genotoxic drugs, such as monoclonal antibodies targeting HSCs, have been used to condition *Fanca*$^{-/-}$ mice[87]. This latter approach would constitute an ideal conditioning method for FA gene therapy, should conditioning would be necessary to treat these patients.

An alternative approach for facilitating engraftment of corrected FA HSCs involves accessory cells. In this respect, previous studies showed that intrabone transplantation of MSCs facilitated homing of transplanted HSCs from wild type animals[88]. A more recent study demonstrated that co-infusion of MSCs with corrected *Fanca*$^{-/-}$ HSCs facilitates the engraftment of these cells in transplanted animals[74], suggesting that MSCs may constitute relevant cell populations that could be considered for inclusion in clinical FA gene therapy strategies.

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6. Experience from previous FA gene therapy trials involving gamma-retroviral vectors

In contrast to the successes achieved in gene therapy for primary immunodeficiencies and hemoglobinopathies[52-55], to date clinical trials involving FA patients have not shown engraftment of gene-corrected HSCs or clinical efficacy for reverting BMF[89-91]. The first trial included three children and one adult with FA-C[89]. HSCs were obtained from BM or PB isolated from these patients after mobilization with G-CSF. Purified CD34$^+$ cells were then transduced over 3 days with a RV carrying *FANCC*. Up to four infusions of corrected cells were given to these patients at intervals of 3 to 4 months. Although transient improvements in PB cell counts were noted, no sustained hematologic responses were observed, and no gene-corrected cells were observed several months post-infusion. Strikingly, in one patient who received radiation therapy for a concurrent gynecological malignancy, cells with the *FANCC* transgene could be detected.

A subsequent clinical trial was conducted in FA-A patients who had not developed BMF[91]. CD34$^+$ cells were pre-stimulated for 3 days, followed by two rounds of transduction with a RV encoding for *FANCA*. No gene-marked cells could be detected in any of the enrolled patients beyond 3 months post-infusion, and only transient improvements in PB cell numbers were observed.

Possible reasons for the defective engraftment of corrected HSCs in these gene therapy trials include defects in the transduction of true HSCs after relatively long transductions with RVs, infusion of limited numbers of transplanted HSCs, or absence of patient conditioning prior to cell infusion.

7. Advances in lentiviral transduction of FA hematopoietic stem cells in xenogeneic transplant models

Based on the conclusions obtained from previous FA gene therapy trials and studies in FA mouse models, gene therapy approaches for FA patients have been progressively optimized. Given the efficacy and safety of LVs relative to first generation RVs, two similar PGK-*FANCA*.Wpre* LVs were developed[83,92]. Both vectors were used to transduce FA HSCs in a short period of time under conditions that protect FA cells from oxidative damage,

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including the addition of N-acetylcysteine to the culture medium and maintenance of a low oxygen atmosphere during cell culture[83, 92-94]. Due to the hypersensitivity of FA HSCs to TNF-α[95] transduction, the culture medium also included the TNF receptor-Fc fusion protein etanercept to prevent cytotoxic effects induced by this growth factor[94]. As deduced from data obtained in clonogenic assays, these conditions showed optimized survival of hematopoietic progenitors in FA patients.

To study the functional properties of human HSCs, *in vivo* analyses of the ability of these cells to repopulate in immunodeficient mice are frequently performed. NSG (NOD/LtSz-scidIl2rg$^{-/-}$) mice that lack T, B and NK cells currently constitute one of the most efficient models to demonstrate human HSC functionality[96-98]. In the case of FA, previous studies showed a lower HSPC content compared to healthy donor HSCs, and also evidenced homing defects when these cells were transplanted into immunodeficient mice[99]. These observations, together with the limited number of HSCs present in the BM of FA patients [100-102], have limited the study of the long-term repopulation capacity of these cells.

