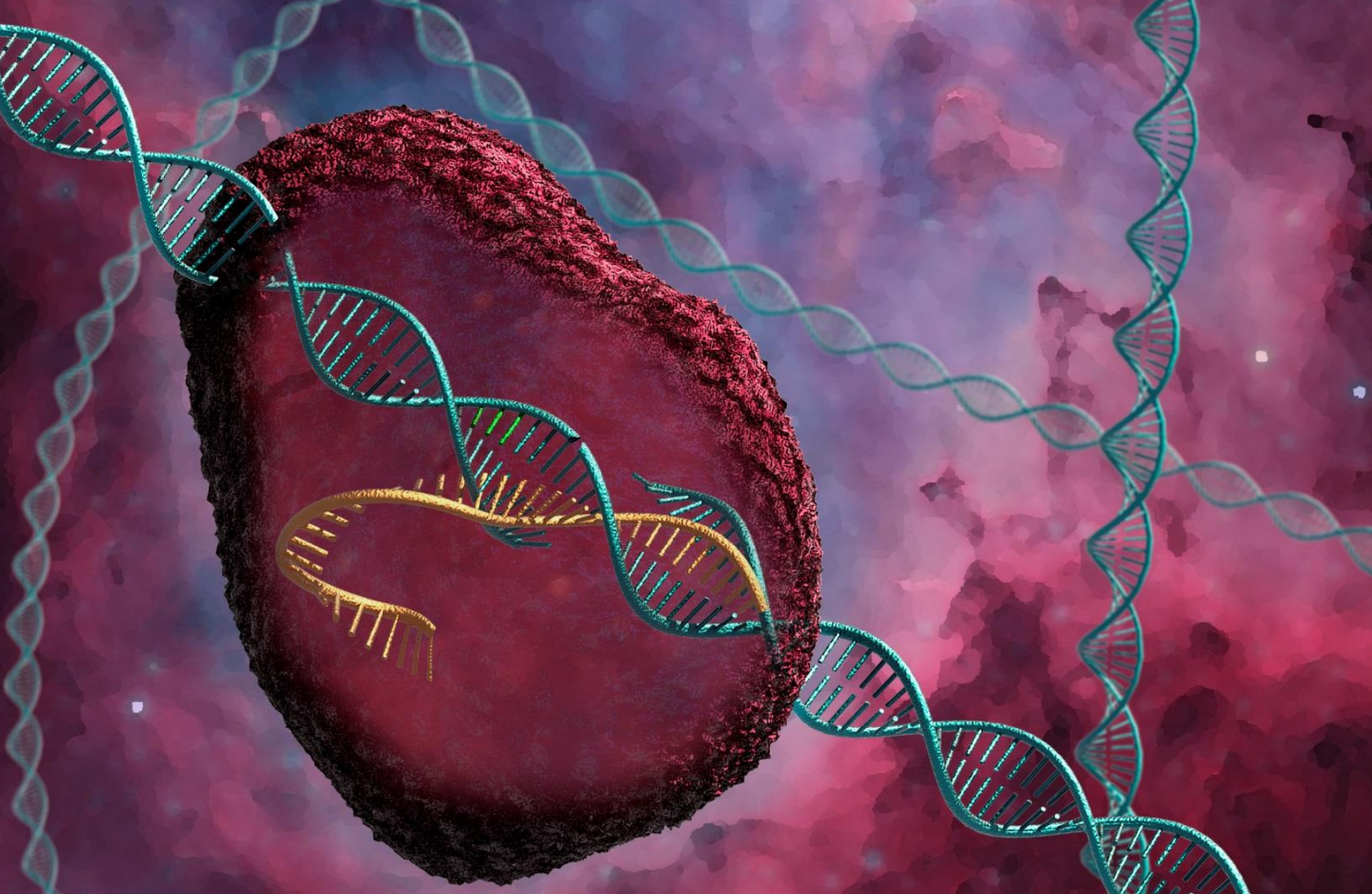
UNIVERSIDAD AUTÓNOMA DE MADRID
Doctorado en Biociencias Moleculares

Gene Editing Mediated by Non-Homologous End-Joining: a Versatile Approach for the Gene Therapy of Hematopoietic Stem Cells from Fanconi Anemia Patients



Francisco José Román Rodríguez

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Gene Editing Mediated by Non-Homologous End-Joining: a Versatile Approach for the Gene Therapy of Hematopoietic Stem Cells from Fanconi Anemia Patients

Tesis Doctoral escrita por Francisco José Román Rodríguez, licenciado en Biotecnología, para optar al grado de Doctor en Biociencias Moleculares con mención internacional por la Universidad Autónoma de Madrid.

Directores de Tesis

Paula Río Galdo

Juan Antonio Bueren Roncero

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CENTRO DE INVESTIGACIONES ENERGÉTICAS, MEDIOAMBIENTALES Y TECNOLÓGICAS (CIEMAT)

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Paula Río Galdo, científica titular de OPIS de la División de Terapias Innovadoras en el Sistema Hematopoyético del Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (CIEMAT), y **Juan Antonio Bueren Roncero**, investigador titular de OPIS y Jefe de la División de Terapias Innovadoras en el Sistema Hematopoyético del CIEMAT, certifican que la presente memoria titulada *Gene Editing Mediated by Non-Homologous End-Joining: a Versatile Approach for the Gene Therapy of Hematopoietic Stem Cells from Fanconi Anemia Patients* ha sido realizada por Francisco José Román Rodríguez bajo la dirección conjunta de los que suscriben, y cumple las condiciones exigidas para optar al título de Doctor en Biociencias Moleculares (R.D. 99/2011) con mención internacional por la Universidad Autónoma de Madrid.

Paula Río Galdo

Juan Antonio Bueren Roncero

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"I don't know half of you half as well as I should like;
and I like less than half of you half as well as you deserve."
— J.R.R. Tolkien, The Fellowship of the Ring.

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Hace mucho tiempo, en una galaxia muy, muy lejana –de cuyo nombre no quiero acordarme– no ha mucho tiempo que vivía un becario en un pequeño estudio de Argüelles. Pero no un estudio húmedo (a pesar de los esfuerzos de la buena señora de arriba), sucio, repugnante, con la cama entre el fregadero y al váter; ni tampoco un estudio, oscuro, lúgubre y ruinoso, sin nada en que sentarse o que comer. Era un agujero-hobbit y eso significa comodidad.

–¿Cómo llegó este becario a un agujero-hobbit en Madrid, si ya vivía en La Comarca (de la Loma)?

El primer punto de inflexión fue la Biotecnología.

–¿Bioqué? –solían preguntar siempre todos aquellos a los que se lo contaba–. Pues eso: quería estudiar Biotecnología.

La Genética había sido algo que siempre me había interesado y (a pesar de lo mucho que me gusta la Historia) la idea de montarme un Jurassic Park en Sevilla me acabó atrayendo mucho más que lo de convertirme en Indiana Jones (¡cuánto daño hace el cine…!).

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Me puedes llamar a mi mv cuanto antes para hablar sobre las becas.

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"This book was written using 100% recycled words."

— Terry Pratchett, Wyrd Sisters.

Summary

Preclinical as well as clinical studies suggest that gene therapy based on the infusion of autologous hematopoietic progenitor and stem cells (HSPCs) previously corrected with lentiviral vectors may constitute a future alternative for treating Fanconi anemia (FA) patients. In this context, gene editing appears to be a new step in the development of safe and precise gene therapy approaches. Since non-homologous end-joining (NHEJ) is the preferred mechanism to repair DNA double-strand breaks in quiescent cells, such as HSPCs, and given that this pathway has been reported to be enhanced in FA cells, we have tested the feasibility of using NHEJ to generate compensatory mutations that may restore the function of FANCA protein, thus mimicking the spontaneous reversions reported in some FA mosaic patients.

Our hypothesis was initially tested in two FA-A patient-derived lymphoblastic cell lines carrying different mutations in *FANCA* using the CRISPR/Cas9 system. Analyses of the targeted sites by next generation sequencing (NGS) revealed the presence of cells harbouring potentially therapeutic repair events whose frequency increased over time. Functional analyses confirmed the re-expression of a new functional FANCA protein capable of correcting the FA cell phenotype. Importantly, transplantation of healthy donor HSPCs after NHEJ-editing in immunodeficient (NSG) mice showed the feasibility of efficiently targeting long-term repopulating HSCs. Moreover, when FA-A patient HSPCs were targeted by our CRISPR/Cas9 nuclease, therapeutic NHEJ-repair events were identified by NGS, showing up to 50-fold *in vitro* expansion after only 9 days in culture. Furthermore, the transplantation of limited numbers of FA-edited $hCD34^+$ cells into an NSG mouse showed a remarkable *in vivo* expansion of corrected cells. Additionally, corrected cells showed the reversion of the hypersensitivity to mitomycin C, defined as a hallmark of FA cells. All together, these results demonstrate for the first time the NHEJ-mediated phenotypic correction of FA HSPCs. The NGS analyses of the top-five *in silico* predicted off-target loci in edited FA HSPCs showed no unspecific activity, confirming the safety of this new approach.

Moving forward to *in vivo* applications of NHEJ-mediated repair approaches, serotype 6 adeno-associated viral vectors (AAVs) were tested *in vitro* and also *in vivo*, demonstrating the possibility of transducing hematopoietic progenitor cells in both settings. Moreover, the delivery of the CRISPR/Cas9 system via an all-in-one AAV confirmed the feasibly of these vectors to edit human HSPCs, opening the possibility of future *in vivo* NHEJ-mediated gene editing approaches in FA.

Resumen

Actualmente, estudios tanto preclínicos como clínicos sugieren que la terapia génica basada en la infusión de células madre hematopoyéticas (CMHs) autólogas previamente corregidas a través de vectores lentivirales representa una alternativa de futuro para el tratamiento de la anemia de Fanconi (AF). En este contexto, la edición génica constituye un avance en el desarrollo de nuevas aproximaciones de terapia génica seguras y precisas. Puesto que la unión de extremos no homólogos (NHEJ, de sus siglas en inglés) es el principal mecanismo de reparación de roturas de doble cadena en el ADN de células quiescentes, como las CMHs, y teniendo en cuanta que esta ruta se encuentra favorecida en células AF, hemos evaluado la posibilidad de utilizarlo con el objetivo de generar mutaciones compensatorias que puedan restaurar la función de la proteína FANCA, emulando las reversiones espontáneas descritas en algunos pacientes mosaicos de AF. Inicialmente, testamos esta hipótesis en dos líneas celulares linfoblastoides derivadas de pacientes AF-A utilizando el sistema CRISPR/Cas9. El análisis de los sitios diana por secuenciación masiva (NGS, de sus siglas en inglés) reveló la presencia de células que contenían eventos de reparación potencialmente terapéuticos cuya frecuencia aumentaba con el tiempo. Los estudios funcionales confirmaron la reexpresión de una nueva proteína FANCA que corrigió el fenotipo de las células con AF. Asimismo, el trasplante de CMHs sanas después de la edición por NHEJ en ratones inmunodeficientes tipo NSG demostró la posibilidad de modificar eficientemente verdaderas CMHs con capacidad de reconstitución a largo plazo. Además, cuando editamos CMHs de pacientes AF mediante CRISPR/Cas9, los análisis por NGS demostraron la presencia de eventos terapéuticos de reparación por NHEJ, cuya frecuencia se incrementó 50 veces después de tan sólo 9 días de cultivo. Las células corregidas demostraron, además, la reversión de la hipersensibilidad a mitomicina C, característica de la AF, así como una significativa expansión *in vivo* tras su trasplante a un ratón NSG, confirmando la funcionalidad de la nueva proteína FANCA. Estos resultados demuestran por vez primera la corrección fenotípica de CMHs de pacientes AF mediada por NHEJ. El análisis por NGS confirmó la seguridad de la estrategia, al no encontrar edición inespecífica en los cinco principales *off-targets* predichos *in silico*. Buscando la posibilidad de aplicar esta estrategia *in vivo*, utilizamos un vector viral adenoasociado de serotipo 6, el cual demostró ser capaz de transducir CMHs de forma eficaz *in vitro* e *in vivo* y así como de mediar la introducción del sistema CRISPR/Cas9 en su versión reducida, abriendo la posibilidad de una futura aplicación *in vivo* de la edición génica por NHEJ en AF.

"Este museo de arcángeles disecados,

este perro andaluz sin domesticar,

este trono de príncipe destronado,

esta espina de pescado,

esta ruina de don Juan."

— Joaquín Sabina, Nos sobran los motivos.

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"Life is too short and DNA is too long."

— Michael Crichton, Jurassic Park.

Abbreviations

A	Adenine
AAV(s)	Adeno-associated viral vector(s)
BM	Bone marrow
BMF	Bone marrow failure
bp	Base pair
C	Cytosine
Cas	CRISPR-associated (genes)
CGD	Chronic granulomatous disease
CRISPR	Clustered regularly-interspaced short palindromic repeats
DDR	DNA damage response
DEB	Diepoxybutane
DSB(s)	Double-strand break(s)
dsDNA	Double-stranded DNA
FA	Fanconi anemia
FAAP	Fanconi anemia associated proteins
G	Guanine
GFP	Green fluorescent protein
gGM10	Guide RNA designed to target the c.295C>T mutation number 10
gGM4	Guide RNA designed to target the c.295C>T mutation number 4
gINS11	Guide RNA designed to target the c.3558insG mutation number 11
gINS8	Guide RNA designed to target the c.3558insG mutation number 8
HD	Healthy donor
HDR	Homology-directed repair
hpe	Hours post-electroporation
hpt	Hours post-transplantation/transduction
HR	Homologous recombination
HSC(s)	Hematopoietic stem cell(s)
HSCT	Hematopoietic stem cell transplantation
HSPC(s)	Hematopoietic stem and progenitor cell(s)
ICL(s)	Interstrand crosslink(s)
IDLV(s)	Integrase-deficient lentiviral vector(s)

Indel(s)	Insertion/deletion event(s)
IVT	*In vitro* transcription/transcribed
LCL(s)	Lymphoblastic cell line(s)
LT	Long term
LV(s)	Lentiviral vector(s)
MMC	Mitomycin C
MOI	Multiplicity of infection
mPB	Mobilized peripheral blood
NER	Nucleotide excision repair
NGS	Next generation sequencing
NHEJ	Non-homologous end-joining
NSG	Immunodeficient Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ mouse
ORF	Open-reading frame
PAM	Protospacer adjacent motif
PB	Peripheral blood
RNP	Ribonucleoprotein
RNP4	Ribonucleoprotein complex assembled with IVT gGM4
RVD	Repeat-variable di-residue
SD	Standard deviation
sgRNA	Small guide RNA
sc	Self-complementary
SCD	Sickle-cell disease
ss	Single-stranded
ssODN	Single-stranded donor oligonucleotide
T	Thymine
TALEN(s)	Transcription activator-like effector nuclease(s)
TLS	Translesion synthesis
UCB(s)	Umbilical cord blood(s)
WT	Wild-type
X-SCID	X-linked severe combined immunodeficiency
ZFN(s)	Zinc-finger nuclease(s)

Dos caracoles un día
tuvieron fuerte quimera
sobre quién mayor carrera
en menos tiempo corría.
Y una rana les decía:
Yo he llegado a sospechar
que sois ambos a la par
algo duros de mover;
antes de echar a correr,
mirad si podéis andar.
– Juan Eugenio Hartzenbusch,
Fábulas en verso castellano.

Introduction

1. Fanconi Anemia

1.1 General features of the disease

Fanconi anemia (FA) is a genetic inherited disorder characterized by the presence of developmental abnormalities, increased cancer predisposition and hematological defects that progressively lead to bone marrow failure (BMF), the main cause of death during the first decade of life (32, 192). It is caused by mutations that impair the function of a DNA damage response (DDR) mechanism known as FA/BRCA pathway. The altered function of this pathway results in genetic instability, the key pathogenic mechanism behind the disease (65). The disease was first described in 1927 by the Swiss pediatrician Guido Fanconi in a family with five children, three of whom developed a severe condition resembling a kind of pernicious anemia that finally had fatal consequences (87). Later studies conducted in peripheral blood (PB) cells from these and other patients evidenced that not only the erythropoiesis, but also all the hematopoietic lineages were affected, being these alterations the main cause of mortality (165).

1.2 Genetics and molecular biology of Fanconi anemia

FA is a genetically heterogeneous syndrome caused by mutations in any of the 22 genes known as FANC genes, traditionally named alphabetically: from *FANCA* to *FANCW*. In the majority of the cases, FA behaves as an autosomal recessive disorder, except for those cases associated with mutations in *FANCB*, which is located on chromosome X (175), and with mutations in the oligomerization domain of *FANCR/RAD51*, which are dominant negative (275). *FANCA* is the most commonly mutated, accounting for approximately 60% of FA patients worldwide, followed by *FANCC* (12%) and *FANCG/XRCC9* (10%) (190). In the case of FA patients from Spain, these numbers increase for *FANCA* (80%) and *FANCG* (15%), while the estimated frequency for *FANCC* is slightly lower (10%) (46).

FA incidence is calculated to be approximately 1-3 cases per 500,000 births (32). However, a higher incidence of the disease has been reported in certain ethnic groups as well as in genetically isolated populations because of founder effects associated with particular mutations (46). One of the most interesting examples is the case of the Spanish Gypsies, which show the highest FA prevalence in the world, with an estimated carrier frequency of 1/64 (40). Strikingly, 30% of FA patients in Spain belong to the Gypsy ethnic group and share the same null mutation in *FANCA* exon 4, in homozygosis: c.295C>T (p.Q99X) (46).

1.2.1 FANC genes and FA pathway

FANC genes encode for proteins that cooperate in the FA/BRCA pathway, a DDR mechanism mainly involved in sensing and resolving DNA interstrand crosslinks (ICLs) (140). Any alteration in this pathway gives rise to genomic instability and cytotoxicity (65, 68).

ICLs are covalent links occurring between opposite strands of double-stranded DNA (dsDNA) due to the presence of bifunctional alkylating agents. These linkages are extremely cytotoxic as they block the replication fork and the transcription process (118). They occur as a consequence of the exposure to either environmental pollutants and chemotherapeutic agents, such as mitomycin C (MMC), diepoxybutane (DEB) (12, 233) or cisplatin (211); or to subproducts of the endogenous metabolism (52), mainly through the generation of reactive aldehydes due to alcohol detoxification (97), histone demethylation and lipid peroxidation (259).

Classically, the FANC proteins are classified into three groups:

1.- The FA core complex: recognizes DNA lesions and monoubiquitinates FANCD2-I heteroduplex. It is constituted by FANCA, -B, -C, -E, -F, -G/XRCC9, -L, -M and –T/UBE2T.

2.- FANCD2-I heteroduplex: trigger the recruitment and activation of the effector proteins.

3.- The downstream/effector proteins that unhook the ICL (FANCP/SLX4 and FANCQ/ERCC4) and conduct DNA repair by homologous recombination (HR) (FANCD1/BRCA2, -J/BRIP1, -N/PALB2, -O/RAD51C, -R/RAD51, -S/BRCA1, -U/XRCC2, -V/REV7 and –W/RFWD3).

Additionally, the so-called FA-associated proteins (FAAPs) constituted by FAAP10/MHF2, FAAP16/MHF1, FAAP20, FAAP24, PAAP100 and FAN1, cooperate in the pathway although mutations in their genes have not been found in any FA patient so far (140).

1.3 FA/BRCA pathway: a master regulator of different DNA damage responses

The resolution of DNA interstrand crosslinks is a complex multi-step process conducted by the coordinated action of FANC proteins together with specific enzymes from other repair pathways. Therefore, a major function of the FA/BRCA is to coordinate each independent DNA repair pathway to orchestrate the removal of the ICLs. The main DNA repair pathways that interact with the FA network are Nucleotide excision repair (NER), Translesion synthesis (TLS), Homology-directed repair (HDR) and Non-homologous end-joining (NHEJ) (72, 155).

1.3.1 Nucleotide excision repair

NER is a versatile pathway involved in the recognition and removal of many different DNA lesions (103). It eliminates damaged DNA bases in the form of 24-32 nucleotide-long oligomers after conducting dual incisions, one on either side of the affected nucleotide (185, 256). This ability is exploited by the FA pathway to promote the unhooking of the ICL (185).

1.3.2 Translesion synthesis

TLS is a DNA damage tolerance mechanism that bypasses DNA lesions through the activity of low fidelity DNA polymerases that allow completion of the replication process without correcting the lesion, preventing prolonged replication stalling. As the unhooking of the ICL generates single-stranded DNA (ssDNA) gaps, the FA pathway requires the action of TLS polymerases to fill these gaps for the repair of the DNA damage (120).

1.3.3 Homology-directed repair

HDR is a mechanism involved in the highly accurate repair of double-strand breaks (DSBs) using the homologous DNA strand as a template. However, as this donor template is only available when cells are dividing, it takes place almost exclusively during S phase (229). FA pathway works in combination with the HDR to correct the DSB generated after the excision of the ICL.

1.3.4 Non-homologous end-joining

NHEJ is an error-prone DSB-repair mechanism consisting of the direct ligation of the two DNA ends without regard for homology. Usually, the processing of both ends occurs prior to the re-joining, which generates insertions and/or deletions (indels) that alter the original sequence. It takes place during the whole cell cycle, when no sister chromatid is available to be the template for HR (161). During S phase, cells face the decision of whether to undergo HDR or NHEJ, and it is suspected that FA pathway plays a role in this choice, promoting the repair of the DSB by the high-fidelity HDR (155) by precluding the interaction of the NHEJ machinery with the free DNA ends (36). In the case of hematopoietic stem and progenitor cells (HSPCs), it was demonstrated that under genotoxic or oxidative stress, the FA pathway coordinates with the poly-ADP-ribose polymerase 1 (PARP1), a major DDR protein, to prevent excessive NHEJ (81).

1.3.5 One pathway to rule them all: how FA/BRCA pathway coordinates ICL repair

When a stalled replication fork is detected, FANCM, in association with FAAP10/MHF2, FAAP16/MHF1 and FAAP24 (51, 248), mediates the translocation and anchoring of the FA core complex, together with FAAP20 and FAAP100, to damaged DNA (Figure 1). Then, the FA core complex monoubiquitinates FANCD2 and FANCI (98, 252) through the coordinated action of FANCL –ubiquitin-ligase (178)– and FANCT/UBE2T –ubiquitin-conjugase (122, 216)–. Monoubiquitination primes the heterodimerization and activation of FANCD2-I, which relocate to the damaged DNA in a process orchestrated by ATR and FANCS/BRCA1 (31) to unhook the ICL from one of the two parental DNA strands, uncoupling one sister chromatid from the other (150). The unhooking takes place through FANCP/SLX4 that acts as a molecular scaffold holding together FANCQ/ERCC4-ERCC1, MUS81-EME1 and SLX1 endonucleases (144, 146). These proteins form the nuclear repair foci that promote nucleolytic cleavage at 3′ and 5′ of the ICL, converting the stalled replication fork into a double-strand break (DSB) (146, 285) and originating ssDNA gaps in one strand of the double helix.

As the next step, the FA core complex recruits TLS polymerases REV1 and REV3-FANCV/REV7 to fill the ssDNA gaps resulting from ICL unhooking (29). The repaired DNA is then used as a template for the resolution of the DSB by HR in a process where FANC and canonical HDR proteins act coordinately (50). One of the triggering steps seems to be the polyubiquitination of the replication protein A (RPA), conducted by FANCW/RFWD3 (174), that primes the recruitment of FANCR/RAD51 (5, 275). FANCD1/BRCA2, FANCO/RAD51C, FANCN /PALB2 and FANCU/XRCC2 also move to the chromatin, promoting the formation of a FANCR/RAD51 ssDNA nucleofilament that invades the sister chromatid to search for the homologous DNA template, forming the characteristic D-loop structure that induces the resolution by HR (24). The process is initiated by the end-resection at the DSB promoted by the recruitment of CtIP induced by FANCD2 and FANCS/BRCA1 (232). The induction of HR is also promoted by FANCC which prevents the interaction between NHEJ factors and the DSB ends (36). The resolution of FANCR/RAD51 nucleofilaments is performed by FANCJ/BRIP1, which unwinds D-loops thanks to its 5′ to 3′ helicase activity (109). The next step is the removal of the unhooked ICL via nucleotide excision repair (NER) mechanism (52). Then, FANCW/RFWD3 induces the replication fork restart (149). Finally, the deubiquitinating USP1-UAF1 complex removes the ubiquitin residues from FANCD2-I heterodimer to inactivate the complex (54, 196).

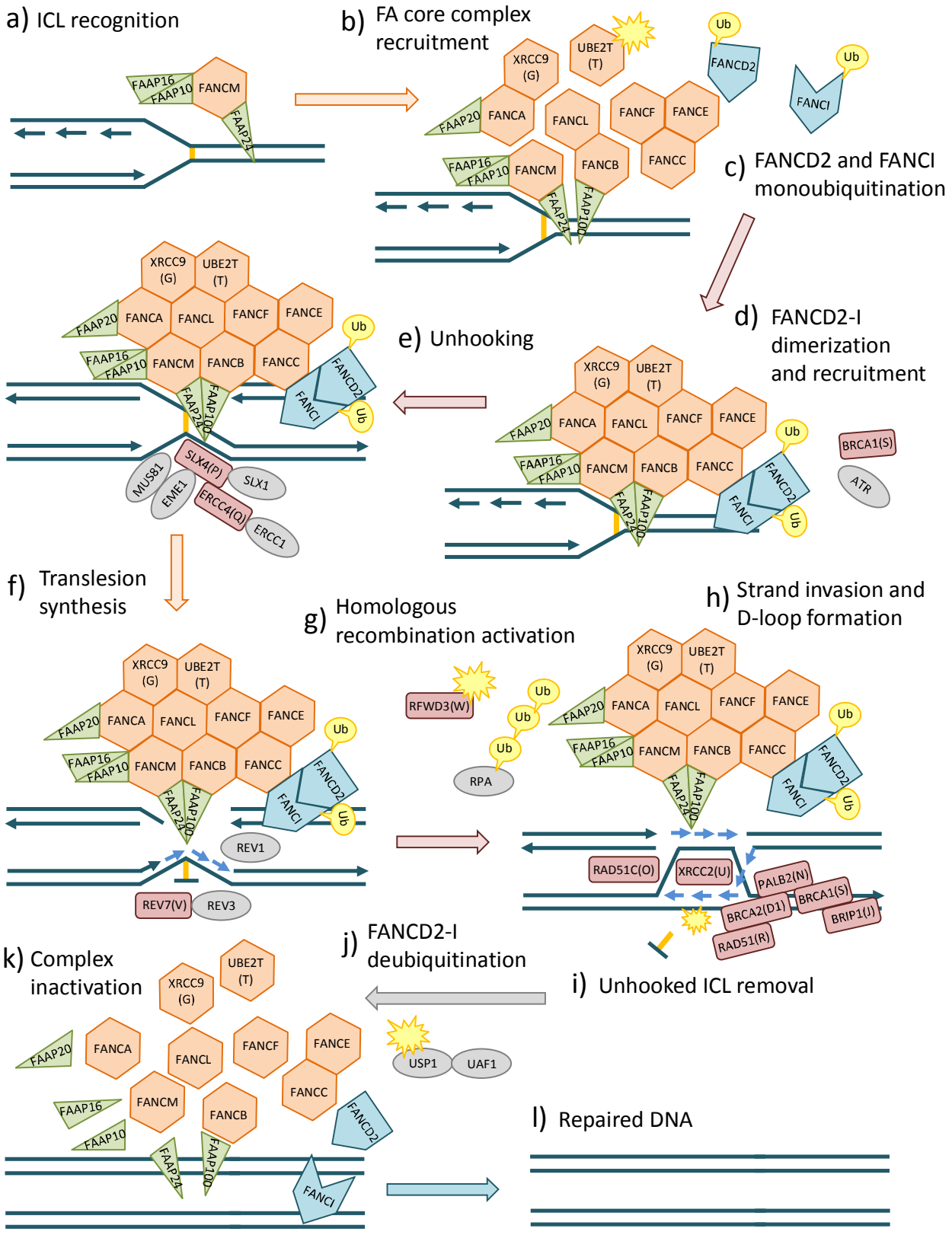


Figure 1.- ICL repair by the FA/BRCA pathway. The most relevant steps of the current model of the FA/BRCA pathway are represented (224, 261). FA core complex protein are coloured in orange, FANCD2-I heteroduplex in blue, downstream effector proteins in pink, FA-associated proteins in green and other proteins involved in the pathway in grey. For those proteins with two names, the FA letter is shown in brackets.

1.4 Phenotypic features of Fanconi anemia

1.4.1 Cellular and molecular phenotype

The presence of ICL-repair defects is the distinctive hallmark of FA (13) and accounts for the cells hypersensitivity to ICL-inducing agents (12), G2/M cell cycle arrest (241) and chromosomal instability (45). In addition, FA cells show a high sensitivity to oxygen (134, 228, 235) and aldehydes, and an exacerbated production of proinflammatory cytokines (226, 238), which are proapoptotic for HSCs (32). Although the FA/BRCA pathway is required to preserve genome stability of every cell type, there are some tissues, such as the hematopoietic system, where the consequences of an impaired FA pathway are more exacerbated due to their continuous proliferative activity. Moreover, in contrast to other monogenic blood disorders, such as sickle cell disease (SCD) or chronic granulomatous disease (CGD) where the defects only appear in matured hematopoietic cells, in the case of FA the primitive HSCs are also heavily affected (49, 130).

1.4.2 Clinical phenotype

The complex cellular and molecular phenotypes associated to FA give rise to a heterogeneous clinical phenotype in FA patients that can be classified into four categories:

- Physical abnormalities such as short stature, abnormal skin pigmentation (*café au lait* macules, hypo- and hyperpigmentation), skeletal malformations (hypoplastic thumbs and radius), microcephaly and ophthalmic and genitourinary tract anomalies. These are present in approximately 75% of FA individuals, who frequently exhibit several of them (80).
- Endocrine defects (hypothyroidism and abnormal insulin and glucose levels) and reproductive alterations (hypospadias, micropenis, cryptorchidism, hypo- or azoospermia and reduced fertility in males; bicornuate uterus and small ovaries in females) (82, 237).
- Cancer predisposition: especially to myelodysplastic syndrome, acute myeloid leukemia and solid tumours, such as head and neck squamous cell carcinomas in esophagus and vulva; cervical cancer, and liver tumours. Additionally, in *FANCC*, *-D1/BRCA2*, *-J/BRIP1*, *-M*, *-N/PALB2*, *-O/RAD51C* and *-S/BRCA1* genetic subtypes, the disease is linked to breast and ovarian cancer predisposition in adult patients and also in monoallelic carriers (190).
- Hematological defects: although blood cell counts are usually normal at birth, the number of HSCs is reduced (49, 138, 269). Progressively, the hematological parameters become

altered, with macrocytosis being the first symptom, followed by thrombocytopenia and neutropenia. Between 5 and 10 years of age, pancytopenia appears and worsens with age (225). Finally, all these defects result in BMF: the decreased production of one or more major hematopoietic lineages, leading to their reduction or even exhaustion (247). BMF is manifested at a median age of 8 years and constitutes the main cause of morbidity and mortality in pediatric FA patients (38).

1.4.3 Somatic mosaicism

Several studies have shown that certain FA mutations have spontaneously reverted in some patients, originating a phenomenon known as somatic mosaicism. This condition has been identified in approximately 20% of FA patients and implies the presence of a mixed FA cell population together with reverted cells that exhibit a healthy phenotype and display proliferative advantage over the diseased ones (106). Although the origin of the reversion has not been completely elucidated, it can take place through different mechanisms such as gene conversions, intragenic crossovers and secondary missense mutations, microdeletions and/or microinsertions that compensate the original mutation (272) (Figure 2).

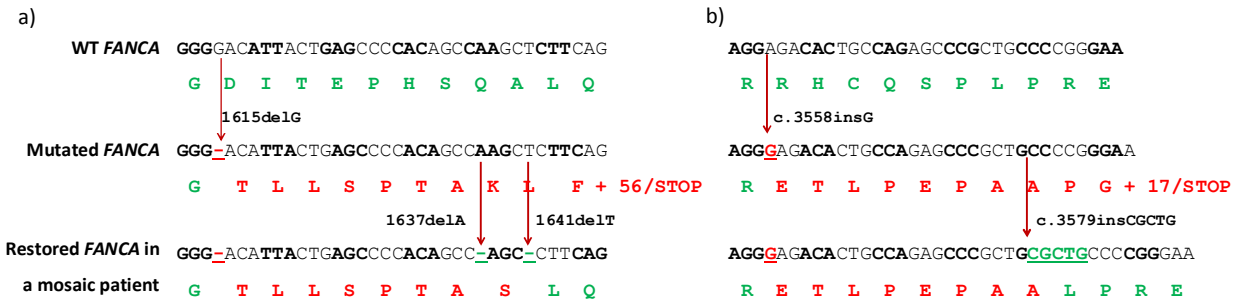


Figure 2.- Reversion events described in two different FA mosaic patients. Schematic representation of the original mutations (signalled using red and underlined characters) that altered *FANCA* gene open-reading frame (ORF) in two different patients (**a** and **b**) and the secondary mutation events that compensated the frameshift (green and underlined). Alternating codons are represented with bold and normal letters. The altered protein sequence is represented in red capital letters and the wild-type one in green. Figure modified from Waisfisz *et al.* Nat. Gen. (1999) (272).

In most of the cases, reversions only take place in committed lineages (T cells mainly), so the recovery is only transiently evidenced in one or two specific cell types (106, 115). However, when it takes place in a pluripotent HSC, the subsequent expansion of this reversion to every cell of the hematopoietic hierarchy can give rise to the improvement of the peripheral blood cell counts in the patient for long periods of time. In fact, in some cases, the proliferative advantage

of the corrected cells counteracts the BMF progression. This phenomenon is considered a natural example of gene therapy (108, 172) and suggests that this approach could be a potential therapeutic alternative for the hematological problem in these patients.

1.4.4 Fanconi anemia diagnosis

The differential test for the diagnosis of FA is the chromosomal breakage analysis of PB T cells in the presence of interstrand crosslinking agents such as MMC or DEB (11, 45, 195), sometimes complemented by G2/M phase cell-cycle arrest in skin fibroblasts when somatic mosaicism is suspected (117, 195). The identification or subtyping of the affected gene is traditionally conducted by complementation analysis: PB T cells are transduced using a battery of vectors –each one containing a healthy copy of a single FANC gene– and then exposed to ICL-inducing drugs. The vector that reverses the hypersensitivity identifies the affected gene, thus determining the patient's complementation group (44). This information is normally confirmed by sequencing. Nowadays, next generation sequencing (NGS) is simplifying the diagnosis, allowing the direct identification of the mutation (102). However, subtyping is still necessary to confirm the pathogenicity of newly-identified mutations.

1.5 Therapeutic aspects of Fanconi anemia

1.5.1 Hematopoietic stem cell transplantation

Currently, the only curative treatment for the BMF and leukemia in FA patients is allogenic HSC transplantation (HSCT) from healthy donors (HDs). Although still challenging, the success of the procedure has improved dramatically in FA patients (26, 83, 271). The high sensitivity of FA patients to conditioning regimens –used to facilitate engraftment of the transplanted cells– compromised their survival, so the amelioration of these preparatory treatments overcame initial excessive toxicities (4). In addition, one of the main difficulties concerning this approach is the availability of HLA-matched donors. It has been estimated that only 25% of FA patients have an HLA-identical donor (84). In this context, a huge effort is being made to improve the outcome of unrelated donor transplant and the use of alternative sources such as HSCs from umbilical cord blood (83, 168). One of the major risks derived from allogeneic transplantation is the development of graft-versus-host disease (GvHD) (26), which also increases the incidence of solid tumours in the long term (4). The main strategy used to prevent this reaction is the use of Fludarabine in conditioning (136, 166) together with T-cell depletion of the donor graft (84, 208).

1.5.2 Pharmacological therapies

When an adequate donor is not available, the only therapeutic alternative is the use of palliative treatments to enhance hematopoiesis and delay BMF. One example is androgen therapy, which have long been used to treat BMF in FA patients when a residual endogenous hematopoiesis still remains. Androgens mainly stimulate erythropoiesis, with lower effects on neutropenia and thrombocytopenia. Those most commonly used nowadays are 17-α-ethinyl testosterone –Danazol– (234), oxymetholone and oxandrolone (39). Approximately 75% of FA patients respond to this therapy. However, the effects are temporary and the prolonged used of oxymetholone and oxandrolone usually produces side effects such as masculinization (82) and increased risk of hepatic tumours (207). Supportive treatments of packed red blood cell transfusions are also important, either alone or combined with androgens to ameliorate the anemia symptoms. However, they are not recommended if the possibility of a transplant exists (82).

1.5.3 Gene therapy

Gene therapy is a therapeutic approach aiming at the cure or prevention of some inherited or acquired disorders by modifying the DNA of the patient. According to the way that the cell genome is modified, gene therapy can be classified into two categories (276):

- "Non-targeted" or "conventional" gene therapy: implies the delivery of a healthy copy of the affected gene (gene addition), which integrates into the cell genome in a non-targeted fashion.
- Targeted gene therapy or gene editing: implies the site-specific therapeutic modification of the genome.

FA disease has been considered a good candidate for gene therapy due to the proliferative advantage observed by the spontaneously reverted cells in mosaic patients and confirmed later on by non-targeted gene therapy (221). Therefore, autotransplantation of the patient's own HSCs, previously corrected by gene therapy, would constitute a potential therapeutic alternative for the treatment of BMF in FA patients.

2. Non-targeted Gene Therapy in HSCs

HSCs are considered ideal gene therapy targets since their self-renewing and long-term (LT) repopulation capacities ensure a permanent functional reconstitution of patients' hematopoiesis after the treatment (188). Furthermore, the use of autologous HSCs previously corrected by gene therapy minimizes side effects associated to allogeneic HSCT, as it implies fewer immunological complications (153).

Nowadays, the procedures to transduce HSCs from mobilized PB (mPB) or BM and their reinfusion into the patient are well established (9, 137), implying that *ex vivo* gene therapy is now frequently used to correct inherited monogenic hematological disorders. It has proven to be not only efficient but also safe in many different clinical trials (2, 27, 42, 47, 112, 113). In these trials, patient HSPCs (including LT-HSCs and multipotent progenitors) were collected by immunoselection of the human CD34 surface marker and a healthy copy of the mutated gene was delivered in a non-targeted way using integrative viral vectors. Although γ-retroviral vectors (γ-RVs) efficiently mediate HSC transduction (153), self-inactivating (SIN) lentiviral vectors (LVs) are the current vectors of choice when modifying HSCs (189) due to the absence of insertional genotoxicity (69, 179, 183) previously shown by RVs (153, 283).

2.1 FA gene therapy

The feasibility of correcting FA cells with different viral vectors has largely been demonstrated *in vitro*. Indeed, complementation analyses conducted in patient cells to identify the affected gene rely on this capacity (255). A wide variety of preclinical studies demonstrated that either γ-RVs (53, 93, 110, 116, 130, 157, 164, 198, 223, 273), LVs (21, 89, 96, 105, 131, 182, 186, 219, 284), adeno-associated viral vectors (AAVs) (274) and foamy viral vectors (246) can successfully transduce not only lymphoblastic cell lines (LCLs) derived from patients (273) but also primary FA fibroblasts (44) and HSPCs (221, 274, 284), delivering a healthy copy of the therapeutic gene. Transduced cells became resistant to ICL-drugs and reversed the G2/M phase cell cycle arrest. Moreover, the development of FA mouse models of *Fanca*$^{-/-}$ (63), *Fancc*$^{-/-}$ (62, 280) and *Fancd1*$^{-/-}$ (191) showed that the transduction of FA murine hematopoietic progenitor cells not only reverted the MMC hypersensitivity in colony forming cell (CFC) assays but also repopulated the BM in these FA models (89, 96, 110, 116, 157, 164, 182, 186, 198, 219, 223, 284), even

demonstrating, in the case of *Fancd1*$^{-/-}$, the *in vivo* proliferative advantage of the corrected HSCs (219).

The main limitation that hampered the development of gene therapy in FA was the difficulties in collecting significant numbers of HSPCs from the patients, as was evidenced in the first clinical trial that evaluated the efficacy and safety of the mobilization and harvesting of HSPCs in FA patients (NCT00271089) (57). Nevertheless, an ongoing mobilization trial (NCT02931071) conducted by the Spanish FA consortium has optimized the collection of significant numbers of HSCs by conducting the harvest in pediatric patients mobilized with G-CSF and Plerixafor. This combination –initially devised for poor mobilizers– (33, 79, 243) has worked efficiently in FA patients, and the preliminary results obtained demonstrate not only its efficacy but also its safety (see NCT02931071 clinical trial).

Concerning the correction of FA patients' HSPCs, three different gene therapy trials using γ-RVs were conducted. They evaluated the efficacy and safety of the reinfusion of autologous FA HSPCs previously corrected by *ex vivo* gene addition of a healthy copy of the mutated gene (*FANCC* in NCT00001399 and NCT00005896, and *FANCA* in NCT00272857 (138)). Although *in vitro* functional gene correction could be observed, none of the trials showed evidence of engrafted, gene-corrected HSCs (57, 163). Furthermore, the hematological improvements registered in the PB cell counts were only transient, thus no complete rescue of the BMF was achieved (1, 220, 290). However, the data they provided paved the way for the advancement of the field. Nowadays, two new gene therapy trials are in progress in the United States (NCT01331018) (1), and in Spain (NCT03157804), both using SIN-LVs carrying a healthy copy of *FANCA* gene under the control of the Phosphoglycerate kinase promoter. Importantly, preliminary results obtained from treated patients in Spain have evidenced the phenotypic reversion of gene-corrected PB and BM cells and the progressive repopulation of the gene-corrected hematopoietic cells even without any conditioning regimen (222). This evidences for the first time that gene therapy could constitute a real therapeutic alternative for FA patients.

3. Gene Editing: Targeted Gene Therapy

Despite the encouraging results showed by the different clinical trials conducted so far (3, 47, 151, 240, 270), huge efforts are still being made in an attempt to further increase the safety of the gene therapy. In this context, two facts gave rise to a paradigm shift in the gene therapy field: the observation that HR could be exploited to introduce exogenous DNA in a sequence-specific manner into the genome (251), and the discovery that nucleases could be engineered to generate targeted DSBs (132), increasing the frequency of the HR more than 1000 fold (67, 212). These observations boosted the sophistication of gene therapy, enabling the targeted correction of the mutations that cause the disease. This targeted gene therapy is also known as gene editing and comprised all those techniques that allow the precise modification of the human genome, thus exploiting the natural cellular mechanisms that repair DNA breaks (16).

3.1 Programmable nucleases

Programmable or engineered nucleases comprise different families of enzymes with the common ability of inducing site-specific DNA cleavages in the genome (141). The most important engineered nucleases used in gene editing approaches are meganucleases, zinc-finger nucleases, transcription activator–like effector nucleases and clustered regularly-interspaced short palindromic repeat and Cas nucleases (230).

3.1.1 Meganucleases

Meganucleases are engineered versions of naturally existing restriction enzymes that have extended DNA recognition sequences (12–45 bp) (250). In contrast to other platforms, DNA binding and cleavage functions are performed by the same domain, so they can only target the region where the restriction site is present. Although hundreds of different natural meganucleases have been identified and chimeric ones have been engineered, the target sites are not so variable as to cover all the human genome, which limits their application in gene therapy (85).

3.1.2 Zinc-finger nucleases

A zinc-finger nuclease (ZFN) is a modular enzyme composed of a nuclease domain derived from the FokI restriction enzyme and a zinc-finger protein (ZFP) domain that mediates DNA

binding selectivity (145). As each domain recognizes a 3-bp DNA sequence (282), normally between 3 and 6 ZFPs are used to increase its specificity, generating a single ZFN subunit that binds to DNA sequences of 9–18 bp. Since the endonucleolytic domain of FokI acts as a dimer, two ZFN monomers are required to form an active nuclease. This dimerization significantly increases the specificity, as it doubles the length of recognition sites which, in addition, must be separated by 5–7 bp spacer sequences (141). However, ZFNs have shown an elevated cytotoxicity due to the off-target activity caused by dimerization of the same monomer (59). Nevertheless, the latest designs have overcome this problem resulting in more specific ZFNs (262). Their main limitation is that they cannot be engineered to target any desired site in the genome, as there is no open-source collection of 64 ZFPs that covers all possible combinations of triplet sites (14). Moreover, the design of the multi-finger domains often requires arduous computational analysis and selection, as the affinity of each ZFP can be altered by the neighbouring ones, making the production of ZFNs laborious and expensive (206).

3.1.3 Transcription activator–like effector nucleases

Transcription activator–like effector nucleases (TALENs) are modular enzymes also used as dimers, as they are also composed of the FokI nuclease. Their DNA-binding domain is a repetitive motif known as transcription activator-like effector (TALE), derived from the plant-pathogenic bacterium *Xanthomonas*. TALEs are composed of tandem arrays of 33–35 amino acid repeats, each of which recognizes a nucleotide in the major groove of the DNA (74). The specificity of each repeat is determined by amino acids at positions 12 and 13, which are called repeat-variable di-residues (RVDs). They can be engineered to target almost any given DNA sequence; the only limitation is the requirement for a Thymine at the 5′ end of the target sequence. Their dimeric structure and relatively long recognition motifs generate a remarkable specificity, showing very rare off-target effects and normally only when the target sequence differs at least 7 nucleotides from any other sequence in the genome (143). Even more, structural modifications have been conducted to reduce the risk of unspecific activity as much as possible (206). TALENs are easier to design than ZFNs but their highly repetitive sequence, together with their length, make their production difficult, as well as hamper packaging into viral vectors, thus limiting their applications (141).

3.1.4 Clustered regularly-interspaced short palindromic repeats and Cas nucleases

Clustered regularly-interspaced short palindromic repeats and Cas nucleases (CRISPR/Cas) are the latest in gene editing tools. In contrast to previous nucleases, their distinctive feature is that the recognition of the targeted sequence is based on RNA-DNA interaction (230).

This gene editing platform derives from CRISPR systems, which are adaptative immune mechanisms used by many bacteria and archaea to protect themselves from foreign nucleic acids, such as viruses or plasmids (19, 181). They are composed of repetitive DNA sequences encoded as arrays within the bacterial genome which are separated by unique spacers of similar length (all together form the so-called CRISPR) and associated Cas (**C**RISPR-**as**sociated) proteins involved in the different stages of the defence mechanism.

Among the wide variety of CRISPR/Cas systems described so far (170), the gene editing tools currently used worldwide derive from Type II. This system incorporates sequences from invading DNAs between the CRISPR repeat sequences of the host genome. The transcription of the CRISPR repeat arrays originate CRISPR RNAs (crRNAs), each containing a variable sequence from the invading DNA –known as "protospacer" sequence– and part of the CRISPR repeat (34). Each crRNA hybridizes with the so-called transactivating CRISPR RNA (tracrRNA) (73) and then, the duplex binds to the Cas nuclease. When the system detects an invading genome containing a sequence complementary to the protospacer-encoded portion of the crRNA, the Cas nuclease catalyses a DSB to avoid infection, but only when this region is adjacent to a precise short sequence known as protospacer adjacent motifs (PAMs) (99).

In 2012, the type II CRISPR system from *Streptococcus pyogenes* was adapted for its *in vitro* use. It was demonstrated that the crRNA, tracrRNA and Cas9 nuclease were enough to induce a targeted DSB in DNA sequences homologous to the crRNA protospacer region, adjacent to the 5'-NGG-3' PAM sequence from *S. pyogenes* Cas9. The system was further simplified by the fusion of the crRNA and tracrRNA into a single chimeric synthetic RNA molecule (named as small guide RNA –sgRNA–) that retains the functions of both individual RNAs (133) and it was suggested that the CRISPR/Cas9 system could be efficiently used as a genome-editing tool (Figure 3). Since that moment, the CRISPR/Cas9 technology has experienced a meteoric expansion. The simplicity and versatility of the system facilitated its delivery in many different ways, either by transfection of the components as plasmid DNA (214), mRNA (277) and ribonucleoprotein (RNP) complex (142), or packaged into adenoviral vectors (78), RVs (64), LVs

(242) and AAVs (213, 215). Moreover, the high activity and specificity of the Cas9 nuclease, together with the low cost of sgRNA design and engineering have made CRISPR/Cas9 one of the preferred genome editing platforms, enabling studies of precise genetic modifications not only for basic science but also for preclinical and even clinical applications (16, 20). In this system, the targeting only depends on the PAM sequence. However, the frequency of the NGG PAM motif from *S. pyogenes* Cas9 is extremely high in the genome. Furthermore, the continuous characterisation of new CRISPR/Cas systems from different prokaryotes (245), together with the generation of improved mutant variants of currently known Cas proteins (7, 148) are broadly expanding their capacity to edit the genome with an efficacy without precedent.

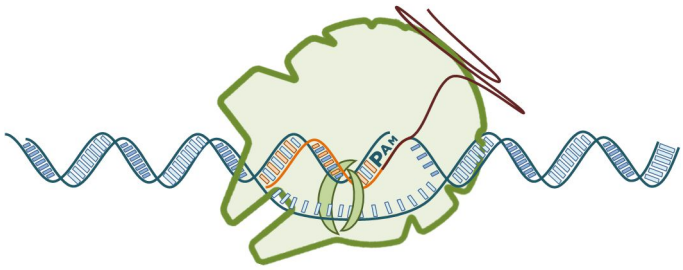


Figure 3.- Schematic representation of the CRISPR/Cas9 system used in gene editing. The sgRNA is composed by the fusion of the crRNA (orange) and tracrRNA (brown) which binds to the Cas9 (green) and guides it to the target site (the complementary region to the crRNA sequence). The Cas9 catalyses the DSB three nucleotides upstream of the PAM.