Although several studies showed the feasibility of correcting the phenotype of hematopoietic progenitors from FA patients (i.e., reduced mitomycin C (MMC)-hypersensitivity of FA colony forming cells (CFCs))[59, 83, 92, 93, 103], evidence of correction in FA repopulating cells has remained elusive. One study showed the presence of a low number of marked cells in a single transplanted mouse after the long-term culture of FA transduced cells[104], an approach that is now not recommended for FA cells. A very recent study demonstrated reproducible engraftment of corrected HSCs from FA patients using a clinically applicable transduction protocol[94]. In this study, CD34^{+} cells from FA-A patients were mobilized with G-CSF and plerixafor, and then transduced for a short period of time with the therapeutic PGK-*FANCA*.Wpre* LV[83]. Notably, human myeloid and lymphoid cells were identified in transplanted mice, suggesting the engraftment of repopulating cells having multipotent differentiation capacity. Moreover, the observation of a marked increase in MMC-resistance of engrafted progenitor cells demonstrated for the first time the phenotypic correction and *in vivo* proliferative advantage of corrected HSCs in FA patients[94]. These observations thus suggested that a similar proliferation advantage of corrected FA HSCs should occur after infusion in FA patients, ideally in the absence of

conditioning. Based on improvements achieved in these experimental approaches, two gene therapy trials with FANCA LVs are ongoing with the aim of demonstrating phenotypic hematopoietic correction in FA patients[57] (clinicaltrials.gov ID: NCT02931071 and NCT03157804).

8. FA gene therapy with non-viral vectors and new pseudotyped lentiviral vectors

Based on the safe integration profile of Sleeping Beauty transposon vectors[105], this family of non-viral vectors was proposed for gene therapy approaches in various tissues and diseases that affect hematopoietic and non-hematopoietic tissues[106]. Recent improvements in the transposition of human HSCs[107] suggest that this simple and relatively inexpensive gene therapy approach will have a significant role in future therapies for hematopoietic diseases, including FA[108].

Since a current drawback in FA gene therapy derives from the difficulties involved in efficiently selecting $CD34^+$ cells from FA patients, several studies have pursued the targeting of HSCs using new pseudotyped vectors, such as the measles virus glycoprotein pseudotyped LV (hemagglutinin and fusion protein LVs [H/F-LVs]) that efficiently transduce non-purified HSPCs from FA patients[109]. Conclusions reached from the use of these vectors in experimental models of gene therapy strongly support the relevance of their production under GMP conditions for clinical applications. In addition to these new *ex vivo* strategies of gene therapy, the *in vivo* transduction of FA HSCs also constitutes an attractive approach to avoid the need for *in vitro* manipulation of these very sensitive cells. Since H/F-LVs are resistant to serum complement, these pseudotyped LVs would also constitute a good alternative for future approaches in FA gene therapy[110]. Under the same objective, recent studies showed the possibility of *in vivo* transduction of HSCs with adenoviral and foamy viral vectors[111, 112], suggesting that these new *in vivo* approaches would also be relevant to the development of future FA gene therapies.

9. Next generation of FA gene therapies based on gene editing

To increase the safety of gene therapy, significant advances have also been achieved in gene targeting, due to the design of specific nucleases that markedly increase the efficiency of homologous recombination (HR) in defined sequences of the genome. The

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use of these nucleases combined with the transfer of donor DNA templates facilitated the correction of specific mutations, as well as the insertion of wild type genes in *safe harbor* loci or immediately downstream of endogenous regulatory sequences (*knock-in* strategies)[113].