As occurs with the other programmable nucleases, the major concern regarding the use of the CRISPR/Cas9 system in clinics is the potential off-target activity, especially because, unlike ZFNs and TALENs, CRISPR/Cas9 acts as a monomer and unspecific activity has been reported in off-target loci differing up to several nucleotides from on-target (56, 66, 94, 124). In some instances, this implies the presence of thousands of potential off-targets in the human genome (154). Nowadays, the different sgRNA design software available evaluate the specificity of a given guide according to the number and probability of the different *in silico* predicted off-target loci. However, different studies have demonstrated that, apart from the number and position of the mismatches in the off-target sequence compared to the on-target one, there are other factors that influence the unspecific activity of a CRISPR/Cas9 nuclease such as the level and duration of the nuclease expression, together with target site accessibility (287). Multiple efforts have been made to assess the real off-target activity using experimental, unbiased methods such as digested genome sequencing (Digenome-seq) (139), high-throughput genome-wide translocation sequencing (HTGTS) (92), circulation for *in vitro* reporting of cleavage effects by sequencing (CIRCLE-seq) (268), and chip-hybridized association mapping platform

(CHAMP) (135). Although these methods have demonstrated that unpredicted off-target activity may occur, their frequency and consequences were not as significant as was initially suggested (129), and especially considering that, in a gene therapy context, the activity of these nucleases would be carefully controlled to avoid any undesired activity by using the lowest efficient dose during the shortest period of time. Even so, a huge effort is being made to increase their specificity and minimize as much as possible off-target risks, such as engineering the Cas9 to enhance its fidelity (147) or modify its activity. Mutated versions of Cas9 have been generated to catalyse nicks instead of DSB. These nickases must be used in pairs, such as ZFNs and TALENs, increasing their specificity (171). In addition, truncated sgRNAs have also demonstrated a higher specificity, as only the 12 nucleotides immediately adjacent to the PAM seem to be crucial to target recognition (95). All these advances are giving rise to the development of the most versatile and probably safest gene editing tool engineered for gene therapy designed up to now.

3.2 Gene editing strategies

The DSBs induced by nucleases are the starting point for gene editing. When a DSB occurs, it is mainly repaired by two different pathways: HDR and NHEJ (Figure 4). These natural-occurring pathways can be exploited in different ways to achieve precise objectives.

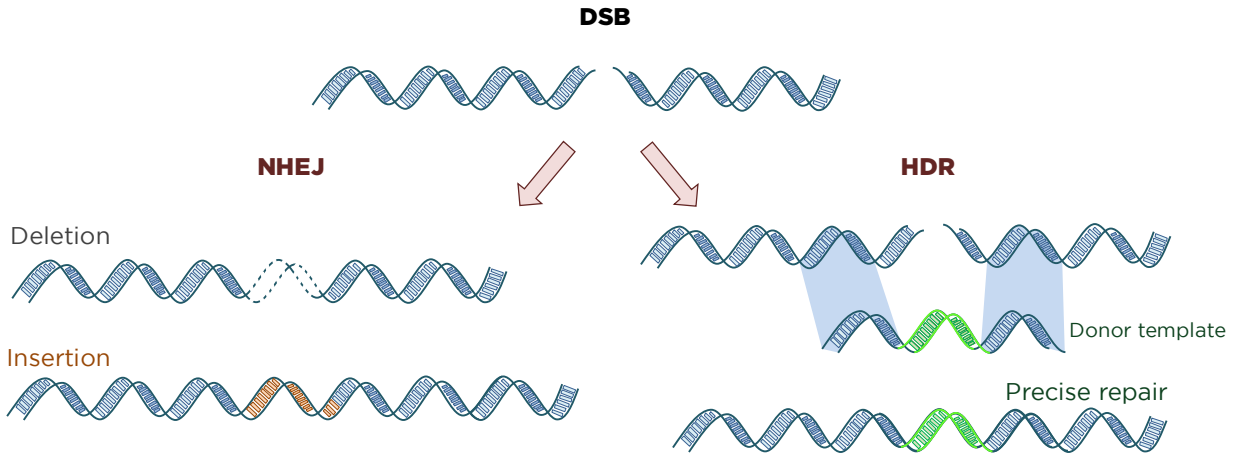


Figure 4.- Schematic representation of a DSB repair. Nuclease targeting generates DSBs that can be repaired by NHEJ or HDR pathways. The error-prone nature of the NHEJ can produce indels of variable length, whereas HDR promotes precise repair when a donor template is available.

3.2.1 Gene targeting

Gene targeting is a gene editing strategy that implies the integration of an exogenous DNA sequence into the genome in a highly specific and precise manner using the HDR pathway. Although this mechanism mainly takes place during S phase, it can be induced by providing a donor template to the cells: to this end, the exogenous DNA sequence must be flanked by homologous arm sequences identical or very similar to the region bracketing the DSB. This precise integration can be used with different goals:

- Introducing a complete transgene into a "safe harbour" locus (167) or to partially modify the endogenous gene (knock in) (75).
- Replacing precise DNA segments to remove (76) or introduce point mutations (128)
- Disrupting (knock out) genes (10).

In contrast to conventional gene therapy, gene targeting markedly limits the risk of insertional mutagenesis as it promotes the precise integration of the therapeutic construct into the desired locus. Furthermore, gene editing now offers the possibility to correct diseases caused by dominant negative mutations that could not be treated by conventional gene addition (152).

Previous results obtained in our laboratory demonstrated for the first time that gene targeting is feasible in FA HSPCs. A healthy copy of the *FANCA* gene delivered by integrase deficient LV was precisely introduced into the adeno-associated virus integration site 1 (*AAVS1*) "safe harbour" locus (167) using ZFNs, giving rise to the phenotypic reversion of the characteristic MMC hypersensitivity of FA cells (77) (see Appendix 3). The main limitation faced in these studies was the efficient targeting of quiescent HSPCs, as HR mainly takes place in diving cells (126). Moreover, the extensive *in vitro* manipulation of the FA HSPCs required for their pre-stimulation, the delivery of the donor sequence and, finally, the introduction of the nucleases compromised the cell viability, thus reducing the overall efficacy of the protocol.

Taking all these facts into account, the main objective of this doctoral thesis was the development of a simpler approach that can efficiently correct FA HSPCs.

3.2.2 NHEJ-mediated gene editing

As previously described in section 1.3.4, NHEJ is an error-prone DNA repair mechanism that directly reconnects the free ends of a DSB, often leading to small indels. When the indel takes place in the coding region of a gene, frameshift mutations normally occur, disrupting its

expression. That is the reason why this DSB-repair mechanism is mainly used for gene knock-out applications. ZFNs have been used to disrupt the C-C motif chemokine receptor type 5 (*CCR5*) expression in human T cells (209) and HSCs (158) to generate artificial resistance to HIV infection. The high efficacy of the strategy gave rise to a phase I/II clinical trial with edited T cells already completed (264) and an ongoing phase I trial with edited HSCs (NCT02500849). However, the error-prone nature of the NHEJ can also be therapeutically exploited in those cases in which slight variations in the protein sequence do not compromise its function. This is the case in Duchenne Muscular Dystrophy (DMD) (203) where Ousterout *et al.* demonstrated that indels generated by targeting the mutation can remove or compensate it, recovering the ORF (204). As the introduction of indels by NHEJ resembles the spontaneous secondary mutation events that cause the reversion in some FA mosaic patients (Figure 2), the possibility of conducting NHEJ-mediated gene editing in FA HSCs has been evaluated in this doctoral thesis as a potential therapeutic approach.

4. *In vivo* Gene Therapy in FA

One of the main challenges in FA gene therapy is the compromised number of HSCs present in the BM of FA patients, as it limits the number and quality of the HSCs available for gene correction and reinfusion (1). Although more efficient mobilization protocols have recently been developed (see NCT02931071 clinical trial), patients in advances stages of the disease cannot benefit from them. Moreover, low CD34 expression leads in general to a low HSPC recovery after the immunoselection procedure (90). In this context, numerous efforts have been made toward the efficient *in vitro* expansion of HSCs (278). However, the modest results obtained so far in healthy donor (HD) cells (88) have precluded its application in FA, especially considering the exacerbated negative impact that the *ex vivo* manipulation of HSPCs has over their viability, self-renewal capacity, homing (291) and repopulation abilities (221). Taking these facts into account, FA treatment would benefit significantly from the development of gene therapy approaches that allow the correction of patient HSPCs *in vivo*. Among the different strategies available nowadays, we have focused on lentiviral vector pseudotyping and adeno-associated viral vectors.

4.1 Lentiviral vector pseudotyping

Pseudotyping is the design and modification of viral envelopes with the aim of improving the transduction efficacy and/or modifying the specificity of the vector (91). In this context, many envelopes have been tested for HSPC transduction: glycoproteins derived from vesicular stomatitis virus (VSV-G) (267), gibbon ape leukemia virus (GaLV) (100), endogenous feline leukemia virus (RD114) (231), baboon endogenous retrovirus (BaEV) (104), cocal vesicular virus (125) and measles virus (MV) (156). Among these, pseudotyping using the GaLV demonstrated to mediate an efficient transduction of FA HSPCs even at low multiplicities of infection (MOI) (130, 131). However, difficulties in good manufacturing practice production hampered its clinical application (266). Additionally, a recent study conducted by Dr. Els Verhoeyen in collaboration with us has demonstrated the remarkable efficiency of MV pseudotyped LVs to target quiescent not pre-stimulated hCD34$^+$ cells without compromising their engraftment and differentiation capacities. Moreover, this envelope demonstrated the capacity to efficiently transduce unfractionated BM samples from FA patients, giving rise to their phenotypic correction (156) (see Appendix 4). This outstanding capacity to mediate the transduction of

HSCs in unfractionated samples makes the MV envelope a promising candidate to be used in a future *in vivo* gene therapy application.

4.2 Adeno-associated viral vectors

AAVs consist of small (25 nm) non-enveloped icosahedral capsids that contains a linear ssDNA genome of 4.7 kb (18). AAVs are one of the most versatile gene delivery systems available to date because, despite their low packaging capacity, their minimalistic genome facilitates its modification. Moreover, they are able to naturally transduce a broad range of target cells with high efficacy, including non-dividing tissues. In addition, their relatively simple capsid organization enables customization of the tropism and efficiency of transduction (119). Derived from non-pathogenic viruses, AAVs display an excellent safety profile due to their non-integrative nature, which avoids the risk of insertional oncogenesis, together with their low immunogenicity. Thanks to these features, AAVs are currently the platform of choice for *in vivo* gene therapy, with the number of clinical trials using this delivery system having increased significantly (107).

Among the 12 different AAV serotypes described so far, AAV2 has been one of the most frequently used in gene therapy trials, mainly because of the broad tropism reported (119). Since AAVs do not integrate in the cell genome, they were initially used to target post-mitotic cells and their use in HSCs was not considered. However, the possibility to deliver donor sequences and also nucleases supported their application in HSC gene editing approaches. Furthermore, serotypes 2 (257) and 6 have demonstrated to be able to transduce $hCD34^+$ cells. Interestingly, the remarkable efficacy shown by AAV6 suggests a tropism for HSPCs (253, 254), presenting another promising candidate to take into account for a future development of FA *in vivo* gene editing approaches.

In this doctoral thesis, the *in vitro* and *in vivo* HSPC transduction efficacy of AAV6 were tested with the aim of developing a future *in vivo* gene editing approach for FA treatment.

"No vamos a conseguir hacer más de lo que podamos.

Pero todos haremos lo posible para que no sea menos de eso."

— Andrzej Sapkowski, La Dama del Lago.

Objectives

The main goal pursued in this doctoral thesis was to investigate whether the error-prone nature of the non-homologous end-joining (NHEJ) repair pathway could be positively exploited to remove/compensate Fanconi anemia associated mutations, leading to the phenotypic correction of hematopoietic stem cells from Fanconi anemia subtype A patients.

To achieve this end, the following objectives were proposed:

1

To design sgRNAs specifically targeting two different mutations in *FANCA* gene with a reported clinical relevance.

2

To determine if the insertions and/or deletions generated as a consequence of the NHEJ-mediated double-strand break repair compensate the pathogenic mutations in lymphoblastic cell lines derived from Fanconi anemia subtype A patients.

3

To verify that NHEJ-mediated gene editing efficiently targets hematopoietic stem cells without compromising their long-term repopulation ability in immunodeficient mice.

4

To correct hematopoietic stem and progenitor cells from Fanconi anemia subtype A patients using the proposed NHEJ-based gene editing approach.

5

To develop a strategy aiming at *in vivo* correction of Fanconi anemia patients' hematopoietic stem and progenitor cells based on this NHEJ-mediated gene editing approach.

"Espada tengo. Lo demás, Dios lo remedie".

— Miguel de Cervantes Saavedra, El casamiento engañoso.

Materials and methods

1. Cell Lines, Primary Cells and Animals

1.1 Cell lines

1.1.1 Lymphoblastic cell lines

Lymphoblastic cell lines (LCLs) from healthy donors (HDs) and FA-A patients were generated by transduction of peripheral blood (PB) cells with Epstein-Barr virus. Three different HD LCLs were used in this study: CP1, CP3 and CP4; and two FA-A LCLs: FA-55 and FA-178. FA patients and HDs were encoded to protect their confidentiality and informed consents were obtained in all cases prior to the generation and use of the cell lines.

The mutation nomenclature used followed the HGVS recommendations and was referred to *FANCA* transcript NM_000135.

LCLs were grown in *Roswell Park Memorial Institute* medium (RPMI, Gibco/Thermo Fisher Scientific, Waltham, Massachusetts, USA), 20% HyClone (GE Healthcare, Chicago, Illinois, USA), 1% penicillin/streptomycin (P/S) solution (Gibco), 0.005 mM β-mercaptoethanol (Gibco), 1 mM sodium pyruvate (Sigma-Aldrich/Merck KGaA, Sant Louis, Missouri, USA), non-essential amino acids (Lonza, Basel, Switzerland) under normoxic conditions (37°C, 21% O_2, 5% CO_2 and 95% relative humidity –RH–) in cell culture flasks (Corning, New York, New York, USA).

1.1.2 293T cell line

293T cells (ATCC: CRL-11268) were used to produce adeno-associated viral vectors (AAVs). This is a human embryonic kidney epithelial cell line that constitutively expresses a temperature-sensitive gene encoding the simian SV40 T-antigen, which renders higher transfection efficiencies. The inserted sequence allows the episomal replication of plasmids that contain the replication origin in the early promoter region of the SV40.

293T cells were grown in *Iscove's Modified Dulbecco's Medium* (IMDM, Gibco), 10% HyClone (Gibco) and 1% P/S solution (Gibco) under normoxic conditions in 150 cm^2 surface cell culture plates (Corning).

1.2 Primary cells

1.2.1 Hematopoietic stem and progenitor cells from healthy donors

HD $hCD34^+$ cells were obtained from umbilical cord blood (UCB) samples provided by the *Centro de Transfusiones de la Comunidad de Madrid* previously scheduled for discard, after

obtaining an informed consent from the mothers. Mononuclear cells from pooled UCBs were purified by Ficoll-Paque PLUS (GE Healthcare) density gradient centrifugation according to manufacturer's instructions. Human $CD34^+$ cells were immunoselected from the mononuclear fraction by immunoselection using the *CD34 Micro-Bead Kit* (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany). Magnetically-labelled cells were selected with a LS column in *QuadroMACS™ Separator* (Miltenyi Biotec) following manufacturer's instructions. Purified $hCD34^+$ cells were then analysed by flow cytometry to evaluate their purity in *LSRFortessa Cell Analyser* (BD, Franklin Lakes, New Jersey, USA) using *FlowJo Software v7.6.5* (FlowJo LLC). Purities ranging from 90-98% $hCD34^+$ cells were routinely obtained.

Cells were grown in *StemSpam* (StemCell Technologies, Vancouver, Canada) supplemented with 1% GlutaMAX™ (Gibco), 1% P/S solution (Gibco), 100 ng/mL human stem cell factor (hSCF), human FMS-like tyrosine kinase 3 ligand (hFlt3-L), human thrombopoietin (hTPO), and 20 ng/mL human interleukin 3 (hIL3) (all obtained from EuroBioSciences GmbH, Friesoythe, Germany) under normoxic conditions.

1.2.2 Hematopoietic stem and progenitor cells from FA-A patients

A small number of mobilized peripheral blood (mPB) $hCD34^+$ cells from patients FA-655, FA-739 and FA-807 that remained in cell collection bags and tubes from the *CliniMACS® System* (Miltenyi Biotec), used for the collection (FANCOSTEM trial; Eudra number CT 2011-006197-88) and the subsequent transduction of $hCD34^+$ cells with LVs (FANCOLEN Trial: Eudra number CT 2011-006100-12) were used. Informed consents were obtained from their parents in all cases and the procedures complied with all relevant ethical regulations and approval by the Ethic Committees at Hospital Vall d'Hebron in Barcelona and Hospital del Niño Jesús in Madrid.

Cells were grown in *StemSpam* (StemCell Technologies) supplemented with 1% GlutaMAX™ (Gibco), 1% P/S (Gibco), 100 ng/mL hSCF, hFlt3 and hTPO, and 20 ng/mL hIL3 (all EuroBioSciences GmbH), 10 μg/mL anti-TNFα (Enbrel-Etanercept, Pfizer, New York, New York, USA) and 1 mM N-acetylcysteine (Pharmazam, Westchase, Florid, USA) under hypoxic conditions (37°C, 5% of O_2, 5% of CO_2 and 95% RH).

One $hCD34^+$ cell sample from FA-807 patient was also obtained from total bone marrow (BM) by immunoselection as indicated for UCB samples and cultured under the previously-described conditions.

Since some of the FA samples used were cryopreserved, in all cases 24 hours of pre-stimulation was used before the gene editing protocol was carried out.

1.3 Animals

Human CD34^{+} cells were transplanted in non-obese diabetic (NOD) immunodeficient Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ mice (NSG). This strain carries two mutations in the NOD/ShiLtJ genetic background: one causing a severe combined immune deficiency (scid) and another inducing a complete absence of the IL2 receptor common gamma chain (*IL2rg*^{null}). The scid mutation takes place in the *Prkdc* gene, which encodes for a DNA repair complex and originates a deficiency in B and T cells. The *IL2rg*^{null} mutation prevents cytokine signalling through multiple receptors, causing NK-cell function deficiency. The severe immunodeficiency allows the mice to be humanized by engraftment of human CD34^{+} cells, patient derived xenografts, or adult stem cells and tissues.

All experimental procedures were conducted according to the European and Spanish regulations in the field: European convention ETS 123, regarding the use and protection of vertebrate mammals used in experimentation and other scientific purposes, Directive 2010/63/UE, Spanish Law 6/2013 and Real Decreto (R.D.) 53/2013 regarding the protection and use of animals in scientific research.

Procedures involving Genetically Modified Organisms were conducted according to the proper European and Spanish regulations: Directive 2009/41/CE, Spanish Law 9/2003 and R.D. 178/2004. Procedures were approved by the CIEMAT Animal Experimentation Ethical Committee according to all external and internal bio-safety and bio-ethics guidelines, and previously authorized by the Spanish Government (Code PROEX #070-15# Cell and Gene Therapy in rare diseases with chromosomal instability).

Mice were housed and bred at the CIEMAT Laboratory Animal Facility (registration number ES280790000183), where they were routinely screened for pathogens in accordance with the Spanish Society for the Laboratory Animal Science (SECAL) and the Federation of European Laboratory Animal Science Associations (FELASA, Tomworth, United Kingdom) recommendations and no pathogens were found.

Mice were provided with food (TEKLAD Global Diet 2918, irradiated with 25 KGy gamma rays) and water (acidified and autoclaved) *ad libitum*, under controlled environmental conditions. Mice were housed during the experimental protocols in individually-ventilated cages type IIL with 25 air cage changes per hour. A maximum of 6 mice were housed in each cage. Room lighting was controlled with light/dark cycles of 13/11 hours, and temperature and humidity were regulated at 20 ± 2ºC and 55 ± 10%, respectively. HEPA air filters were present in all rooms.

2. CRISPR/Cas9 Nucleases

2.1 Plasmids

Gene editing experiments in LCLs were performed using the pX330-U6-Chimeric_BB-CBh-hSpCas9 (55), kindly provided by Dr. Feng Zhang (Addgene plasmid #42230) (Figure 5). Guide RNAs to target c.295C>T and c.3558insG mutations and compatible with *Staphylococcus pyogenes* PAM sequence (5'-NGG-3') were designed (Table 1) using the *CRISPR Design* bioinformatics tool from MIT (http://crispr.mit.edu/). The sgRNAs were selected according to the closest proximity of the cutting site to the mutation while offering the lowest off-target probability score.

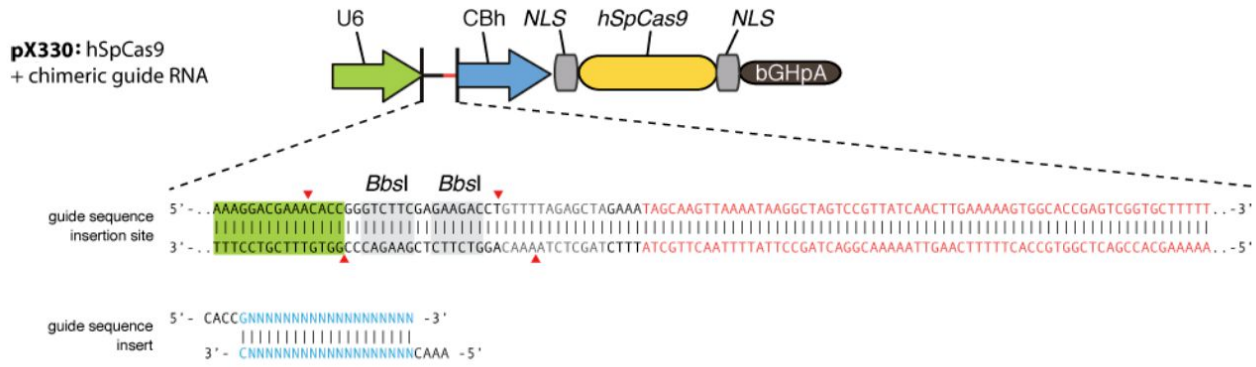


Figure 5.- pX330 plasmid backbone. Plasmid pX330 was used to clone our designed sgRNAs according to the strategy showed. The expression of the sgRNA is driven by the U6 promoter (green arrow). Cas9 (yellow) expression is controlled by a hybrid version of the chicken β-actin promoter (blue arrow) flanked by two nuclear localization signals (grey). The figure was obtained from the Addgene webpage.

Table 1.- Designed sgRNA sequences.

Target mutation	sgRNA	Sequence (5' to 3')	Orientation
c.3558insG	gINS8	CTCCACCGGCAGAGCAGCAC	Antisense
	gINS11	ACTGCCAGAGCCCGCTGCCC	Sense
c.295C>T	gGM4	CACGGGAACCCCCAGCCTTG	Antisense
	gGM10	AGGATCAAGCCTCAAGGCTG	Sense

Guide RNAs were ordered as oligos from Sigma-Aldrich (Table 2), annealed and cloned into pX330 plasmid after *BbsI* (Ref #R3539, New England Biolabs, Ipswich, Massachusetts, USA) restriction enzyme digestion. The obtained plasmids were named pX330gGM4, pX330gGM10, pX330gINS8 and pX330gINS11, respectively, and delivered the sgRNA and the Cas9 nuclease into the same construct.

Table 2.- Sequences of the oligos used for sgRNA cloning.

Name	Primer	Sequence (5' to 3')
gINS8	Fw	CACCGCTCCACCGGCAGAGCAGCAC
	Rv	AAACGTGCTGCTCTGCCGGTGGAGC
gINS11	Fw	CACCGACTGCCAGAGCCCGCTGCCC
	Rv	AAACGGGCAGCGGGCTCTGGCAGTC
gGM4	Fw	CACCGCACGGGAACCCCCAGCCTTG
	Rv	AAACCAAGGCTGGGGGTTCCCGTGC
gGM10	Fw	CACCGAGGATCAAGCCTCAAGGCTG
	Rv	AAACCAGCCTTGAGGCTTGATCCTC

2.2 Ribonucleoprotein complex

Cas9 protein was purchased from PNA Bio (#CP01, PNA Bio, Thousand Oaks, California, USA) and used in combination with *in vitro* transcribed (IVT) or synthetic sgRNAs from Synthego (Synthego, Menlo Park, California, USA). The sgRNA was mixed with the commercially acquired Cas9 protein and incubated for 10 minutes at room temperature (RT). After that, cells and RNP were mixed for the electroporation process.

To generate IVT sgRNAs, the T7 promoter sequence was incorporated to the sgRNAs by PCR (Table 3). pX330gGM4 / gINS11 plasmids were used as PCR templates.

PCR products were purified using the *NucleoSpin® Gel and PCR Clean-up* (Ref 740609, MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) and directly used for the *in vitro* transcription of the sgRNAs using the *HiScribe™ T7 High Yield RNA Synthesis Kit* (# E2040S, New England Biolabs) according to manufacturers' instructions. The IVT sgRNAs were purified

using *RNeasy® Plus Mini Kit* (Ref 74134, QIAGEN, Hilden, Germany) and quantified using *NanoDrop Spectrophotometer ND-1000* (Thermo Fisher Scientific).

Table 3.- Primer sequence for sgRNA *in vitro* transcription.

sgRNA	Primer	Sequence (5' to 3')	Tm (°C)	PCR product size (bp)
gINS11	Fw	GAAATTAATACGACTCACTATAG**ACTGCCAGAGCCCGCTGCCC**	60	122
	Rv	AAAAGCACCGACTCGGTGCC		
gGM4	Fw	GAAATTAATACGACTCACTATAG**CACGGGAACCCCCAGCCTTG**	62	122
	Rv	AAAAGCACCGACTCGGTGCC		

Normal letters indicate the T7 promoter sequence added to the forward primer sequence (signalled in bold).

2.3 Adeno-associated viral vectors

Two different strategies were developed to package the CRISPR/Cas9 system into an AAV particle.

2.3.1 Two-component system

The expression cassette of the sgRNA from pX330 plasmid carrying the gGM10 guide sequence was subcloned into the backbone of a self-complementary AAV (scAAV) and produced as an AAV6 (scgGM10-AAV6) (Figure 6a).

The expression cassette of the human codon optimized *S. pyogenes* Cas9 from pX330 plasmid was subcloned into the backbone of a single-stranded AAV (ssAAV) and produced as an AAV6 (ssSpCas9-AAV6) (Figure 6b).

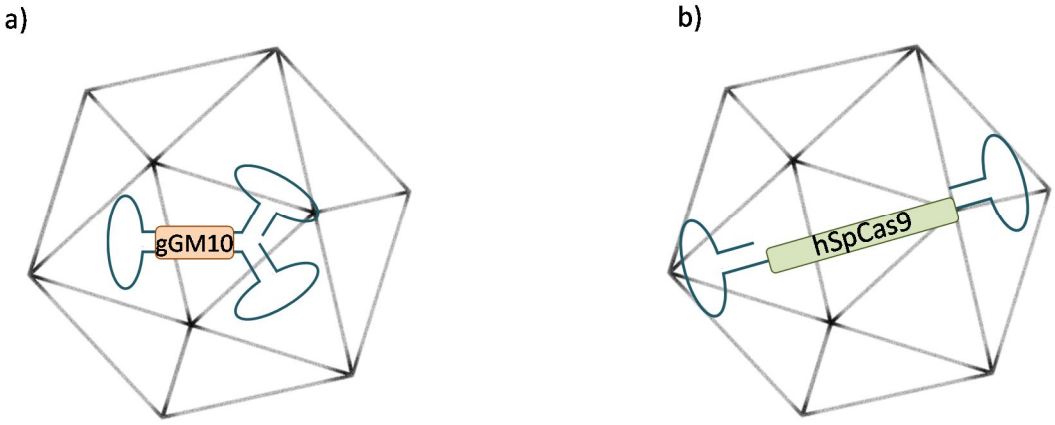


Figure 6.- Schematic representation of the AAV constructs used. (**a**) The sgRNA expression cassette was delivered in a scAAV6. (**b**) The expression cassette of the Cas9 was delivered in an ssAAV6.

2.3.2 All-in-one system

A new sgRNA compatible with the *S. aureus* Cas9 PAM (5'-NNGRRN-3') sequence was designed and cloned into the pX601-AAV-CMV::NLS-SaCas9-NLS-3xHA-bGHpA;U6::BsaI-sgRNA plasmid (213), kindly provided by Dr. Feng Zhang (Addgene plasmid # 61591) (Figure 7a). The sgRNA was designed using the *Breaking Cas* bioinformatic web tool (199) from Centro Nacional de Biotecnología (http://bioinfogp.cnb.csic.es/tools/breakingcas) (Madrid, Spain). The smaller size of the *S. aureus* Cas9 allowed the packaging of the construct into a single AAV particle (Figure 7b) that was produced as an AAV6 (ssgGM2SaCas9-AAV6) by the Unidad de Producción Viral from Universidad Autónoma de Barcelona/ Instituto de Investigación Vall d'Hebrón (https://www.viralvector.eu/) (Barcelona, Spain).

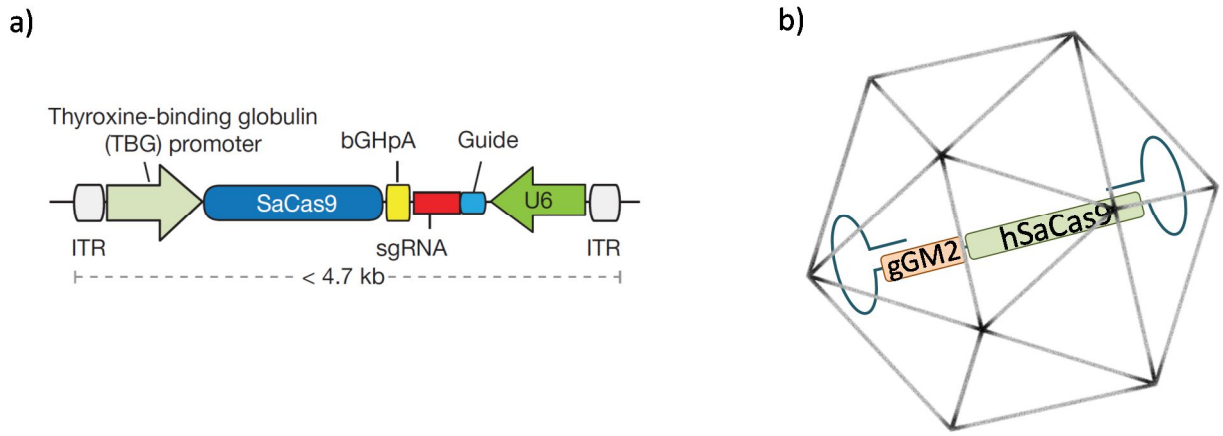


Figure 7.- Schematic representation of the all-in-one AAV system used. (**a**) Plasmid pX601 backbone acquired from Addgene. The expression of the sgRNA is driven by the U6 promoter (green arrow). The *S. aureus* Cas9 (dark blue) expression is controlled by the thyroxine-binding globulin promoter (light green arrow). The figure was obtained from the Addgene webpage. (**b**) Both CRISPR/Cas9 components (sgRNA and Cas9) were delivered in an ssAAV6.

2.3.3 Control AAVs

To evaluate the transduction efficacy of the AAVs in HSPCs and as a control to the different AAV-constructs carrying the CRISPR/Cas9 system, the green fluorescence (GFP) gene was packaged under two different conformations (self-complementary and single-stranded) in an AAV2 and AAV6 (Figure 8).

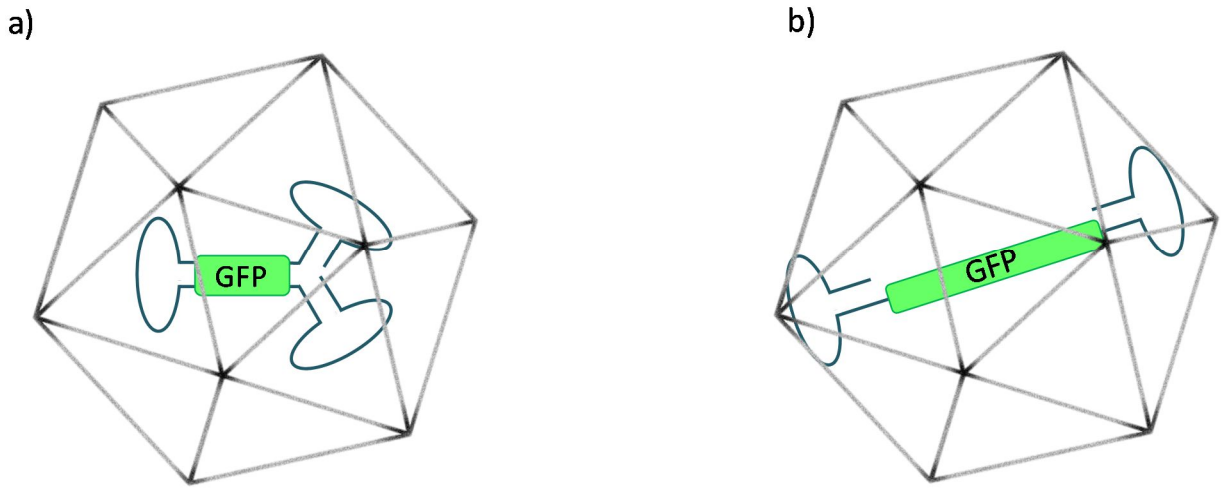


Figure 8.- Schematic representation of the control AAV construct used. To initially test the ability of the AAV2 and 6 serotypes to transduce HSPCs, and as a control to the AAV-constructs carrying the CRISPR/Cas9 system, the GFP gene was packaged into self-complementary (**a**) and single-stranded (**b**) AAVs.

3. TALENs

3.1 Plasmids

A pair of TALEN monomers to target the c.295C>T mutation was kindly designed and provided by Dr. Claudio Mussolino from the Institute of Cell and Gene Therapy at University Medical Center in Freiburg, Germany.

The DNA binding domain of each monomer of the TALEN pair is composed of a tandem array of 17.5 repeats, each one consisting of 34 highly conserved residues, except the last module which contains 20 amino acids and is therefore referred to as "half-repeat" (30). The DNA binding specificity of each repeated unit is driven by the repeat-variable di-residues (RVDs) at positions 12 and 13, according to the canonical code (NG = T; HD = C; NI = A; NK = G) (184).

The TALEN backbones were cloned by Dr. Claudio Mussolino using the assembly system and plasmids from Thomas Lahaye (184). Two different plasmids were generated, one for the left and another for the right monomer. The TALEN backbone of both monomers contains an N-terminal domain composed of 153 residues with a nuclear localization signal (NLS), the "0 repeat" binding to the T in position -1 of the target site-nucleotide, the 17.5 repeats, and the C-terminal domain composed of 17 residues fused to the catalytic domain of the FokI

endonuclease type II (187). There is a 14-bp spacer sequence between both monomer binding sites.

4. Plasmid Amplification and Purification

Plasmids containing the different constructs used for AAV production and gene editing with CRISPR and TALENs were transformed in TOP10 bacteria (Invitrogen) for their amplification. Plasmid DNA was purified using *NucleoBond® Xtra Midi EF kit* (MACHEREY-NAGEL GmbH & Co. KG) and verified by restriction enzyme digestion. All enzymes used were obtained from New England Biolabs.

5. AAV Production

The AAV production protocol was taught by Dr. Hildegard Büning during my internship in the Hannover Medical School (Hannover, Germany) under her supervision.

5.1 Transfection

A day before transfection, 15 150 mm^2 surface cell culture (P150) plates were seeded with $17.5x10^6$ of 293T cells in 18 mL final volume of IMDM (Gibco), supplemented as previously described in section 1.1.2.

Two hours before transfection, the culture medium was replaced by fresh medium.

Transfection was conducted following the $CaCl_2$ DNA precipitation method when cells reached 80% of confluence. The amounts of plasmids used for a P150 plate (Corning) of 293T cells were: 7.5 µg of the corresponding transfer plasmid, 7.5 µg of capsid plasmid and 22.5 µg of helper plasmid, at a 1:1:1 molar ratio (114). This mixture was prepared in a final volume of 1 mL of 0.1X Tris-EDTA buffer (10 mM Tris pH 8; 1 mM EDTA pH 8; diluted 1:10 with still distilled H_2O) per plate and incubated for 15 minutes at RT in agitation in order to allow the correct homogenization of the mixture.

Then, 100 µL of 2.5 M $CaCl_2$ were added and the mixture was again incubated for 15 minutes at RT in agitation.

As the next step, 1 mL of 2X HBS buffer (100 mM HEPES, 281 mM NaCl, 1.5 mM Na_2HPO_4, pH 7.15) was added dropwise while vortexing at full speed, allowing the formation of Ca^{2+}/DNA^- precipitates. The total volume was immediately added to 293T cells, which will subsequently phagocyte the precipitates.

After 24 hours, culture medium was replaced by fresh medium supplemented with 1% P/S but only 2% HyClone (Gibco).

5.2 Harvesting

Forty-eight hours after the transfection, 293T cells were scraped, harvested and pelleted by low-speed centrifugation (15 minutes at 3,000 rpm).

Cell pellet was resuspended in lysis buffer (150 mM NaCl; 50 mM Tris/Cl pH 8.5; 1 mM $MgCl_2$) and freeze-thawed 3 times using liquid nitrogen and a water bath at 37°C.

The lysate was then treated with Benzonase (Merk KGaA, Darmstadt, Germany) for 60 min at 37°C. Cell debris was spun down at 3,000 rpm for 20 min at 4°C and the supernatant collected.

5.3 Purification

The purification of the AAVs was conducted by iodixanol density gradient centrifugation. The supernatant was loaded into an *OptiSeal™ Polypropylene Tube* (#362183, Beckman Coulter, Brea, California, USA). The gradient was layered using a peristaltic pump P-1 (GE Healthcare), starting from 15% density solution (15 mL), followed by 25% (4.5 mL), 40% (3.5 mL) and finally 60% (3.5 mL) with a Sterican long needle (Ø 0.80 x 120mm 21G x 4 ¾'', Braun GmbH, Kronberg, Germany). The iodixanol used was *OptiPrep Density Gradient Medium* (#D1556-250mL, Sigma-Aldrich) and the different density solutions were prepared as shown in Table 4.

Table 4.- Preparation of the iodixanol solutions.

	OptiPrep™ Iodixanol	10x PBS-MK	5 M NaCl	H_2O	Phenol Red (0.5%)
15%	12.5 mL	5 mL	10 mL	22.5 mL	75 µL
25%	20 mL	5 mL	-	25 mL	75 µL
40%	33.3 mL	5 mL	-	11.7 mL	-
60%	49 mL	-	-	-	25 µL

PBS-MK = 50mL 10xPBS + 500µL 1M $MgCl_2$ + 500µL 2.5M KCl.

The tubes were loaded into a 70 Ti fixed-angle rotor (Beckman Coulter) and centrifuged at 63,000 rpm for 1 hour at 18 °C, maximum acceleration and slow brake.

After centrifugation, packaged AAV6 particles retained in the 40% iodixanol layer were extracted by piercing the tube with a Sterican needle (Ø 0.90 x 40mm 20G x 1 ½´´, Braun GmbH) slightly above the 60% layer.

5.4 Titration

Titration was performed by quantitative PCR (Q-PCR). DNA was obtained from a 10 µL aliquot of the purified AAVs using the *DNeasy® Blood & Tissue Kit* (QIAGEN).

Q-PCR amplification was performed using specific primers for the eGFP gene or the CMV promoter (depending on the construct) in a 7500 fast real-time PCR system (Applied Biosystems/Invitrogen, Foster City, California, USA) using *FastStart Universal SYBR Green Master PCR mix* (Roche, Basel, Switzerland). The amplification program used was:

95°	15 min	
94°	15 sec	x 40
60°	30 sec	
72°	30 sec	
95°	15 sec	plus 1°C
60°	1 min	
95°	15 sec	

Quantification of viral genomes was performed by interpolating the cycle threshold values (Ct) from the DNA sample to the Ct of a standard curve with five-fold dilutions of a known concentration of DNA from an AAV plasmid.

Titers ranging from 10^8 (self-complementary constructs) to 10^{10} (single-stranded constructs) viral particles per microliter were routinely obtained.

6. Gene Editing Experiments in LCLs

6.1 Electroporation

6.1.1 CRISPR/Cas9

HD and FA-A patient-derived LCLs were electroporated with increasing doses of plasmid pX330gGM4 / gGM10 / gINS8 / gINS11 (from 1 to 10 µg) using the *SF Cell Line 4D-Nucleofector® X Kit* for *Amaxa 4-D device* (Lonza). Two hundred thousand cells were resuspended in a total of 20 µL nucleofection solution according to manufacturer's instructions and electroporated in strip wells using program EW-113. After the pulse, electroporated cells were incubated for 10

minutes at 37°C. Then, 180 µL of pre-warmed medium were added and cells were passed to a cell culture plate. Cell viability was assessed by flow cytometry 24 hours post-electroporation using 4', 6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific).

6.1.2 TALENs

The two plasmids encoding for each TALEN monomer were simultaneously electroporated into the LCLs. Total amounts of 3, 5 and 10 µg were used (1.5, 2.5 and 5 µg of each plasmid) under the same previously described conditions in section 6.1.1.

6.2 LCLs single clone isolation

Five hundred cells were plated in methylcellulose (StemMACS™, Miltenyi Biotec) to isolate individual clones and incubated for three weeks under normoxic conditions. Individual colonies were picked and transferred to a 96-well plate for their *in vitro* amplification and characterisation.

6.3 Functional analyses in LCLs

6.3.1 FANCA protein expression by Western-blot

Protein extracts were isolated from at least $6x10^6$ cells in lysis buffer (Hepes 20 mM pH 7.5, NaCl 100 mM, $MgCl_2$ 20 mM, EGTA 10 mM, β-glycerol phosphate 40 mM, 1% de Triton X-100, PMSF 1 mM, protease inhibitors 1X −*Complete Mini Protease Inhibitor Cocktail*, Roche− and phosphatase inhibitors 1X −*PhosSTOP*, Roche−). Cell lysates were centrifuged at 13,000 rpm for 5 minutes at 4°C and supernatants collected. Quantification was conducted by *Bradford colorimetric assay* (BioRad Laboratories, Hercules, California, USA) in a spectrophotometer (Eppendorf BioPhotometer, Hamburg, Germany) according to manufacturer's instructions. Fifty micrograms of each sample were incubated for 5 minutes at 95°C in redox and denaturing conditions using β-mercaptoethanol (Gibco) and Laemmli buffer (BioRad) in a 1:9 ratio. Gel electrophoresis was conducted in 4-15% polyacrylamide gels (BioRad) using BioRad running buffer (Tris 25 mM, Glycine 192 mM, SDS 0.1%; pH 8.3) for 2 hours, following manufacturer's instructions. Proteins were transferred using the *Trans-Blot Turbo Transfer* device from BioRad using BioRad's Transfer Buffer with 20% absolute ethanol. Membranes were blocked for 1 hour at RT and agitation with 5% w/v non-fat dry milk in 0.1% Tween-20 PBS. Membrane was incubated with rabbit anti-hFANCA antibody (ab5063, Abcam, Cambridge, England) 1:1,000

dilution in 2.5% milk PBS-T, mouse anti-β-actin antibody (ab6276, Abcam) 1:4,000 dilution in 2.5% milk PBS-T or mouse anti-vinculin (ab73412, Abcam) 1:500 dilution in 2.5% milk PBS-T over night at 4°C. β-actin and vinculin served as loading controls. Membrane was washed with PBS buffer three times and then was incubated with 1:5,000 dilution in 2.5% milk PBS-T of the secondary anti-rabbit (pAb to Rb IgG-HRP, ab6721-1, Abcam) and anti-mouse (pAb to Ms IgG-HRP ab6808, Abcam) antibodies during 1 hour at RT. Finally, membranes were washed three times with PBS. Blots were visualized with *ChemiDoc MP System* (BioRad) using *Clarity Western ECL substrate* (BioRad).

6.3.2 Nuclear FANCD2 foci immunofluorescence

One million cells were seeded in chamber slides (Nunc, Sigma) previously coated with 20 μg/cm² *Retronectin*® (Takara Bio inc, Kusatsu, Shiga, Japan). Twenty-four hours later cells were incubated in the absence or presence of 40 nM MMC (Sigma). Cells were washed with PBS and fixed with 3.7% paraformaldehyde (Sigma) for 15 minutes at RT. Then cells were washed 3 times with TBS (20 mM Tris-HCl; 140 mM NaCl) and permeabilized using 0.5% Triton X100 (Sigma) for 5 minutes, washed and blocked with blocking solution (0.1% Nonidet-P40 –Sigma–, 10% HyClone –Gibco– in TBS) for 4 hours. Cells were incubated with the primary antibody against human FANCD2 (ab2187-50, Abcam) as previously described (77) in a humidity chamber at 4°C overnight. Cells were washed 3 times with TBS and then incubated during one hour with the secondary antibody against rabbit IgG, conjugated with AlexaFluor 594 (ab150080, Abcam) 1:1,000 dilution and DAPI (Thermo Fisher Scientific). Finally, cells were washed 3 times in TBS and slides were mounted with Mowiol (Sigma-Aldrich). Samples were visualized with a fluorescence microscope *Axioplan 2 imaging* (Zeiss, Oberkochen, Germany) with an objective of 0.17 mm and 100x/1.45 magnification. Two hundred cells were counted and cells with more than 10 foci were scored as positive. Images were captured with *AxioCam MRm* (Zeiss) and processed with *AxioVision 4.6.3* (Zeiss) and *Corel Photo-Paint 11* (Corel, Ottawa, Ontario, Canada).

6.3.3 MMC sensitivity test

Two hundred and fifty thousand cells were exposed to increasing concentrations of MMC (from 0 to 1,000 nM). Cell viability was measured at days 5, 10 and 15 by flow cytometry using

DAPI (Thermo Fisher Scientific) vital marker in an *LSRFortessa Cell Analyser* (BD). Off-line analysis was performed with *FlowJo Software v7.6.5* (FlowJo LLC).

6.3.4 Analysis of reactive oxygen species

Detection of intracellular reactive oxygen species (ROS) was conducted using the *CellROX® Deep Red Reagent* (C10422, Life Techonlogies/Thermo Fisher Scientific, Carlsbad, California, USA). *The CellROX® Deep Red dye* is a fluorogenic compound that enters the cells in a reduced state as a non-fluorescent probe, exhibiting strong fluorogenic signal (640 nm excitation and 665 nm emission) after oxidation by the presence of ROS. The analysis was performed in FA-A patient LCLs (gene-edited and untreated) following manufacturer's instructions: $5x10^4$ cells were incubated with *CellROX® Deep Red Reagent* at a final concentration of 5 µM in PBS during 20 min at 37°C. After incubation, cells were washed with PBA and DAPI (Thermo Fisher Scientific) was added. Quantification of ROS production was conducted in *LSRFortessa Cell Analyser* (BD) and data were analysed with *FlowJo Software v7.6.5 (*FlowJo LLC*)*.

7. Gene Editing Experiments in HD and FA-A HSPCs

7.1 Electroporation

In gene editing experiments, freshly purified HD $hCD34^+$ cells were immediately electroporated without pre-stimulation or pre-stimulated for 24 hours. In the case of cryopreserved FA HSPCs, a 24-hour pre-stimulation step was always conducted.

HD and FA HSPCs were electroporated with the RNP complex composed of 9 µg of Cas9 + 12 µg of gGM4 / gINS11, using the *P3 Primary Cell 4D-Nucleofector® X Kit* for *Amaxa 4-D device* (Lonza). Two hundred thousand cells were resuspended in a total of 20 µL nucleofection solution according to manufacturer's instructions and electroporated in strip wells using program EO-100. The RNP complex was previously assembled by mixing the sgRNA and the Cas9 protein and incubating for 10 minutes at RT. After the pulse, electroporated cells were incubated for 10 minutes at 37°C. Then, 180 µL of pre-warmed medium were added and cells were incubated in a cell culture plate. Cell viability was assessed by flow cytometry 24 hours post-electroporation and gene editing efficacy was evaluated five days post-electroporation by *Surveyor assay* (IDT) and/or next generation sequencing (NGS).

7.2 Colony Forming Unit Assay

Nine hundred HD or 10,000 FA-A hCD34$^+$ cells were resuspended in 3 mL of enriched methylcellulose medium (StemMACS™ HSC-CFU complete with Epo, Miltenyi Biotec). In the case of FA cells, 10 ug/mL anti-TNFα and 1mM N-acetylcysteine were added. Each mL of the triplicate was seeded in a M35 plate and incubated under normoxic (HD hCD34$^+$ cells) or hypoxic (FA hCD34$^+$ cells) conditions. To test MMC sensitivity in hematopoietic colonies obtained from FA-A patients, 3 and 10 nM of MMC was added to the plate. After fourteen days, colonies were counted using a *Nikon Diaphot* inverted microscope with a 4X (Nikon, Minato, Tokio, Japan) and CFUs-GMs (granulocyte-macrophage colonies) and BFU-Es (erythroid colonies) were identified.

7.3 Gene-edited HD HSPC reconstitution experiments in NSG mice

HD hCD34$^+$ cells from UCB were purified as previously described and freshly electroporated with the pre-assembled IVT gGM4 sgRNA and Cas9 ribonucleoprotein complex (RNP4) using the *P3 Primary Cell 4D-Nucleofector® X Kit* for *Amaxa 4-D device* (Lonza) on the same day. A schematic representation of the protocol can be seen in Figure 9. Three groups of cells were established: untreated cells (control); electroporated cells without nuclease (mock), and electroporated cells with the RNP4 complex (RNP4). Twenty-four hours later, cell viability was assessed by flow cytometry and cells were transplanted through the tail vein into NSG mice previously irradiated with a submyeloablative dose (1.5 Gy) in an X-ray device.

The department of Basic Research at CIEMAT has a category 2 radioactive installation which houses an X-ray device. It is mainly used for the irradiation of laboratory animals, biological samples and other materials, according to Spanish laws such as R.D. 35/2008 (regulation on nuclear and radioactive facilities) and R.D. 783/2001 (regulation on health protection against ionizing radiation).