Although the role of certain FA proteins such as BRCA1 and BRCA2 in HR is clear, the relevance of other FA genes in DNA repair processes is less certain [114]. Thus, the question of whether or not gene editing in FA cells could be feasible has long remained unclear. However, initial studies carried out using fibroblasts from FA-A[115] as well as FA-C and FA-G[116, 117] patients showed the possibility of using gene editing for FA cells. Significantly, a more recent study also confirmed the correction of specific mutations in primary fibroblasts from a BRCA2-deficient (FA-D1) patient[118]. Once gene editing was shown to be feasible in FA cells, editing in LCLs and $CD34^+$ cells from FA-A patients was demonstrated[119]. Designed zinc finger nucleases (ZFNs) and a PGK-*FANCA* sequence flanked by the *AAVS1* homology arms targeted the *FANCA* gene at the *AAVS1* locus with efficiencies of up to 10%[119]. Although the efficiency of gene editing in $hCD34^+$ cells from healthy donors has markedly increased with the use of improved editing tools[113], whether gene-edited FA $CD34^+$ cells will also have *in vivo* repopulating ability, as was already demonstrated in lentivirally-transduced FA-A HSCs, is unknown.

Finally, as was the case with conventional gene therapy, safety concerns must be considered for editing of FA HSCs, particularly regarding the potential off-target activity of nucleases used in these approaches. Although ZFNs used in *AAVS1*-targeting studies showed no off-target activity in any of the top five off-target sites found in *in silico* analyses[119], additional studies will be required prior to the use of these approaches in clinical FA gene therapy.

10. Advantages and current limitations of cell reprogramming in FA gene therapy

Soon after cell reprogramming was described[120], different groups began to use these techniques in regenerative medicine. Given that one aspect that limited hematopoietic gene therapy for FA was the very low number of HSCs present in FA patient BM, the possibility of generating these cells from other non-hematopoietic tissues was thus

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considered. Theoretically, two alternatives can be used for this purpose. Cell types such as skin fibroblasts or keratinocytes could be reprogrammed to generate induced pluripotent stem cells (iPSCs). These pluripotent cells could be then differentiated towards the hematopoietic lineage and the FA genetic defect corrected afterwards. An alternative approach focuses on genetic correction of non-hematopoietic FA cells, followed by reprogramming and re-differentiation of the cells. Previous studies showed that stable FA iPSCs could not be generated from non-corrected FA cells, indicating the involvement of the FA pathway in cell reprogramming[121]. One of the first events that occur during the acquisition of pluripotency is generation of DNA damage[122], as shown in different studies that found very low reprogramming efficiency of uncorrected FA cells due to the inherent DNA repair defects of these cells. Alternative strategies were then used to increase the efficiency of FA cell reprogramming[123-129]. In contrast to these approaches, gene complementation overcame the technical difficulties in generating *bona fide* iPSCs from FA patients [115, 121, 130] and FA mouse models[131, 132].

Despite the great potential of cell reprogramming in regenerative medicine, applications of these technologies in HSCT are currently limited by difficulties in repopulating hematopoietic cells in transplanted animals with either human or mouse iPSC-derived hematopoietic cells[121, 131]. Although some studies have shown the possibility of engrafting hematopoiesis in mice using reprogrammed cells[133], further improvements are required prior to considering use of these techniques for transplantation of patients with monogenic diseases affecting the hematopoietic system, such as FA.

11. Concluding remarks

The last 10 years have seen marked advances in the development of efficient and safe gene therapy approaches for the treatment of various monogenic diseases. Here we summarized the significant achievements and perspectives related to gene therapy involving FA HSCs. In 1999, five years after the first proof of concept for RV-mediated gene therapy using FA cells was described, the first gene therapy trials involving these vectors in BM and G-CSF-mobilized CD34$^+$ cells were conducted. Since 2002, the efficacy of LVs for the treatment of HSCs, either from FA animal models or FA patients, was demonstrated. During the last three years, improved LV-transduction protocols have been developed, and

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evidence of *in vivo* proliferative advantages of gene-corrected FA HSCs was demonstrated in xenogeneic transplantation models. These observations suggested that gene-corrected FA HSCs may progressively replace hematopoiesis in FA patients treated with gene therapy, even in the absence of conditioning. Thus, the first clinical trials with LVs were initiated with the hope that these low toxicity therapies based on the reinfusion of autologous cells will soon be a true alternative to allogeneic transplantation. New *ex vivo* and *in vivo* gene therapy approaches with LVs pseudotyped to specifically target HSCs, or based on gene editing have also been proposed, and may provide additional avenues for the development of gene therapies for FA and other monogenic diseases that affect the hematopoietic system.