Two different cell doses were infused in the transplantation: $1.5x10^5$ cells/mouse and $3.25x10^5$ cells/mouse. A CFC-assay was also conducted and the remaining cells were pelleted for DNA extraction and NGS analysis to evaluate basal gene editing.

Human engraftment was measured monthly by flow cytometry analysis of the percentage of hCD45$^+$ cells (anti-hCD45-APC-Cy7, BioLengend, San Diego, California, USA). Multilineage

reconstitution was also evaluated using antibodies against hCD34 (anti-hCD34-APC, BD) for HSPCs, hCD33 (anti-hCD33-Pe-Cy7, eBioscience/Thermo Fisher Scientific) for myeloid cells, hCD19 (anti-hCD19-Pe-Cy7, BioLegend) for B cells and hCD3 (anti-hCD3-APC, BioLegend) for T cells. The remaining cells were pelleted for DNA extraction and NGS analysis to evaluate the presence of gene edited cells.

At days 30 and 60 after transplantation, BM samples were obtained by intra-bone aspiration, while at day 90, mice were euthanized and PB, spleen, thymus and hind legs were extracted. BM samples were obtained from hind legs by intra-bone perfusion; PB was extracted by exsanguination, and spleen and thymus were mechanically disaggregated prior to the analysis. Human engraftment was evaluated by flow cytometry according to the percentage of $hCD45^+$ cells in the different hematopoietic organs. Multilineage reconstitution was determined using antibodies against hCD34 for HSPCs, hCD33 for myeloid cells, hCD19 for B cells and hCD3 for T cells in PB, spleen and BM; and using hCD3 for T cells and hCD8 (anti-hCD8-Pe, Immunotec, Vaudreuil-Dorion, Quebec, Canada) and hCD4 (anti-hCD4-APC, Miltenyi Biotec) to distinguish between cytotoxic and helper T cells in thymus samples. The presence of progenitor cells was further evaluated in BM samples according to the presence of hCD133 (anti-hCD133-Pe, MACS, Miltenyi Biotec) and hCD90 (anti-hCD90-APC, BD) markers.

To evaluate the long-term engraftment capacity of $hCD34^+$ cells after NHEJ-mediated gene editing, the bone marrow cells extracted from primary recipients were transplanted into secondary ones, mouse to mouse, so the same groups of secondary recipients were established. The remaining BM cells were pelleted for DNA extraction using *DNeasy® Blood & Tissue Kit* (QIAGEN) and NGS analysis to evaluate the presence of gene edited cells.

The human engraftment follow-up in secondary recipients was conducted as previously described: BM aspiration and flow cytometry were performed at 30 and 60 days post-transplantation and hematopoietic organ extraction after euthanasia at day 90 post-transplantation. DNA from BM cells was obtained to evaluate the presence of gene edited cells by NGS at the different time points.

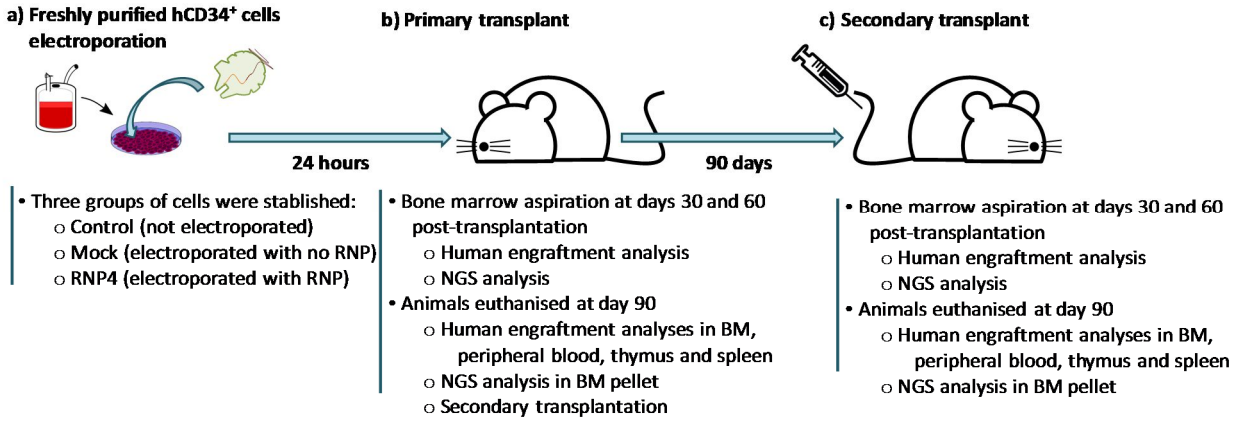


Figure 9.- Timeline of the gene-edited HD HSPC reconstitution experiments in NSG mice. (**a**) Fresh HD hCD34+ cells were purified from UCBs and electroporated with the RNP complex preassembled with gGM4 sgRNA. (**b**) Twenty-four hours later, primary recipients were transplanted. (**c**) When primary recipients were euthanized at day 90, a secondary transplantation was conducted with bone marrow cells extracted.

8. Gene Editing Analyses

8.1 *Surveyor Assay*

DNA was extracted 5 days after electroporation, using *NucleoSpin® Tissue Kit* (MACHEREY-NAGEL GmbH & Co. KG) or the *Proteinase K Lysis protocol* (61), when a limiting number of cells was obtained. Then, a PCR using *Herculase II fusion DNA polymerase* (Agilent, Santa Clara, California, USA) was performed to amplify a 350-500 bp region containing the target sequence in which the CRISPR/Cas9 nucleases cut. Primers used in the PCR are listed on Table 5.

Table 5.- Primers used in *Surveyor assay*.

Region	Primer	Sequence (5' to 3')	Tm (°C)	PCR product size (bp)
c.3558insG	Fw	TGTAGTGGCCTGTAGGAGCA	60	394
	Rv	CCCAGTAGTTGGGATTACAG		
c.295C>T	Fw	TGCTCCTTTTGTGTCATGGGA	60	422
	Rv	TGCTGGTGTCTTACTCTCTGC		

A schematic representation of the *Surveyor assay* protocol can be seen in Figure 10.

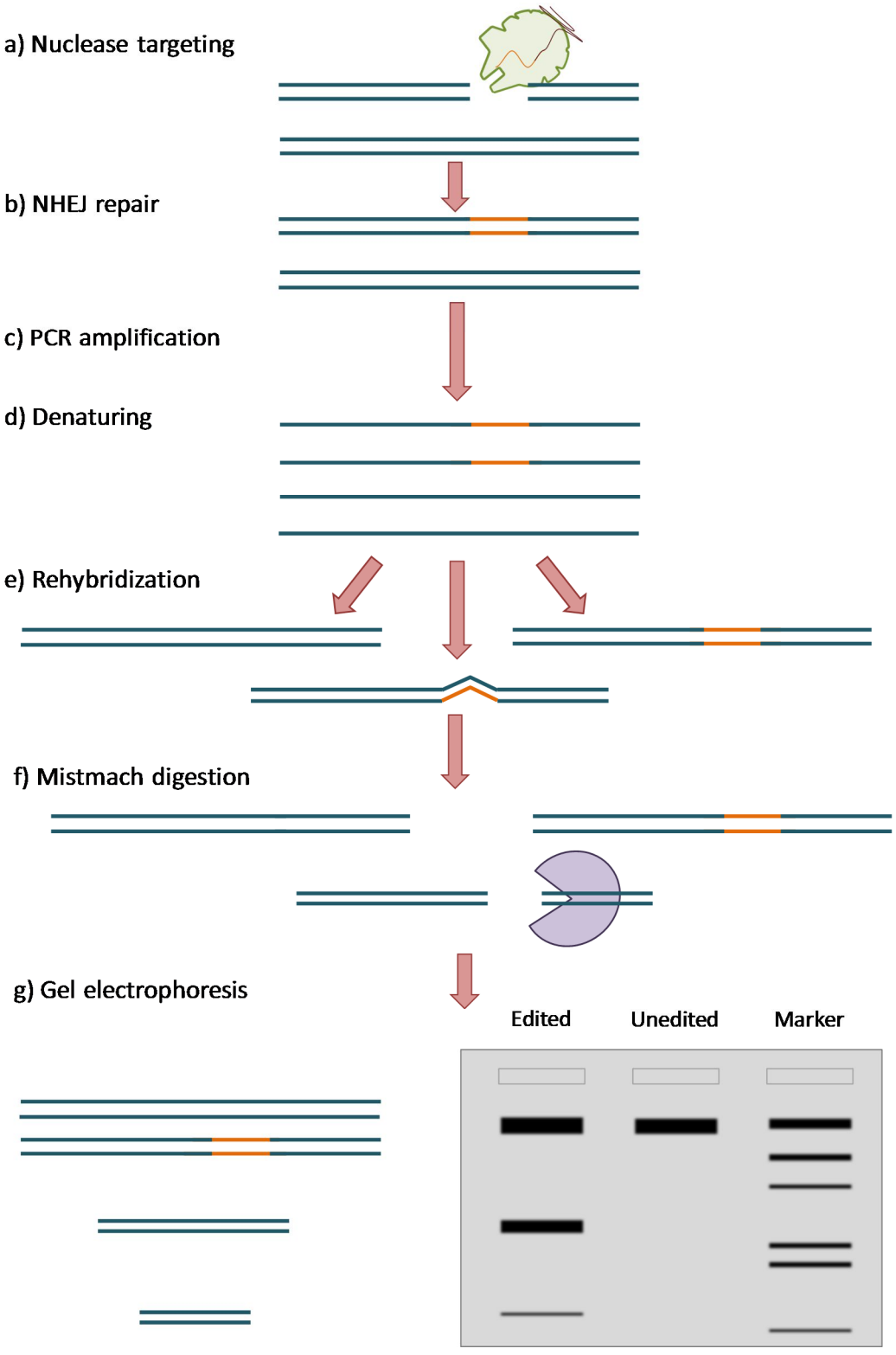


Figure 10.- ***Surveyor assay*** **protocol.** (**a**) The nuclease catalyses a double-strand break (DSB) at the target sequence. (**b**) Cell machinery repairs the DSB by NHEJ, generating some insertions and/or deletions (indels). (**c**) DNA from targeted cells is extracted and the targeted locus is amplified by PCR. (**d**) PCR is denatured by heating. (**e**) The rehybridization of the denatured strands by cooling-down generates mismatches. (**f**) Renatured DNA is digested by an endonuclease that detects and degrades mismatches. (**g**) Gel electrophoresis demonstrates the presence or absence of genome editing in the targeted cells.

The PCR was performed as follows:

95°	10 min	
94°	45 sec	x 40
60°	45 sec	
72°	45 sec	
72°	10 min	
4°	-	

The PCR product was then dehybridized and rehybridized in order to obtain heteroduplexes. If any indels (integration and/or deletion events) have occurred, the corresponding hairpin will be recognized by the *Surveyor® nuclease* (*Surveyor® mutation detection Kit*; IDT, Coralville, Iowa, USA), generating a band pattern that was visualized on 10% TBE gels 1.0 mm (Invitrogen) and analysed using Image J software. Percentages of cleavage were determined using the equation:

$$\%\text{NHEJ} = \frac{(Cleavaged\ band1 + Cleavaged\ band2) - (2xBackground)}{(Cleavaged\ band1 + Cleavaged\ band2 + Parental\ band) - (3xBackground)}\ x\ 100$$

8.2 NHEJ-repair events characterisation by Sanger sequencing

DNA obtained from the isolated gene edited LCL clones and the CFCs from HD or FA HSPCs was used as template to amplify the CRISPR/Cas9 target sites by PCR using *Herculase II fusion DNA polymerase* (Agilent) in the same conditions described for *Surveyor assay* (IDT) and using the same primer pairs. PCR products were cloned using *Zero Blunt PCR Cloning Kit* (Thermo Fisher Scientific) and plasmids transfected into TOP10 competent cells by heat-shock. The transformation was plated in selective Luria-Bertani medium (Sigma) plates supplemented with 50 μg/mL kanamycin and incubated overnight at 37°C. At least 3 individual colonies were picked to obtain products coming from both alleles and grown overnight in selective LB medium in a shaker at 37°C. Plasmids were extracted using *NucleoSpin® Plasmid Kit* (MACHEREY-NAGEL GmbH & Co. KG) and sent to Stabvida (Caparica, Portugal) to conduct Sanger sequencing using M13 primers. Chromatograms were visualized using Finch TV (Digital World Biology LLC, Seattle, Washington, USA).

8.3 CRISPR/Cas9-induced gene editing analysis by next generation sequencing

Genomic DNA from edited LCLs or hCD34$^+$ cells was extracted using the *NucleoSpin Tissue kit* (MACHEREY-NAGEL GmbH & Co. KG) or the *Proteinase K Lysis protocol* (61), depending on the cell number. Target sites were amplified by PCR using *Herculase II fusion DNA polymerase* (Agilent) in the same conditions previously described, generating amplicons of approximately 200 bp surrounding the potential CRISPR binding site. Primers used in these PCRs are listed in Table 6. PCR products were purified by Stabvida using the AxyPrep PCR Clean-Up (Axygen/Corning), quantified using a *Qubit fluorometer* (Thermo Fisher Scientific), and used for library construction with the *KAPA Library preparation Kit* (Kapa Biosystems, Wilmington, Massachusetts, USA) for Illumina platforms. The generated DNA fragments (DNA libraries) were sequenced by Stabvida with v3 chemistry in the *lllumina MiSeq platform*, using 250-bp paired-end sequencing reads.

The analysis of the sequence raw data obtained was carried out using *CLC Genomics Workbench 9.5.4* (QIAGEN Bioinformatics) and those high-quality sequencing reads were mapped against the reference sequences using a length and a similarity fraction of 0.80. A low frequency variant calling was performed, obtaining a list of variants with a low representation within each sample following two criteria: a minimum frequency of 0.01% (meaning a probability of 0.0001); and a forward/reverse sequence read balance ≥30% combined with localization in homopolymeric regions ≤2 nucleotides. Those sequences containing indels of ≥1 bp located within a region encompassing ±5 bp from the cleavage site were considered as CRISPR-induced genome modifications. The frequency of each indel was calculated by the analysis software according to the total number of reads.

Table 6.- Primers used in the on-target NGS analyses.

Region	Primer	Sequence (5' to 3')	Tm (°C)	PCR product size (bp)
c.3558insG	Fw	ATCTCAGCCACCCTCATCTG	60	176
	Rv	ATCTCACCACCCACACGTAC		
c.295C>T	Fw	CCTTTGCATCTATTCTCCCCGT	60	234
	Rv	TGCAGATCTGTCCCACGCTA		

8.4 Next generation sequencing analysis of putative off-targets

The most probable off-target loci for gGM4 sgRNA were predicted *in silico* using the *CRISPR Design* web tool from MIT (http://crispr.mit.edu/). Among the different off-targets that were identified, the top-five were analysed in our study.

Genomic DNA from FA patient hCD34$^+$ cells targeted using RNP4 complex was extracted 5 days after electroporation using the *NucleoSpin® Tissue Kit* (MACHEREY-NAGEL GmbH & Co. KG) or the *Proteinase K Lysis protocol* (61). The putative off-target sites were amplified by PCR including Illumina adapters in the primer sequence using *Herculase II fusion DNA polymerase* (Agilent) in the same previously described conditions, generating amplicons of approximately 200 bp surrounding the potential CRISPR targeting site. Primers used in these PCRs are listed in Table 7. The following procedure was conducted by Servicio de Genómica y Bioinformática from Universidad Autónoma de Barcelona (Barcelona, Spain). PCR products were purified using the *AxyPrep PCR Clean-Up* (Axygen), quantified using a *Qubit fluorometer* (Thermo Fisher Scientific), and used for library construction using the *KAPA Library preparation Kit* (Kapa Biosystems) for Illumina platforms. The generated DNA fragments (DNA libraries) were sequenced on *MySeq platform*, using 150-bp paired-end sequencing reads. The raw data results were analysed and interpreted using the *CRISPResso* bioinformatics tool (210) (http://crispresso.rocks/). Those sequences containing indels of ≥1 bp located within a region encompassing ±5 bp from the cleavage site were considered as CRISPR-induced genome modifications.

Table 7.- Primers used in the off-target NGS analyses.

Off-target	Primer	Sequence (5' to 3')	Tm (°C)	PCR product size (bp)
OT1	Fw	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GCAAGTGGGAAAACACAGGT**	62	232
	Rv	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**AGACCCCTCTTCAGGAAGTC**		
OT2	Fw	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**TCCAGGGCTGGAATGTAGTC**	62	193
	Rv	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GCTTCTGAGCTGCAAGGTCT**		
OT3	Fw	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**AGTGCCACCAGAGGTTAGGT**	62	161
	Rv	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**CTCACCCTCCATATGGGACA**		
OT4	Fw	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**CGCTTCCTGACATGATGGT**	62	168
	Rv	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**AGGCACAGGTGCTTTACCTC**		
OT5	Fw	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GGGAACAGCCAGTTCTCATC**	62	183
	Rv	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**TGCATGTGGAGGTGGTACAT**		

Normal letters indicate the *Illumina* adaptor sequences added to the actual primer sequence (signalled in bold).

9. AAV Transduction Experiments

9.1 HD HSPC transduction

9.1.1 Optimization of the transduction conditions

Before testing the gene editing capacity of the different AAV-constructs carrying the CRISPR/Cas9 system and to evaluate the transduction efficacy of the AAVs in HSPCs, the green fluorescence (GFP) gene was packaged under two different conformations (self-complementary and single-stranded) in an AAV2 and AAV6.

Purified hCD34$^+$ cells from HD UCBs were pre-stimulated for 24 hours with the corresponding hematopoietic cytokines (see section 1.2.1) at a density of 10^6 cells/mL. Then, 10^5 cells were transduced with the corresponding AAV at different multiplicities of infection (MOI) ranging from 10^2 to 10^6 in a 96-well culture plate in a final volume of 100 µL with 0, 10 or 30 µg/mL dimethyl prostaglandin E2 (dmPGE2, StemCell Technologies). Twenty-four hours after transduction, cells were collected and washed in order to eliminate the viral particles that remained in the medium. Transduction efficacy and toxicity were analysed by flow cytometry using an *LSRFortessa Cell Analyser* (BD) in terms of the GFP fluorescence and DAPI (Thermo Fisher Scientific) positive cells, respectively. The GFP signal and the viability of the cells were monitored at 48, 72, 96 hours and 7 days after transduction. Off-line analysis was performed using *FlowJo Software v7.6.5* (FlowJo LLC).

9.1.2 AAV-mediated gene editing experiments

Human CD34$^+$ cells purified from HD UCBs were pre-stimulated for 24 hours, with the corresponding hematopoietic cytokines described in section 1.2.1, at a density of 10^6 cells/mL. Then, 10^5 cells were transduced with the ssgGM2SaCas9-AAV6 construct (MOI 10^5) or co-transduced with the ssSpCas9-AAV6 construct (MOI 10^5) in combination with scgGM10-AAV6 (MOI 10^3), in a final 100 µL volume with 10 µg/mL dmPGE2. Twenty-four hours after transduction, cells were collected and washed, and the toxicity of the transduction evaluated by flow cytometry using an *LSRFortessa Cell Analyser* (BD) in terms of DAPI (Thermo Fisher Scientific) positive cells. Off-line analysis was performed with *FlowJo Software v7.6.5* (FlowJo LLC). Editing efficacy was measured in terms of the percentage of indels detected by *Surveyor assay* (IDT) 72 hours after transduction, as previously described in section 8.1.

9.2 *In vivo* transduction

9.2.1 NSG mice transplantation with HD HSPCs

Prior to *in vivo* inoculation, NSG mice were transplanted through the tail vein with $hCD34^+$ cells purified from HD UCBs after a submyeloablative 1.5 Gy dose irradiation. Each mouse received $3x10^5$ cells. One month after transplantation, human engraftment was evaluated by bone marrow aspiration as previously described in section 7.3. All animals used showed percentages of human engraftment superior to 70%.

9.2.2 *In vivo* inoculation

Once the presence of human engraftment was verified, recipient mice were inoculated with $2x10^{11}$ viral particles of scGFP-AAV6 construct through the tail vein (200 µL) or with $4.4x10^{10}$ by intrafemoral injection (20 µL). Forty-eight hours later, mice were euthanized and peripheral blood and total bone marrow from the injected femur and the contralateral one were extracted. Transduction efficacy was evaluated in terms of the percentage of GFP^+ cells within human and murine $CD45^+$ cells (hCD45-APC-Cy7 and mCD45-PE-Cy7, respectively) using an *LSRFortessa Cell Analyser* (BD). Off-line analysis was performed with *FlowJo Software v7.6.5* (FlowJo LLC).

10. Statistical Analyses

Statistical analyses were performed using *GraphPad Prism* software package for Windows (version 6.0, GraphPad Software, San Diego, California, USA). For the analyses of experiments in which $n < 5$, a nonparametric two-tailed Mann–Whitney test was performed when two variables were compared, or Kruskal-Wallis with Dunn's multiple comparison test when more than two variables were compared. In the experiments in which $n \geq 5$, a Kolmogorov-Smirnov test was done to test the normal distribution of the samples. If samples showed a normal distribution, a parametric two-tailed paired t-test was performed when two variables were compared or an ANOVA with Tukey's multiple comparisons test when more than two variables were compared. If samples did not follow normal distribution, the previously mentioned nonparametric tests were used. Significances are indicated in the figures and legends.

"It's a dangerous business, Frodo, going out your door. You step onto the road, and if you don't keep your feet, there's no knowing where you might be swept off to."

— J.R.R. Tolkien, The Fellowship of the Ring.

Results

1. Therapeutic NHEJ-mediated Gene Editing in Fanconi Anemia A Lymphoblastic Cell Lines

According to the objectives proposed, the first step was to verify if NHEJ repair pathway could be used to correct *FANCA* mutations. As a proof of principle, the biallelic insertion of a Guanine in *FANCA* exon 36 (c.3558insG) was selected. This insertion provokes a 28 amino acid frameshift and, as a consequence, a premature stop codon afterwards (p.R1187EfsX28), truncating the C-terminal end of the protein (46). This mutation was chosen because spontaneous reversions have been described in several patients (272).

Due to their advantageous characteristics, CRISPR/Cas9 nucleases were the gene editing tools chosen to target the mutation (see section 3.1.4 in Introduction). The design and selection of the sgRNAs was done according to two criteria: the highest score possible (which implies high specificity and, as a consequence, less probable off-target activity), and the closest distance to the mutation, to minimize the region affected by the indels generated. Two sgRNAs were selected (gINS8 and gINS11, Figure 11) and cloned separately into the pX330 plasmid, carrying the humanized codon optimized *Streptococcus pyogenes* Cas9 nuclease for the simultaneous delivery of both components.

Figure 11.- Schematic representations of *FANCA* exon 36 region where c.3558insG mutation takes place. The insertion is coloured in red. Target sequences recognized by the two sgRNAs designed are marked with a coloured line. The expected double-strand break (DSB) sites are marked with a coloured triangle. PAM sequences are underlined in black. Codons are represented by alternating bold and normal letters.

To test the efficacy of the selected sgRNAs, a lymphoblastic cell line (LCL) from a FA-A patient (FA-178) bearing the c.3558insG mutation in homozygosis was electroporated using the all-in-one plasmid carrying either gINS8 or gINS11. Different amounts of plasmid DNA were used, ranging from 1 to 10 μg, in order to find the most suitable editing condition. Toxicity of the electroporation was evaluated 24 hours later by flow cytometry and the targeting efficacy was measured by *Surveyor assay* at day 5 (Figure 12). Three micrograms of plasmid gINS11 recurrently resulted to be the most efficient dose, allowing a 22.3 ± 5.9% editing rate with the

lowest toxicity, while cells edited using gINS8 construct could not be amplified. Therefore, subsequent analyses were conducted in LCLs edited with 3 µg of gINS11 plasmid.

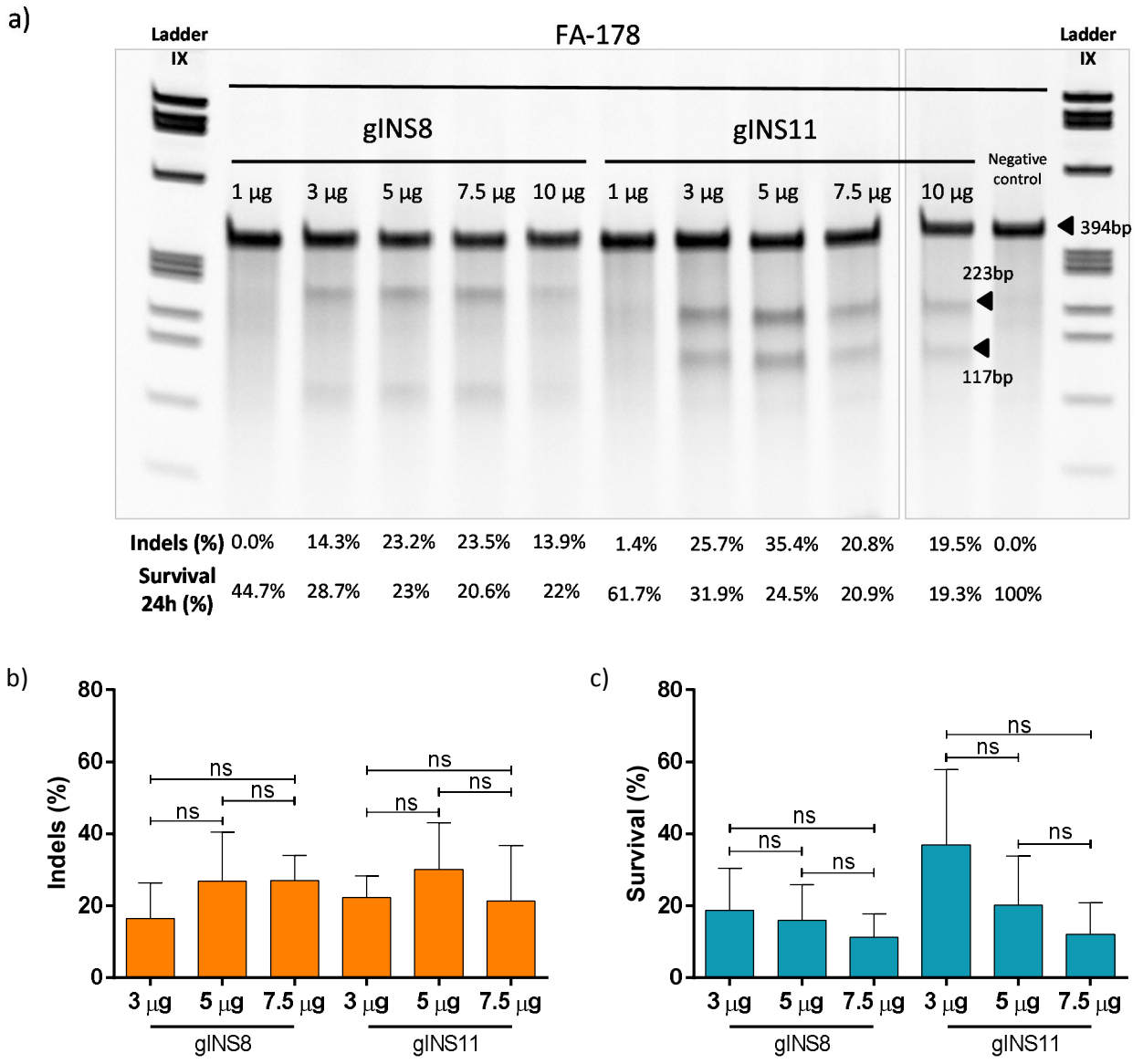


Figure 12.- Characterisation of the editing efficiency in FA-178 LCL using CRISPR/Cas9 nucleases. A FA-A LCL bearing the c.3558insG mutation (FA-178) was electroporated with all-in-one plasmids carrying specific sgRNAs to target the mutation: gINS8 and gINS11. (**a**) CRISPR/Cas9 efficacy was assessed by *Surveyor assay* and the toxicity of the electroporation was determined by the analysis of DAPI positive cells by flow cytometry. A representative image is showed. (**b**) Quantification of the percentage of indels at day 5 post-electroporation. (**c**) Cell survival 24 hours after electroporation. Bars represent mean ± standard deviation (SD) of three different analyses. ns = not significant.

Five days after electroporation, next generation sequencing (NGS) analysis was conducted with the aim of characterising the different NHEJ-repair events that had taken place after the DSB induced by the gINS11 construct. The results showed that a 7.62% from a total 9.94%

editing rate (measured in terms of the percentage of indels detected) corresponded to potentially therapeutic frame-restoring NHEJ-repair events (Figure 13a), as these editing events compensated the frameshift provoked by the original mutation. Interestingly, as small deletions affecting nucleotides surrounding the cutting site are the most frequent NHEJ-repair events detected after a DSB (41), the deletion of the 5'-Guanine immediately adjacent to the Cas9 cutting site (c.3579delG) showed to be extremely favoured, with a frequency more than 10-times higher (6.67%) than the second most frequent indel (c.3572_3578del7 –0.50%–), (Figure 13b). To determine if these events really restored the functionality of *FANCA*, different approaches were conducted.

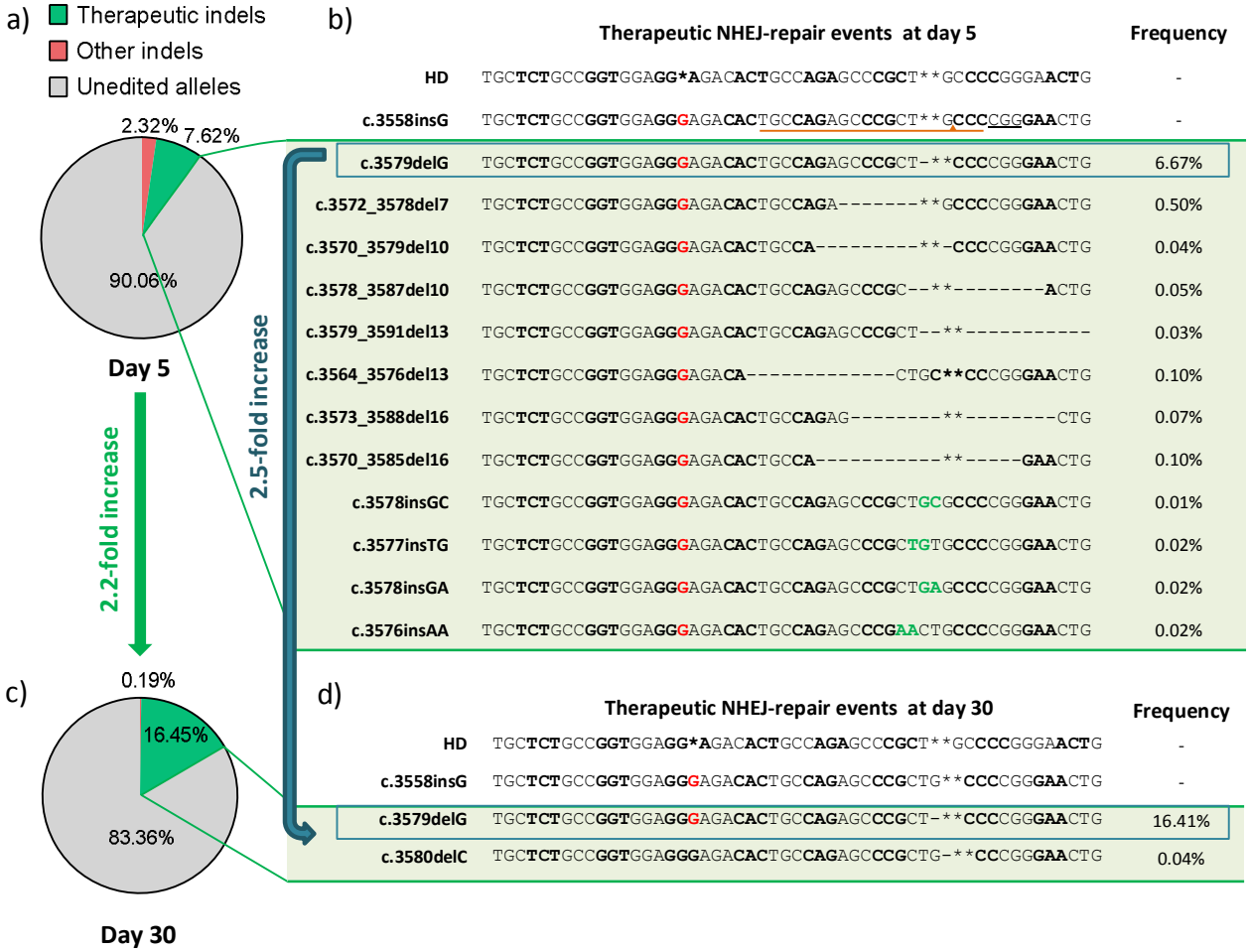


Figure 13.- Percentage of indels identified by NGS at day 5 and 30 after editing. The percentage of editing was measured in terms of the indels detected after a variant calling. Sequences obtained both at 5 (**a**) and 30 days (**c**) were classified into three groups: those that were not edited (unedited alleles –grey–); sequences suffering a DSB that was repaired by NHEJ but did not restore the ORF (other indels –red–), and those in which a NHEJ-repair event that compensated the mutation, restoring the ORF, occurred (therapeutic indels –green–). The therapeutic NHEJ-repair events found at 5 (**b**) and 30 (**d**) days after electroporation and their frequencies are listed in the adjacent tables. Guide gINS11 recognition sequence and cutting site are signalled with an orange line and a triangle, respectively. PAM sequence is underlined in black. The c.3558insG mutation is signalled in red and therapeutic insertions in green. Codons are represented by alternating bold and normal letters. The symbols stand for: "-" = deletion; "*" = insertion.

First of all, edited cells were amplified in culture and NGS analysis was conducted 30 days after electroporation. This analysis showed a marked enrichment of the potentially therapeutic NHEJ-repair events (16.45%) (Figure 13c). However, this enrichment was mainly due to the expansion of the c.3579delG indel, whose frequency experienced a 2.5-fold increase over time, from the initial 6.67% determined at day 5 to 16.41%. In contrast to this observation, the other editing events not only did not increase but even disappeared (Figure 13b and d). This evidence strongly suggested that many of the putative therapeutic indels were not functional, so the open-reading frame (ORF) restoration was necessary but not sufficient to recover the gene function. Accordingly, the expansion experienced by the c.3579delG indel was generated due to a significant proliferative advantage conferred to the bearing cells, and not only because it was the majoritarian NHEJ-repair event initially obtained.

To further confirm this hypothesis, a FANCA protein BLAST analysis was conducted using the bioinformatic tool hosted on the Uniprot webpage (https://www.uniprot.org/blast/). Available FANCA protein amino acid sequences from different vertebrates were compared in order to identify the most conserved regions. The results obtained showed that exon 36 sequence is one of the most conserved ones, which implies that very few variations could be admitted without compromising the structure/functionality of the protein. Nevertheless, Figure 14 shows that the sequence comprised between the c.3558insG mutation (where the frameshift starts –red arrow–) and the c.3579delG generated by NHEJ after the DSB (where the ORF is restored –green arrow–) was not conserved, and gINS11 was targeting this less conserved sequence. This fact perfectly explains why the deletion of the G next to the Cas9 cutting site is the only editing event which conferred proliferative advantage among the wide variations of therapeutic NHEJ-repair events initially obtained in edited FA-178 LCL: the single-nucleotide deletion originated an immediate recovery of the frameshift, upstream of the conserved domain placed downstream to the DSB. Conversely, the other potentially therapeutic indels generated larger deletions that affected conserved amino acids, implying an impairment in the functionality of the protein. In addition, these results also explain why cell cultures electroporated with gINS8 construct could not be expanded, as this sgRNA was targeting the highly conserved region marked with an orange arrow in Figure 14.

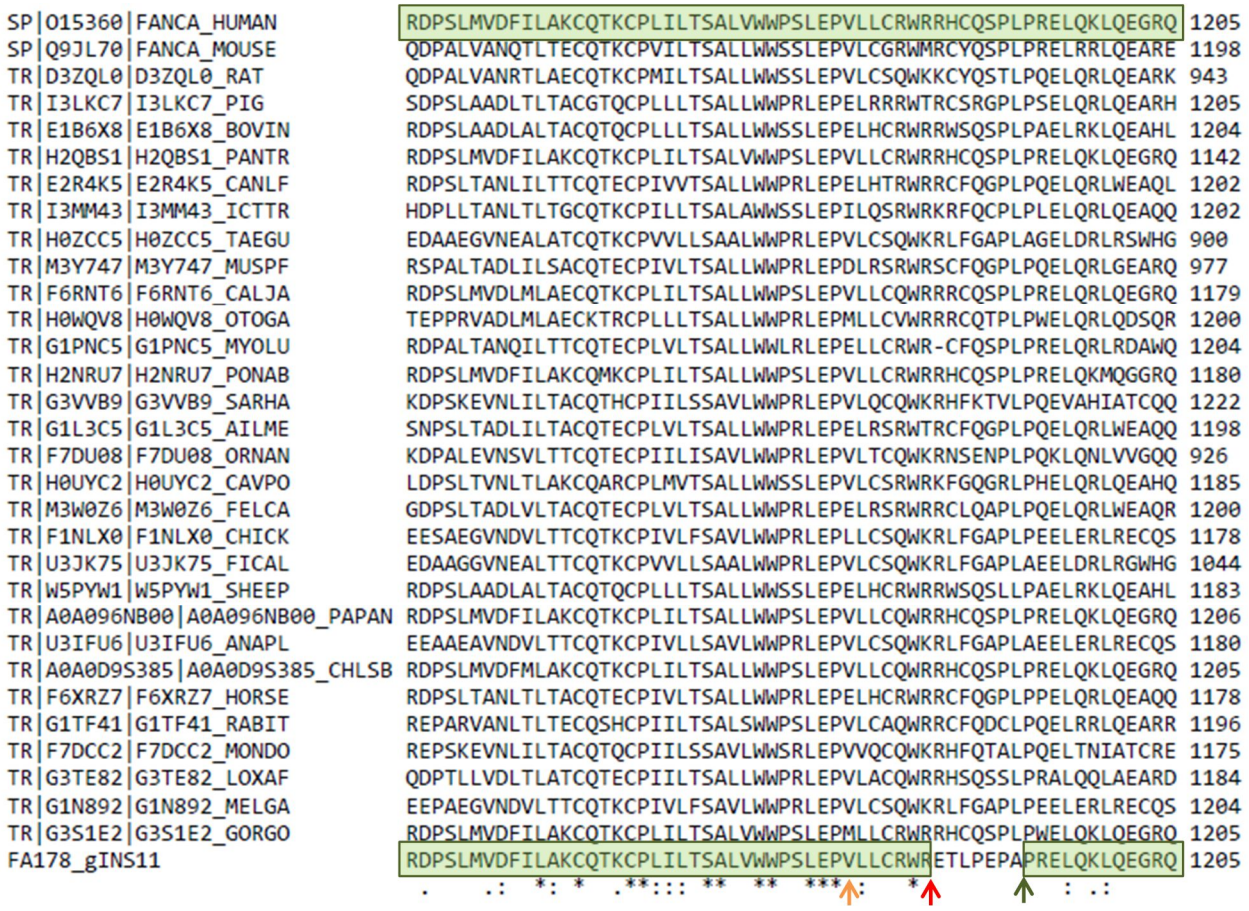


Figure 14.- FANCA protein BLAST analysis corresponding to exon 36. Alignment of the FANCA protein sequence corresponding to part of exon 36 from different vertebrates is shown. FA178_gINS11 = FANCA protein sequence of the most frequent therapeutic NHEJ-repair event obtained after editing with gINS11 construct: the c.3579delG indel. Red arrow indicates the beginning of the frameshift provoked by the insertion c.3558insG. Green arrow indicates restoration of the original frame due to the deletion c.3579delG. Orange arrow indicates gINS8 target site. According to *Uniprot protein BLAST analysis tool* an "*" (asterisk) indicates positions which have a single, fully conserved residue; a ":" (colon) indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix and a "." (period) indicates conservation between groups of weakly similar properties - scoring =< 0.5 in the Gonnet PAM 250 matrix. Amino acids without symbol: not conserved. Sequence marked in green indicates the wild-type FANCA sequence.

The proliferative advantage of the cells bearing therapeutic indels strongly suggested the re-expression of a functional FANCA protein. With the aim of confirming the stable expression of the new edited protein, a Western-blot assay against FANCA was conducted (Figure 15). As a result, edited FA-178 LCL showed the re-expression of this protein, reaching comparable levels to a healthy donor (HD) cell line, in contrast to unedited FA-178 control cells in which FANCA protein was totally absent.

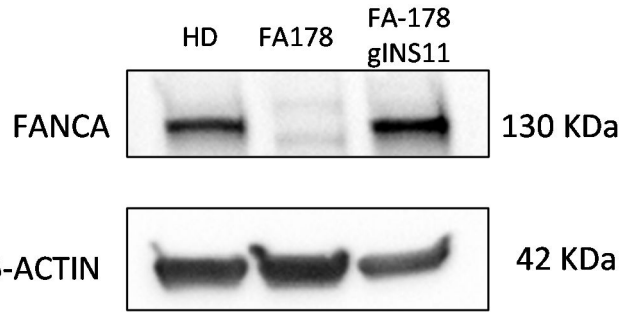


Figure 15.- FANCA protein re-expression in gene edited LCLs. Western-blot analysis was conducted 60 days post-electroporation in edited FA-178 LCL in comparison with healthy donor (HD) and unedited FA-178 LCL. β-actin was used as a loading control.

In order to further confirm that the newly expressed FANCA protein was functional, an immunofluorescence assay against FANCD2 protein was conducted (Figure 16). As was described in the Introduction at section 1, FANCD2 is another member of the FA/BRCA pathway which is recruited by FANCA to signal the lesions on the DNA and trigger their repair. When a HD LCL is exposed to an ICL-inducing agent such as mitomycin C (MMC), FANCD2 foci can be observed, in contrast to FA-A LCLs where the recruitment cannot take place. FANCD2 immunofluorescence showed the recovery of the foci formation in edited FA-178 LCL, confirming the functionality of the new FANCA protein and demonstrating the restoration of the FA pathway.

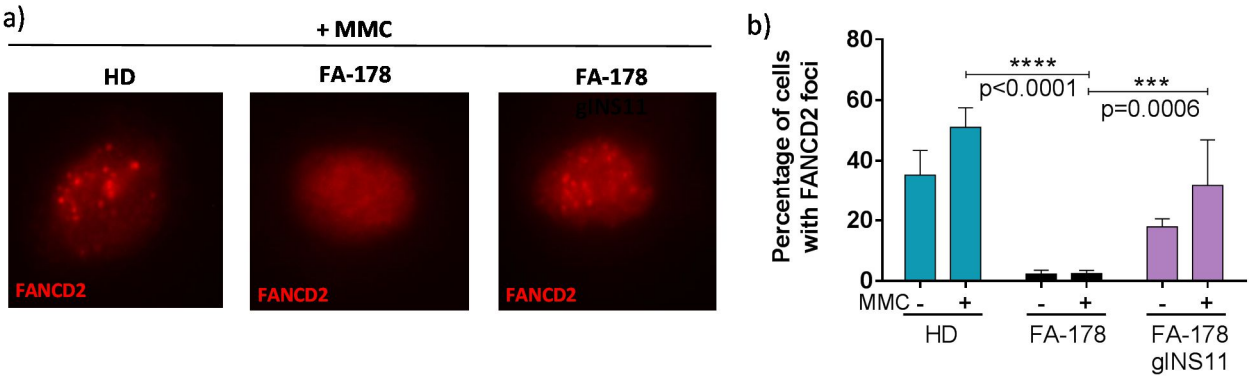


Figure 16.- NHEJ-edited FA-178 LCL restored the formation of FANCD2 foci. (**a**) Representative immunofluorescence against FANCD2 protein (Alexafluor-549nm) microphotographs in LCLs. (**b**) Percentage of cells with FANCD2 foci in the presence or absence of MMC. Cells with more than 10 foci were scored as positive. Two hundred cells were counted for each condition. Bars represent mean ± SD of five different analyses. A two-way ANOVA was performed followed by Tukey's post hoc test.

Finally, edited cells were cultured in the presence of increasing concentrations of MMC and cell viability was analysed 5, 10 and 15 days afterwards. Edited FA-178 LCLs using gINS11 construct showed the reversion of the FA-characteristic MMC hypersensitivity, which was similar to a HD LCL, in contrast to the untreated FA-178 LCL (Figure 17).

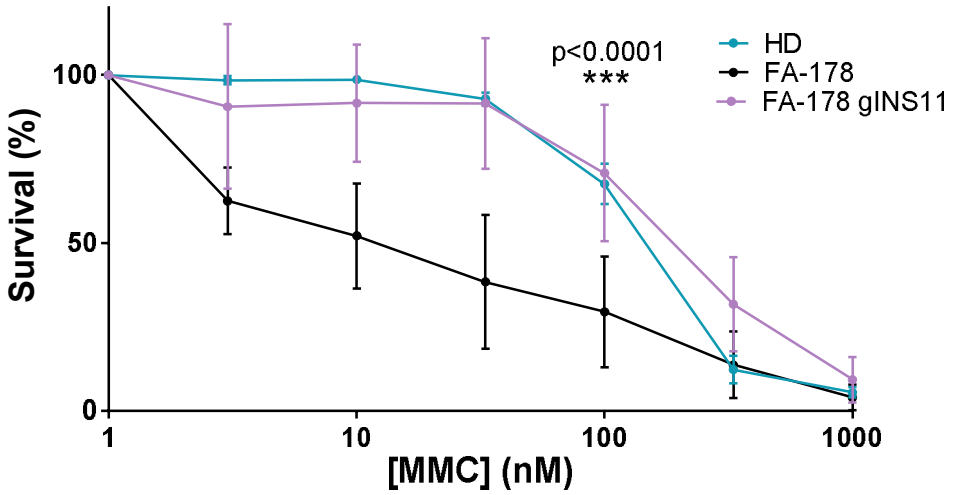


Figure 17.- Reversion of MMC hypersensitivity in edited FA-178 LCL. Edited FA-178 cells were cultured in the presence of increasing MMC doses. Cell viability was measured by flow cytometry in terms of the percentage of DAPI negative cells and compared with HD and FA-178 LCLs. Data are shown as mean ± SD from at least 5 different experiments. A two-way ANOVA analysis was performed, followed by Tukey's post hoc test. Statistically significant differences were observed in the survival at 100 nM MMC of HD and edited FA-178 cells compared to unedited FA-178 LCL.

As the generation of reactive oxygen species (ROS) was described as increased in FA cells (134), differences in ROS production between edited and untreated FA-178 LCL were analysed, as shown in Figure 18. The fluorogenic test showed a ROS level reduction in the edited cells compared to unedited ones.

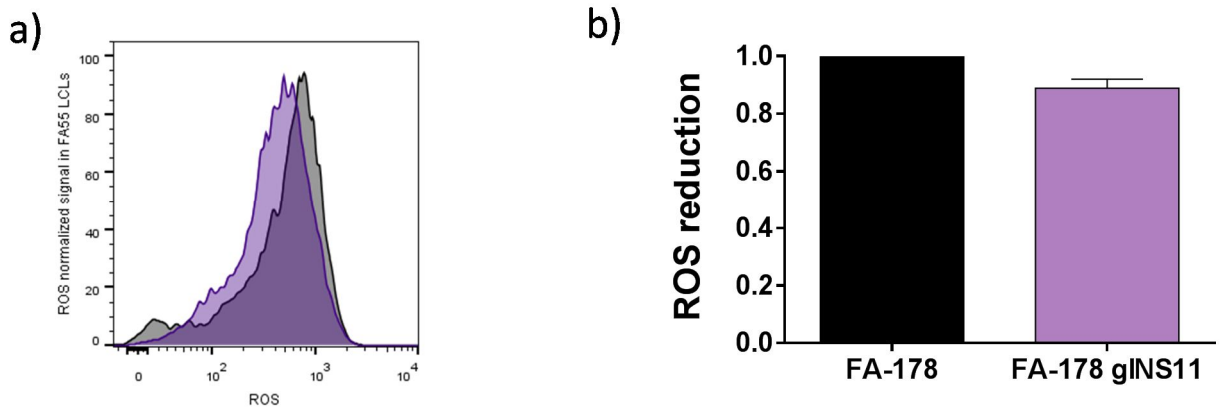


Figure 18.- Cell ROS production was reduced in edited LCL compared to untreated FA-178 LCL. ROS production was measured in FA-178 LCLs after NHEJ-mediated editing and compared with untreated FA-178 control LCLs. (**a**) Representative ROS quantification histogram. (**b**) ROS reduction calculated as the ratio of the ROS production measured in edited cells compared to untreated ones. Bars represent mean ± SD of three different analyses.

Taken together, these results demonstrate for the first time that NHEJ-mediated gene editing is a viable strategy to phenotypically correct FA-A LCLs carrying the c.3558insG mutation in *FANCA*.

2. NHEJ-mediated Gene Editing Can Be Applied to Correct a Variety of *FANCA* Mutations

To further study the extent of this approach, we focused on a more challenging FA-associated mutation: the homozygous c.295C>T substitution in *FANCA* exon 4 that generates a premature stop codon leading to a non-functional truncated protein (p.Q99X) (46).

As we had previously done, two different sgRNAs were designed to target the mutation (Figure 19) and cloned separately into the all-in-one pX330 plasmid.

Figure 19.- Schematic representation of the *FANCA* exon 4 region showing c.295C>T mutation. The mutation is coloured in red. The expected DSB sites and the recognition sequences of the sgRNAs are marked with a coloured triangle and a line, respectively. PAM sequences are underlined in black. Codons are represented by alternating bold and normal upper-case letters, and intronic sequence is showed in lower-case letters.

Different amounts of plasmid DNA, from 1 to 10 µg, were used (Figure 20a). The toxicity of the electroporation was determined by analysis of DAPI positive cells by flow cytometry 24 hours afterwards and the targeting efficacy was evaluated by *Surveyor assay* 5 days post-electroporation, showing mean editing efficacies up to 21.8 ± 5.4% for gGM4, and 48.7 ± 11.6% for gGM10 (Figure 20b). As a result, 5 µg of plasmid electroporation was demonstrated to be the condition that rendered the highest gene editing capacity without compromising cell survival for both sgRNAs. Cells electroporated with this dose were amplified for further experiments.