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Conflict of interest

The Hematopoietic Innovative Therapies Division receives funding from Rocket Pharma and has licensed the PGK-FANCA-Wpre* LV to Rocket Pharmaceuticals. J. A. Bueren is a consultant for Rocket Pharmaceuticals.

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FIGURE LEGEND

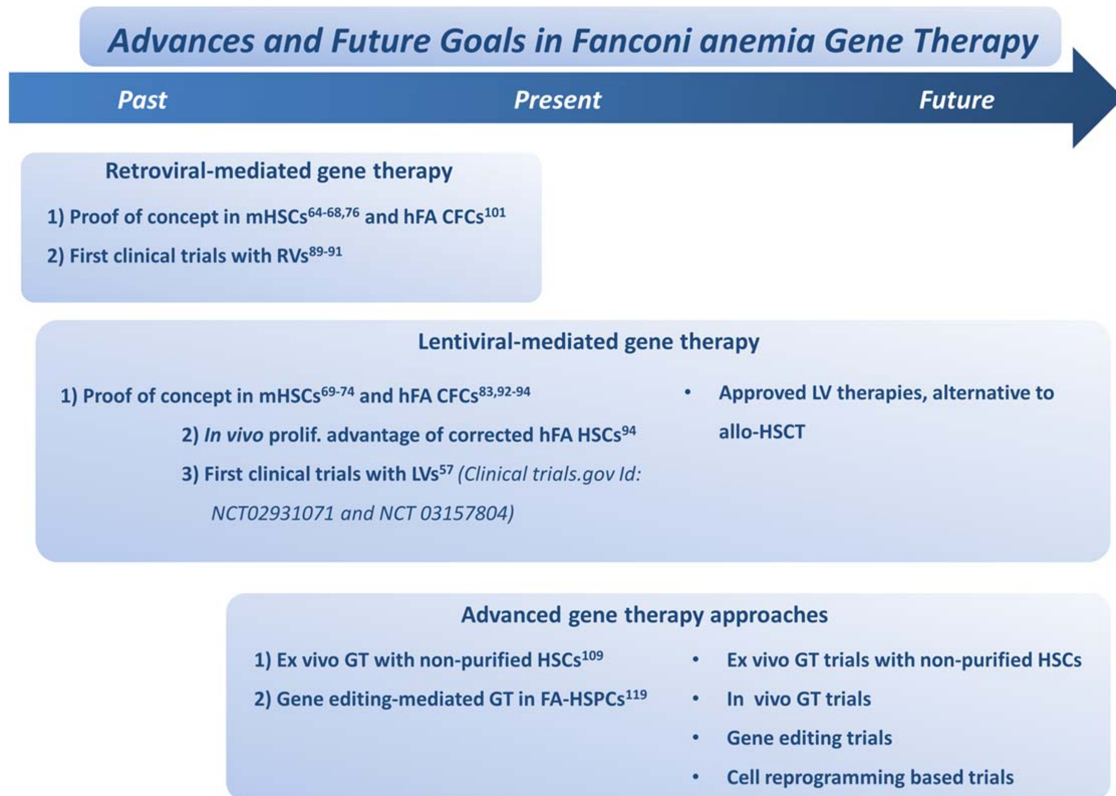

Figure 1: Advances in Fanconi anemia gene therapy during the last 25 years. The figure shows the main achievements obtained and future perspectives in FA gene therapy. GT: Gene Therapy; CFCs: Colony forming cells; HSCs: Hematopoietic stem cells; RV: Retroviral Vector; LV: Lentiviral vector; HSPCs: Hematopoietic stem and progenitor cells; BMT: Bone marrow transplantation

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