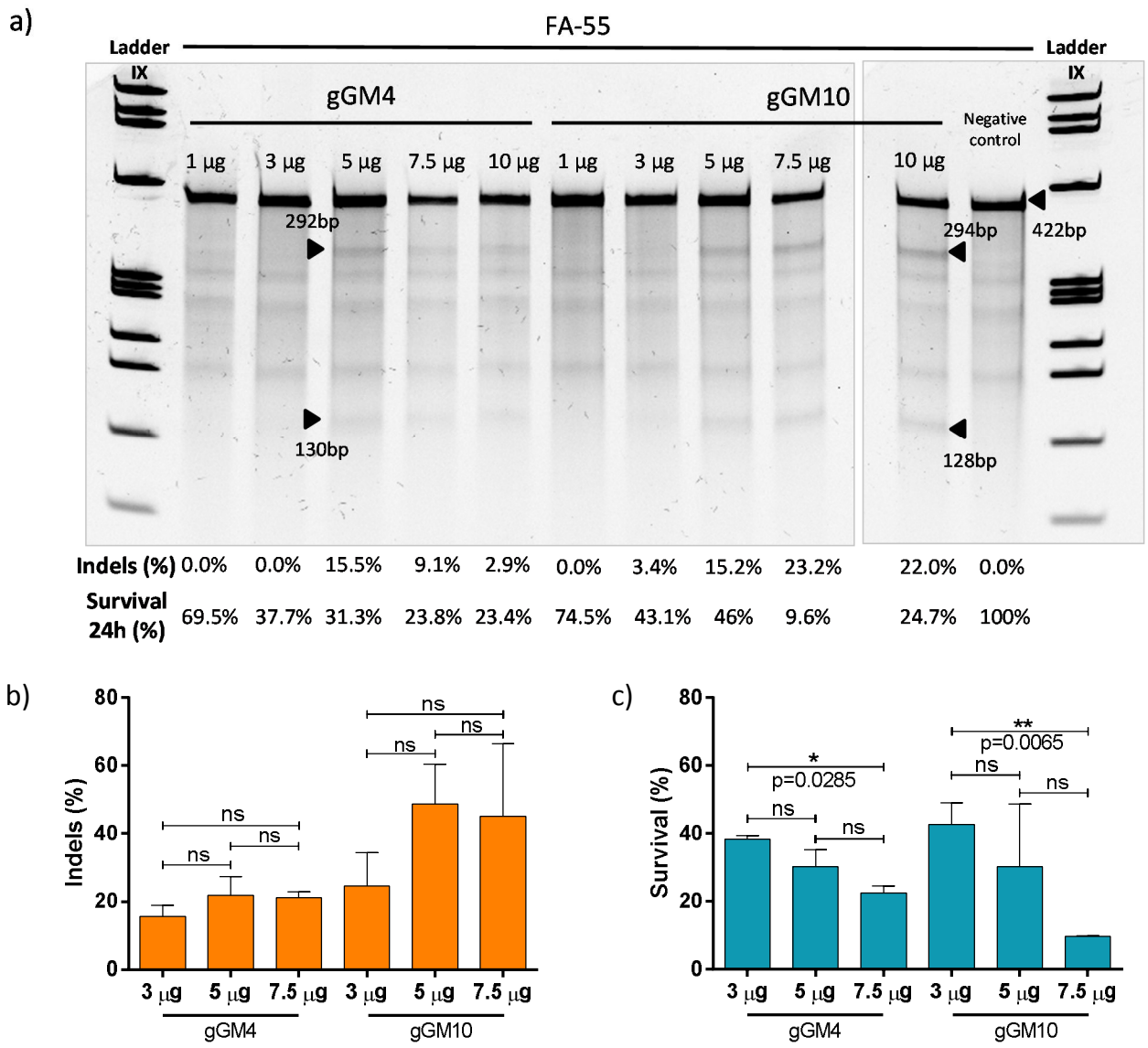


Figure 20.- Characterisation of the editing efficiency in FA-55 LCL using CRISPR/Cas9 nucleases. A FA-A LCL bearing the c.295C>T mutation in homozygosis (FA-55) was electroporated separately with the all-in-one plasmids carrying specific sgRNAs designed to target the mutation: gGM4 or gGM10. (**a**) CRISPR/Cas9 efficacy was assessed by *Surveyor assay* and the toxicity of the electroporation was determined by analysis of DAPI positive cells by flow cytometry. A representative image is showed. (**b**) Quantification of the percentage of indels at day 5 post-electroporation. (**c**) Quantification of the cell survival 24 hours after electroporation. Bars represent mean ± SD of three different analyses. ns = not significant.

NGS analysis of the NHEJ-repair events 5 days after electroporation showed a much lower initial percentage of therapeutic events than the observed with gINS11 in c.3558insG mutation: 0.29% for gGM4 (Figure 21a) and 0.41% for gGM10 construct (Figure 22a), consistent with the challenging nature of this mutation compared to the previous one. However, once again, a marked enrichment of the potentially therapeutic indels could be observed in both cases when the editing was analysed after 30 and 60 days in culture. This enrichment was particularly relevant in the case of the FA-55 LCL edited by the gGM10 construct where the therapeutic

NHEJ-events experienced a 36.4-fold and a total 115.9-fold increase after one and two months in culture, respectively (Figure 22a-c), compared to their initial frequency at day 5. In the case of gGM4, therapeutic events experienced 29.3-fold increase during this period (Figure 21a-c).

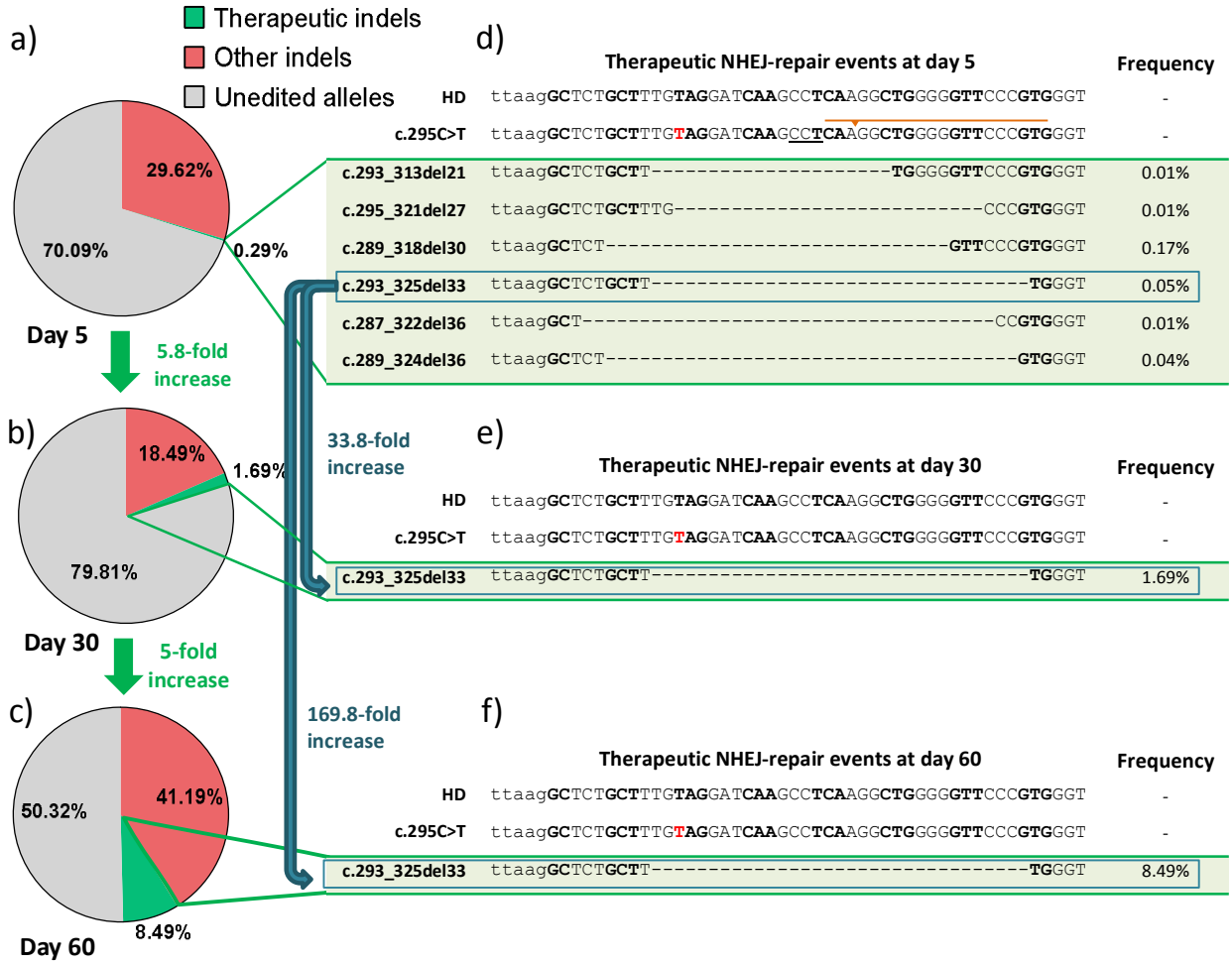


Figure 21.- Percentage of indels identified by NGS 5, 30 and 60 days after editing using gGM4. The percentage of editing is measured in terms of the indels detected after a variant calling. Sequences obtained at 5 (**a**), 30 (**b**) and 60 days (**c**) were classified into three groups: those that were not edited (unedited alleles –grey–); sequences suffering a DSB that was repaired by NHEJ but did not removed the stop codon (other indels –red–), and those in which a NHEJ-repair event removed the premature stop codon and preserved the ORF (therapeutic indels –green–). The therapeutic NHEJ-repair events found at 5 (**d**), 30 (**e**) and 60 (**f**) days after electroporation and their frequencies are listed in the adjacent tables. Guide gGM4 recognition sequence and cutting site are signalled with an orange line and a triangle, respectively. PAM sequence is underlined in black. The c.295C>T mutation is signalled in red. Codons are represented by alternating bold and normal upper-case letters, and intronic sequence is showed in lower-case letters. The symbol "-" stands for deletion.

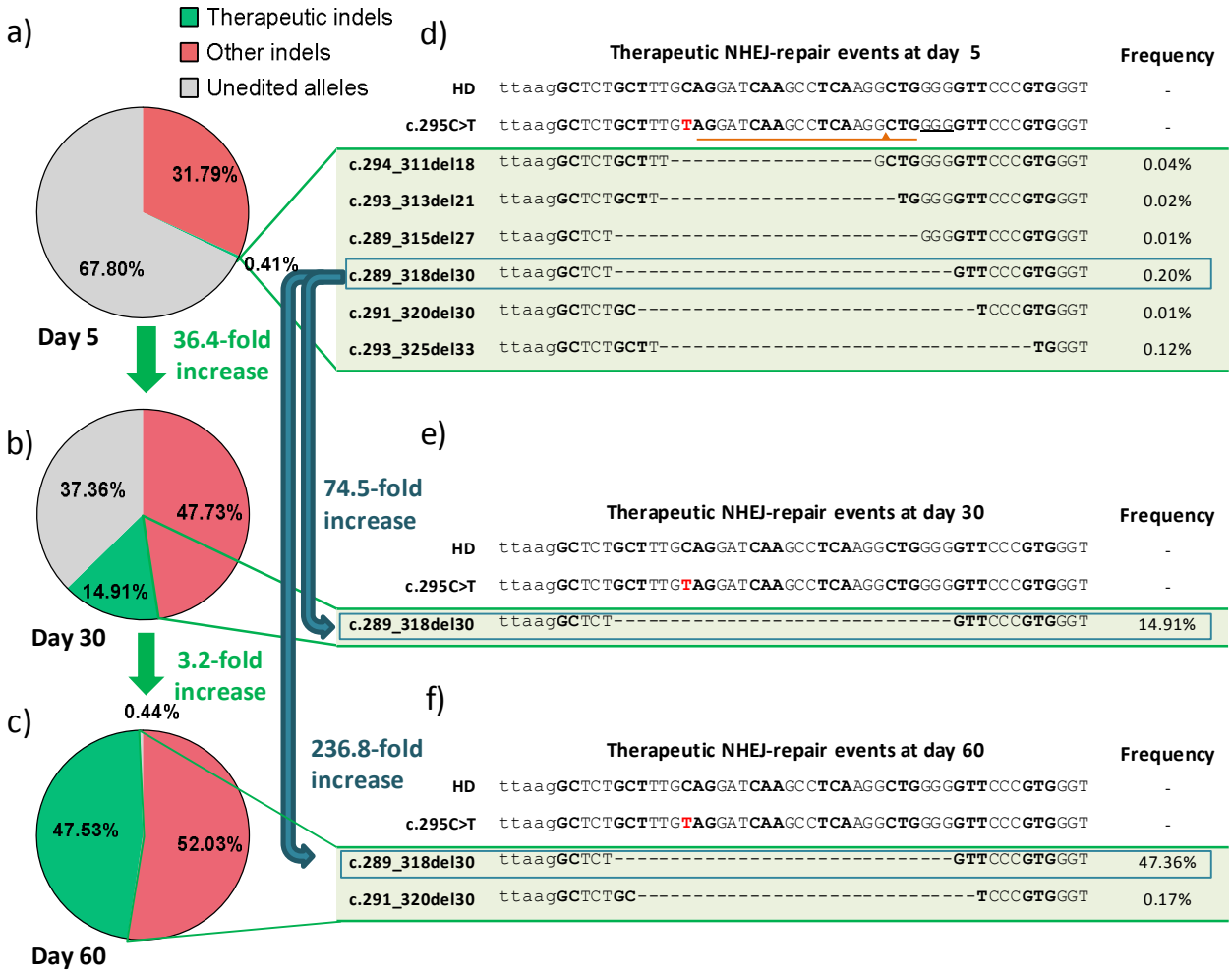


Figure 22.- Percentage of indels identified by NGS 5, 30 and 60 days after editing using gGM10. The percentage of editing is measured in terms of the indels detected after a variant calling. Sequences obtained at 5 (**a**), 30 (**b**) and 60 days (**c**) were classified as in Figure 21. The therapeutic NHEJ-repair events found at 5 (**d**), 30 (**e**) and 60 (**f**) days after electroporation and their frequencies are listed in the adjacent tables. Guide gGM10 recognition sequence and cutting site are signalled with an orange line and a triangle, respectively. PAM sequence is underlined in black. The c.295C>T mutation is signalled in red. Codons are represented by alternating bold and normal upper-case letters, and intronic sequence is showed in lower-case letters. The symbol "-" stands for deletion.

As previously observed in the NHEJ-repair events obtained in edited FA-178 LCL, deep sequencing analyses showed that not all the putative therapeutic indels initially obtained at day 5 conferred equal proliferative advantages. In the case of the FA-55 LCL edited by the gGM4 construct, only a 33-bp deletion (c.293_325del33) was maintained over time (Figure 21d-f). In this case, its frequency experienced a 33.8-fold increase after the first month in culture and a total 169.8-fold increase after two months.

Concerning FA-55 cells edited by gGM10 construct, the only NHEJ-indel whose frequency not only increased over time, but also became the predominant detectable event was a 30-bp

deletion (c.289_318del30), increasing 74.5 times within 30 days and 236.8 times during the 2-month follow-up after editing (Figure 22d-f).

Although FANCA protein BLAST analysis demonstrated that *FANCA* exon 4 is not conserved among vertebrates (Figure 23), the NGS results obtained evidenced that not all the potentially therapeutic NHEJ-editing events obtained were functional, in spite of having removed the stop codon and preserved the ORF.

```
SP|O15360|FANCA_HUMAN           RSHQDLNALLLEVEGPLCKK-LSLSKVIDCDSSEAYANHSSSFIGSA-QDQASRLGVPVG 110
SP|Q9JL70|FANCA_MOUSE           RYHQNMHDLLLEVEEPQCKR-LRLSELIDRDSADASSDRSASFIRSAFRDQASRLGVPVG 110
TR|D3ZQL0|D3ZQL0_RAT            ------------------------------------------------------------
TR|I3LKC7|I3LKC7_PIG            RSHLNLSDLFLEVEGPQFAE-PRLHRWIEWGGPEADARLSSSFVGSALQDQASRLGVPVA 110
TR|E1B6X8|E1B6X8_BOVIN          RSHLNLNDLILEVEGPPCDQ-PCLRQLIDCDGPEAHTNLSSSLIGSALRDQATRLGVPVA 110
TR|H2QBS1|H2QBS1_PANTR          ------------VEGPLCKK-LSLSKVIDCDSSEAYANHSSSFIGSALQDQASRLGIPVG 47
TR|E2R4K5|E2R4K5_CANLF          RRHLNLDDLLLEVEGPACKT-LCLNQLGD---PGASANLSSSFIGSALRDQASRLGVPVA 107
TR|I3MM43|I3MM43_ICTTR          RRHQSLSDLLLEVEAPPCGK-PRLDAVMGCDSDQGSTGGSCSFIGSAFQDQALKLGVPVG 110
TR|H0ZCC5|H0ZCC5_TAEGU          ------------------------------------------------------------
TR|M3Y747|M3Y747_MUSPF          ------------------------------------------------------------
TR|F6RNT6|F6RNT6_CALJA          RSRQDLSALLLEVEGVQCKK-LCLSNLIDCGSSEAHANHSSSFIGSALQDQASRLGVPVG 84
TR|H0WQV8|H0WQV8_OTOGA          RSHQDLNNLLLEVRVLLCRE-SKAGELMDCFSFFFFSHVFSFTCSVLQDQASRLGFPVG 110
TR|G1PNC5|G1PNC5_MYOLU          RRHVNLSQLLLEVEGPAPAE-PPRSRGTD---GWMDRGSPGALVGSALRDQASRLGVPVA 106
TR|H2NRU7|H2NRU7_PONAB          RRHQDLNALLLEVEGPLCKK-LSLSKVIDCDSSEACANHSSSFIGSALQDQASRLGVPVG 110
TR|G3VVB9|G3VVB9_SARHA          KRHQNLNDLFLEVGGIQCKKTLCFTSLIDHEISGVPGTHSKSFIVSTVQEQASKLGVPVG 112
TR|G1L3C5|G1L3C5_AILME          RRHLNLNDLLLEVEGPACKT-LCLHQLMD---PEASADLSSSLIGSALRDQASRLGVPVA 107
TR|F7DU08|F7DU08_ORNAN          ------------------------------------------------------------
TR|H0UYC2|H0UYC2_CAVPO          RRHQNLEDLLLEVEDPPCKK-LCPSQLIDCDSAEACSDWSFIFLSSALRDQALRLGVPVA 94
TR|M3W0Z6|M3W0Z6_FELCA          RRHLNLNDLLLEVEGAPRKT-LCLNQLID---PEACTNLSSSLIGSALRDQASRLGVPVA 107
TR|F1NLX0|F1NLX0_CHICK          DRHQNLSDLLLEVEGSAKPAG--------EGGREHQELSSESFIVSVLQEQASRLGVPTA 89
TR|U3JK75|U3JK75_FICAL          ------------------------------------------------------------
TR|W5PYW1|W5PYW1_SHEEP          RRHLNLNDLILEVEDPPCDQ-LCLRQLIDCDGPEAHTKLSSSLIGSALGDQATRLGIPVA 108
TR|A0A096NB00|A0A096NB00_PAPAN  RSHQDLTALLLEVEGPPCKK-LSLSKLIDCDSSEACANHSSSFIGSALQDQASRLGVPVG 110
TR|U3IFU6|U3IFU6_ANAPL          DRHQNLNDLLLEVEDLVKPAG--------ESGKEHQEALSESFIVSVLQEQASRLGVPAA 77
TR|A0A0D9S385|A0A0D9S385_CHLSB  RSHQDLNALLLEVEGPPCKK-LSLSKLIDCDSSEAYANHSSSFIGSALQDQASRLGIPVG 110
TR|F6XRZ7|F6XRZ7_HORSE          RSHLDWSGLLLEVEGPPCKK-LCLGPLIDHDGPEACSDLSTSFIGSALRDHASQLGVPVA 85
TR|G1TF41|G1TF41_RABIT          SGRQDVGGLLVEVGRAGSAF---RGDEDRASGSEARAQRLGTLVGSALRDEASRLGVPAG 112
TR|F7DCC2|F7DCC2_MONDO          KCYQNLNDLFLEVDSTQCKKTLCFSNLSDHESPGVPGTHSKSFVVSALQEQASKIGVPVG 84
TR|G3TE82|G3TE82_LOXAF          RSRQNLDDLMLEVEGPQCKK-LCLSKFIDCDSSEAYTNPSNSLIGSALQDQASRLGVPVG 97
TR|G1N892|G1N892_MELGA          DRHQKLSELLLEAEGPVKPAG--------EGGIEHQELSSEPLIISVLQEQASRLGVPAA 101
TR|G3S1E2|G3S1E2_GORGO          RSHQDLNALLLEVEGPLCKK-LSLSKVIDCDSSEAYANHSSSFIGSALQDQASRLGVPVG 110
```

Figure 23.- FANCA protein BLAST analysis corresponding to exon 4. Alignment of the FANCA protein sequence corresponding to part of exon 4 from different vertebrates is shown. The amino acid affected by the c.295C>T mutation appears in a red square. According to *Uniprot protein BLAST analysis tool* an "*" (asterisk) indicates positions with a single fully conserved residue; a ":" (colon) indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix and a "." (period) indicates conservation between groups of weakly similar properties - scoring =< 0.5 in the Gonnet PAM 250 matrix. Amino acids without symbol: not conserved.

Remarkably, MMC treatment induced a selective pressure that primed the expansion of the therapeutic NHEJ-repair events, particularly in the case of the LCL edited using gGM4 construct (Figure 24a) where the initial proliferative advantage was lower.

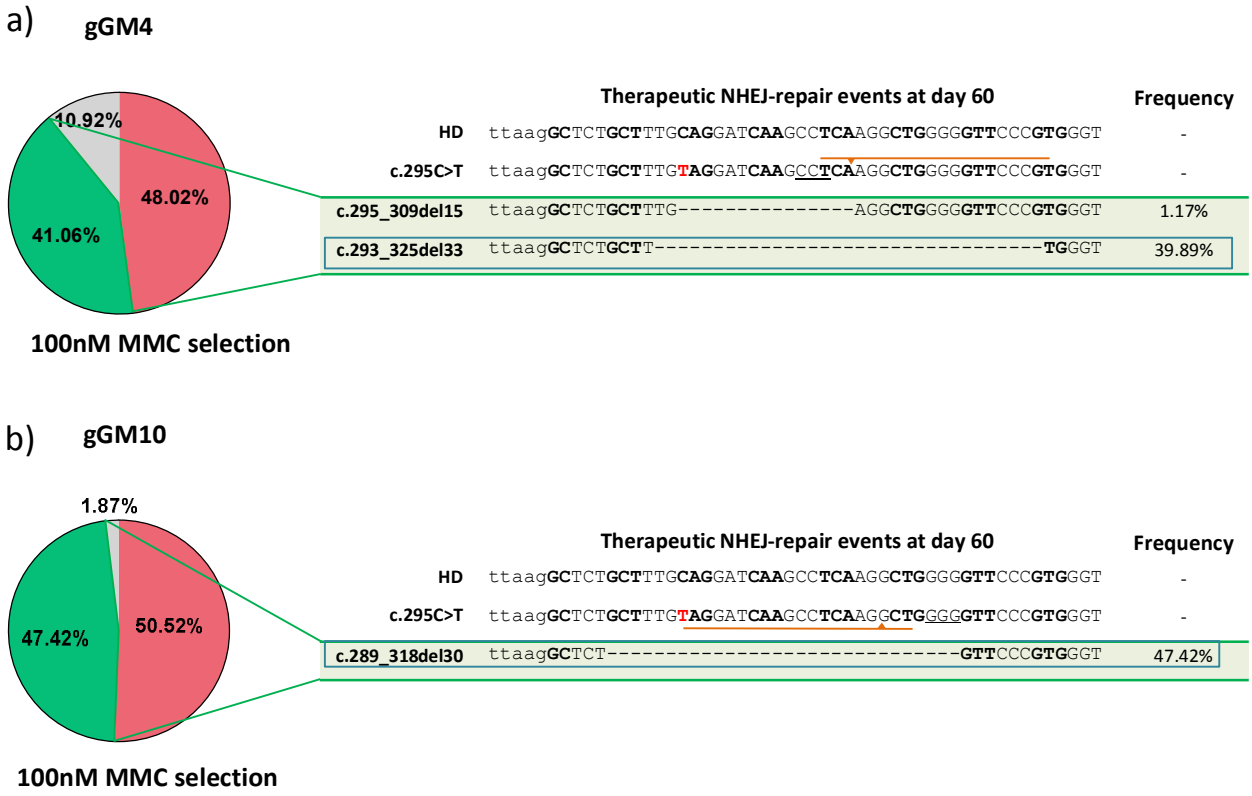


Figure 24.- Selection of therapeutic indels in edited FA-55 LCL after treatment with MMC for 5 days. Indels identified by NGS 60 days after editing in cells treated with 100 nM of MMC when gGM4 (**a**) and gGM10 (**b**) were used. Sequences obtained were classified as in Figure 21. The different therapeutic indels identified are listed on the right. Guide recognition sequences and cutting sites are signalled with an orange line and a triangle, respectively. PAM sequence is underlined in black. The c.295C>T mutation is signalled in red. Codons are represented by alternating bold and normal upper-case letters, and intronic sequence is showed in lower-case letters. The symbol "-" stands for deletion.

Strikingly, deep sequencing analyses showed that the percentage of non-therapeutic indels experienced a parallel increase together with the therapeutic indels (Figure 21a-c, Figure 22a-c and Figure 24). This phenomenon could be explained in cases where CRISPR/Cas9 nuclease had targeted both *FANCA* alleles but only one suffered a therapeutic NHEJ-repair event, while the other experienced a non-corrective one. To verify this hypothesis, single clones were isolated and Sanger sequencing confirmed that both alleles suffered a DSB that was repaired by NHEJ, but only one of them carried a therapeutic editing event that removed the stop codon while preserving the ORF (Table 8). The proliferative advantage conferred by the single corrected allele explained the parallel increase of corrected and uncorrected alleles observed in the NGS analyses.

Table 8.- Characterisation of FA-55 edited clones.

LCL	Clone	Allele	Editing	Sequence
FA-55 gGM4	2	1	c.295_309del15	ttaag**GC**TCT**GCTTT**G----------AGG**CTG**GGG**GTT**CCC**GTG**GGT
		2	c.300_320del21	ttaag**GC**TCT**GCTTT**G**T**AGGA----------**T**CCC**GTG**GGT
	3	1	c.295_309del15	ttaag**GC**TCT**GCTTT**G----------AGG**CTG**GGG**GTT**CCC**GTG**GGT
		2	c.300_320del21	ttaag**GC**TCT**GCTTT**G**T**AGGA----------**T**CCC**GTG**GGT
	4	1	c.295_309del15	ttaag**GC**TCT**GCTTT**G----------AGG**CTG**GGG**GTT**CCC**GTG**GGT
		2	c.300_320del21	ttaag**GC**TCT**GCTTT**G**T**AGGA----------**T**CCC**GTG**GGT
	9	1	c.293_325del33	ttaag**GC**TCT**GCT**T----------**TG**GGT
		2	c.311delG	ttaag**GC**TCT**GCTTT**G**T**AGGAT**CAA**GCC**TCA**A-G**CTG**GGG**GTT**CCC**GTG**GGT
FA-55 gGM10	9	1	c.289_318del30	ttaag**GC**TCT----------**GTT**CCC**GTG**GGT
		2	c.304_311del8	ttaag**GC**TCT**GCTTT**G**T**AGGAT**CAA**--------G**CTG**GGG**GTT**CCC**GTG**GGT
	10	1	c.289_318del30	ttaag**GC**TCT----------**GTT**CCC**GTG**GGT
		2	c.304_311del8	ttaag**GC**TCT**GCTTT**G**T**AGGAT**CAA**--------G**CTG**GGG**GTT**CCC**GTG**GGT
	11	1	c.289_318del30	ttaag**GC**TCT----------**GTT**CCC**GTG**GGT
		2	c.304_311del8	ttaag**GC**TCT**GCTTT**G**T**AGGAT**CAA**--------G**CTG**GGG**GTT**CCC**GTG**GGT

Editing events identified in each allele. Targeted loci were amplified by PCR and cloned into TOPO-TA system to sequence both alleles from each isolated clone. The c.295C>T mutation is signalled in red. Corrective NHEJ-repair events are marked in green while non-corrective ones are shown in red. Codons are represented by alternating bold and normal upper-case letters, and intronic sequence is showed in lower-case letters. The symbol "-" stands for deletion.

To verify whether the editing allowed the re-expression of FANCA protein, Western-blot analyses were conducted in the edited pool and in the isolated clones. As previously observed with c.3558insG mutation, both edited FA-55 LCLs and the different clones isolated re-expressed FANCA protein (Figure 25).

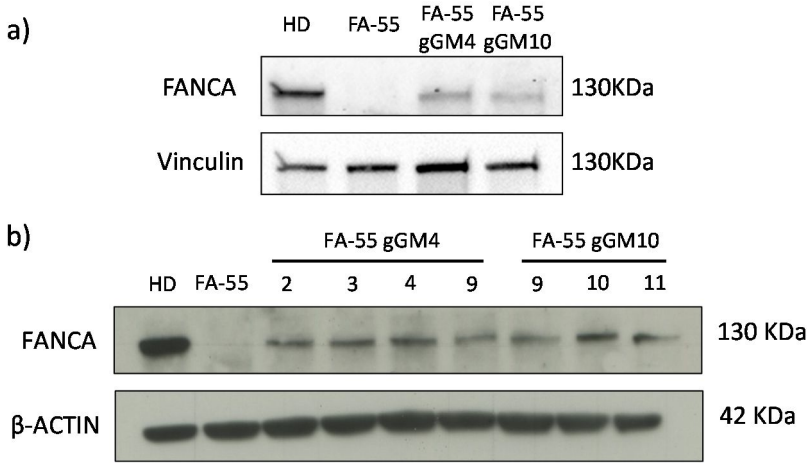


Figure 25.- Gene edited FA-55 LCL recovered the expression of FANCA. (**a**) FANCA Western-blot analysis in gene edited FA-55 LCLs compared to HD and unedited Fanconi LCLs. Vinculin was used as a loading control. (**b**) FANCA Western-blot analysis in the gene-edited FA-55 isolated clones. β-actin was used as a loading control.

In order to confirm the functionality of the new FANCA proteins generated, an immunofluorescence against FANCD2 was performed (Figure 26). As a result, the recovery of the FANCD2 foci formation could be observed in both edited FA-55 LCLs.

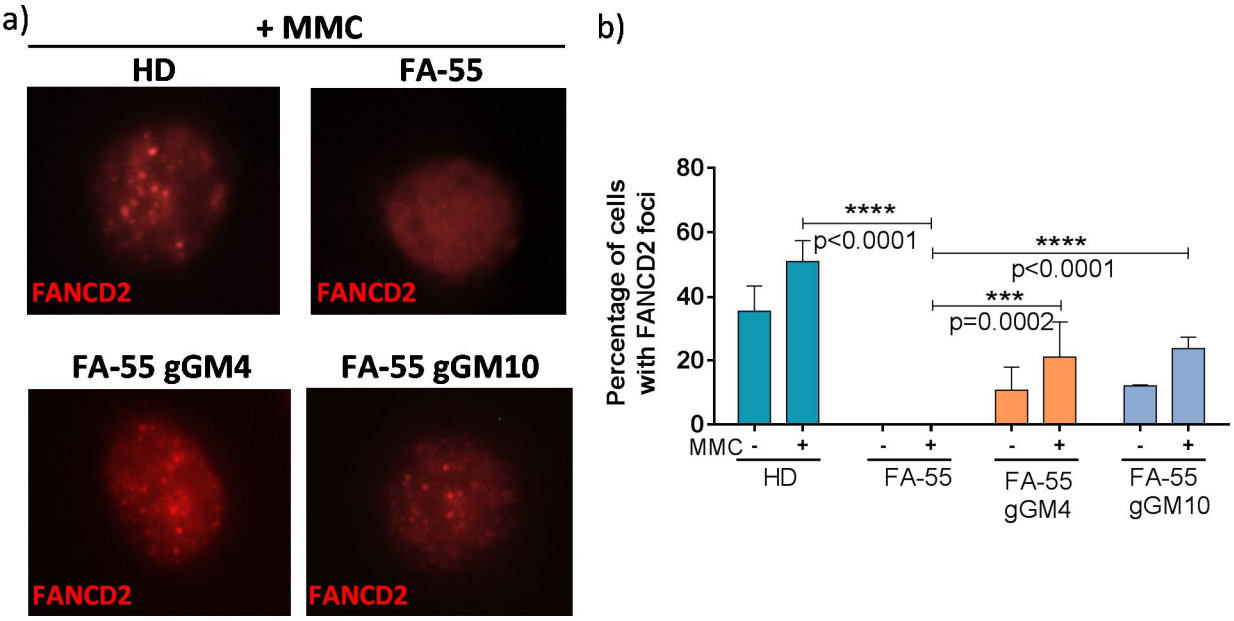


Figure 26.- Edited FA-55 LCLs recovered the formation of FANCD2 foci. (**a**) Representative immunofluorescence against FANCD2 protein (Alexafluor-549nm) microphotographs in LCLs. (**b**) Percentage of cells with FANCD2 foci in the presence or absence of MMC. Cells with more than 10 foci were scored as positive. Two hundred cells were counted for each condition. Bars represent mean ± SD of three different analyses. A two-way ANOVA was performed followed by Tukey's post hoc test.

Moreover, FA-55 LCLs edited either by gGM4 or gGM10 construct showed similar MMC resistance than a HD LCL (Figure 27).

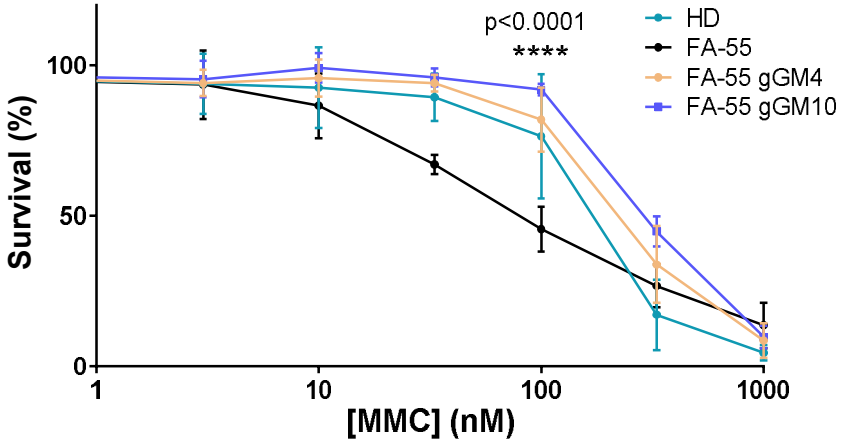


Figure 27.- MMC survival curve in edited FA-55 LCLs. Edited FA-55 cells were cultured under increasing MMC concentrations. The viability of the culture was measured by flow cytometry in terms of the percentage of DAPI negative cells and compared with the HD and the FA-178 LCLs. Data are shown as mean ± SD from at least 5 different experiments. A two-way ANOVA analysis was performed, followed by Tukey's post hoc test. Statistically significant differences were observed in the survival at 100 nM MMC of HD and edited FA-55 cells compared to unedited FA-55 LCLs.

Finally, ROS production was assessed in gene edited FA-55 LCLs in comparison to untreated FA-55 LCL and a reduction in the level of these compounds was observed (Figure 28).

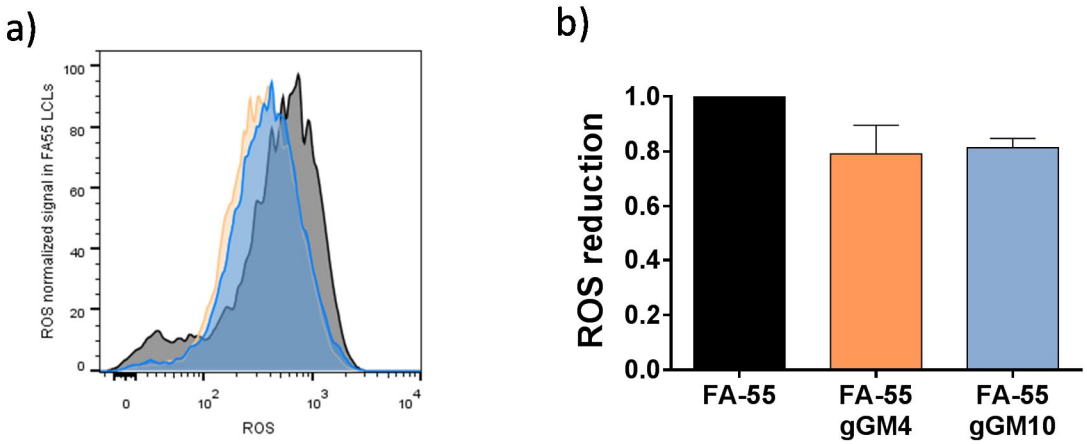


Figure 28.- Edited LCLs showed a reduction in cell ROS production compared to untreated FA-55 LCL. ROS production was measured in FA-55 LCLs after NHEJ-mediated editing and compared to untreated FA-55 control LCLs. (**a**) Representative ROS quantification histogram. (**b**) ROS reduction calculated as the ratio of the ROS production measured in edited cells compared to untreated ones. Bars represent mean ± SD of three different analyses.

These results, together with the proliferative advantage observed in edited FA-A LCLs, demonstrate that NHEJ-mediated gene editing is a feasible approach to correct the phenotype of different FA-A associated mutations, even those challenging ones where a stop codon must be removed.

Considering that TALENs have been described to be more specific than CRISPR/cas9 system (206) and thinking about a future clinical application, we aimed to test the efficiency of these nucleases to specifically target the c.295C>T mutation. To this end, a pair of TALEN monomers was designed to specifically target mutated alleles, as the c.295C>T mutation was included in the recognition sequence of the left monomer –TALEN-L– (Figure 29).

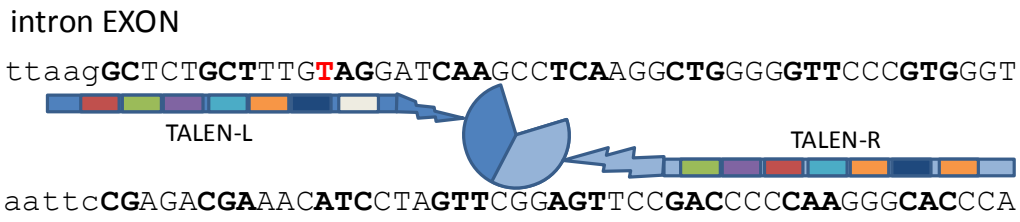


Figure 29.- Schematic representation of the *FANCA* exon 4 region showing c.295C>T mutation and TALEN target site. The mutation is coloured in red. The 18-bp sequences recognised by both TALEN monomers are marked with a multicoloured rectangle. The FokI nuclease domain is represented between both monomers in dark and light blue. The DSB is expected inside the 14-bp spacer sequence. Codons are represented by alternating bold and normal upper-case letters and intronic sequence is showed in lower-case letters.

HD and FA-55 LCLs were electroporated with different doses of the plasmids encoding these two monomers simultaneously. However, the TALEN-mediated gene editing turned out to be not only more toxic than the one obtained using CRISPR/Cas9 nucleases but also completely unspecific, as the TALEN targeted both HD and FA-55 cell lines with comparable efficacies (Figure 30).

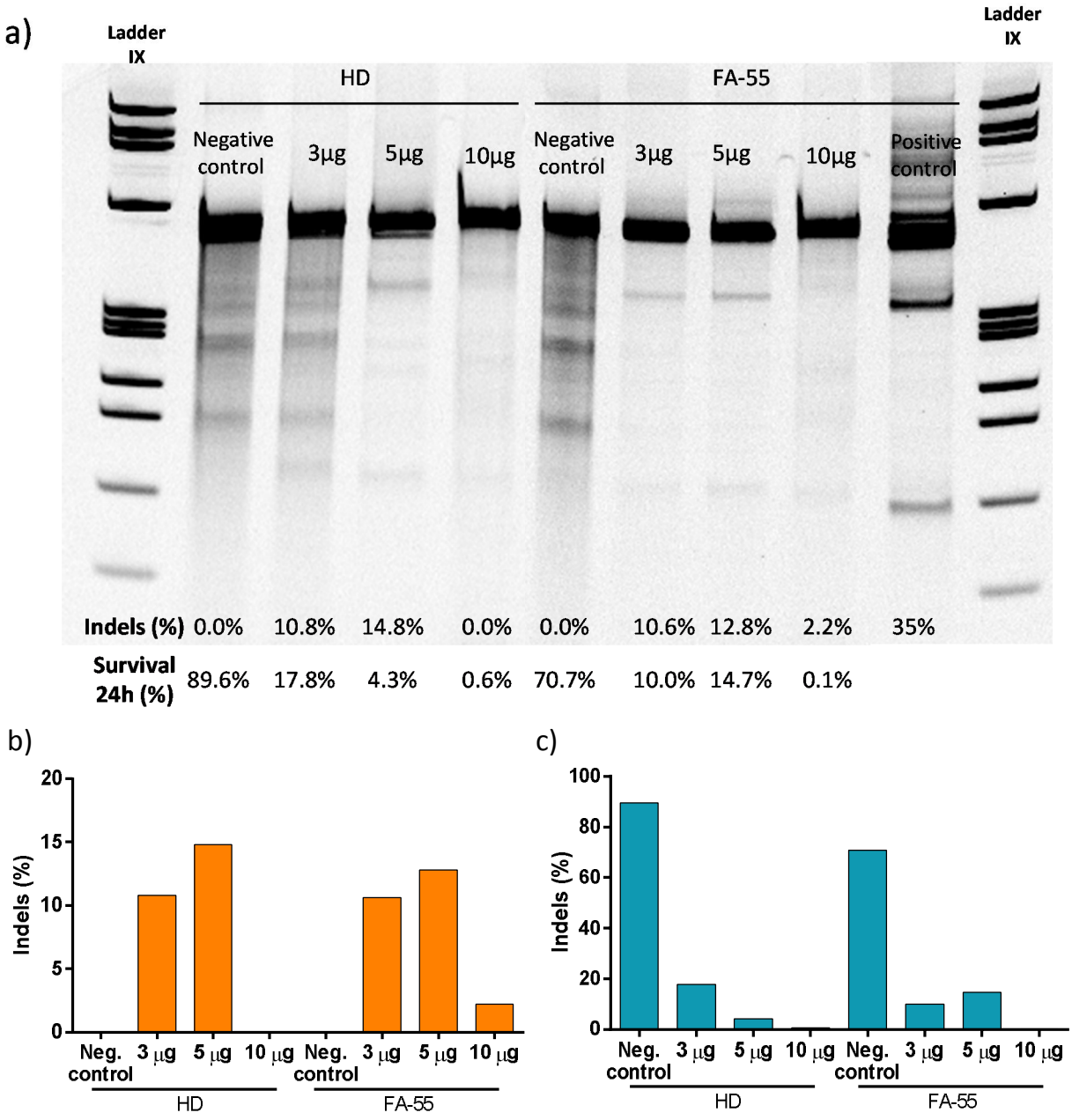


Figure 30.- Evaluation of TALEN nuclease specificity. A HD and a FA-A LCL bearing the c.295C>T mutation in homozygosis (FA-55) were electroporated with two plasmids carrying the TALEN monomers designed to target the c.295C>T mutation: TALEN-L and TALEN-R. (**a**) TALEN efficacy was assessed by *Surveyor assay* and the toxicity of the electroporation was determined by the analysis of DAPI positive cells by flow cytometry. A representative image is shown. Positive control = NHEJ-corrected FA-55 LCL using CRISPR/Cas9 nucleases. (**b**) Quantification of the percentage of indels at day 5 post-electroporation. (**c**) Quantification of the cell survival 24 hours after electroporation.

3. Moving from Gene Editing in LCLs to Human Hematopoietic Stem and Progenitor Cells

Once demonstrated that NHEJ-mediated gene editing can be applied to correct *FANCA* mutations, we moved to the real target of our gene therapy approach: human hematopoietic stem and progenitor cells (HSPCs).

Due to the limited availability of FA HSPC samples, human CD34$^+$ (hCD34$^+$) cells purified from HD umbilical cord bloods (UCBs) were used to establish the most efficient gene editing conditions for these clinically relevant cells. Different delivery strategies were tested using CRISPR/Cas9 system (Table 9).

In contrast to LCLs, plasmid electroporation was extremely toxic, even when low doses were used. Toxicity could be reduced by electroporation of the *in vitro* transcribed (IVT) Cas9 mRNA and sgRNA, but no gene editing was achieved. The only method that allowed efficient hCD34$^+$ cell gene editing was ribonucleoprotein (RNP) complex electroporation, in which the IVT sgRNA was pre-assembled with a commercial Cas9 protein (142), consistent with Liang and co-workers' results (160).

In order to explore different delivery approaches, transduction strategies were also studied. When the CRISPR/Cas9 system was packaged in an integrase-deficient lentiviral vector (IDLV), the viability of the cells was improved but no gene editing was observed (Table 9).

Table 9.- CRISPR/Cas9 nuclease delivery systems used in hCD34$^+$ cells.

Delivery	gRNA	Cas9	Viability	Indels (%)
Nucleofection	All-in-one plasmid		23.6 ± 9.5%	0.0%
	IVT gRNA	IVT mRNA	68.8 ± 5.4%	0.0%
	IVT gRNA + Protein (RNP complex)		51.7 ± 12.1%	18.4 ± 12.2%
Transduction	IDLV-gRNA-SpCas9		73 ± 8.5%	0.0%

Data represents mean ± SD of three different experiments.

Once the best condition to modify HSPCs by NHEJ-mediated gene editing was established, our next goal was to test the repopulation ability of hCD34$^+$ cells after gene editing. To this end, HD hCD34$^+$ cells from UCBs were electroporated with RNP4 (the RNP version of the gGM4 construct previously used in FA-55 LCLs) and transplanted into sublethally irradiated non-

obese diabetic (NOD) immunodeficient Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice 24 hours later through the tail vein.

Three groups of transplanted mice were established: those receiving untreated cells (control); mice receiving electroporated cells without nuclease (mock), and those receiving gene edited cells (RNP4). Two different cell doses were used: 1.5x10^5 and 3.25x10^5 cells per mouse.

Human engraftment was measured at days 30 and 60 after transplantation by bone marrow (BM) aspiration and by BM perfusion when mice were euthanized at day 90. In both cases, flow cytometry analyses of the percentage of hCD45$^+$ cells were conducted. These results demonstrated that the repopulation capacity of edited cells remained unchanged and always comparable to the one showed by control and mock cells (Figure 31). Mean hCD45$^+$ values determined in BM three months after infusion of 3.25x10^5 cells/mouse were 85.6 ± 1.8% (control), 76.0 ± 8.8% (mock) and 81.0 ± 7.7%. No statistically significant differences were observed among the three groups established after a two-way ANOVA followed by Tukey's post hoc test.

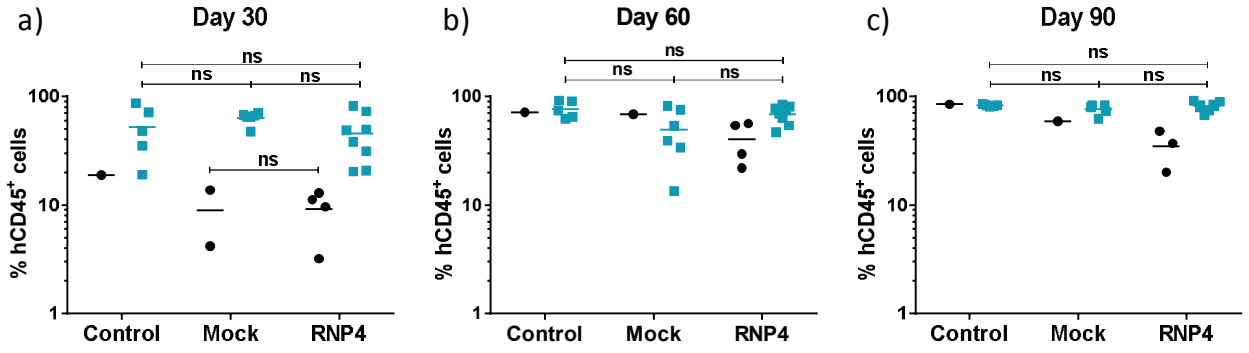


Figure 31.- Human engraftment at days 30, 60 and 90 post-transplantation in primary recipients. Black circles = mice transplanted with 1.5x10^5 cells/mouse. Blue squares = mice transplanted with 3.25x10^5 cells/mouse. A total of 28 mice were used. Mean values are represented with a horizontal line. A two-way ANOVA was performed followed by Tukey's post hoc test. ns = not significant.

At day 90, human engraftment was also evaluated in peripheral blood (PB), spleen and thymus, and multilineage differentiation was also investigated in all the different hematopoietic organs. BM multilineage reconstitution was evaluated using antibodies against hCD34 for HSPCs, hCD33 for myeloid cells, hCD19 for B cells and hCD3 for T cells (Figure 32). The presence of the different HSPC subpopulations was analysed using antibodies against hCD90 and hCD133, the cells with the highest stemness potential being the triple positive (hCD34$^+$ hCD90$^+$ hCD133$^+$), followed by double positive (hCD34$^+$ hCD90$^+$ hCD133$^-$) and hCD34$^+$ (hCD90$^-$ hCD133$^-$) cells. Only a slight decrease in the percentage of hCD19$^+$ population was observed in

control mice in comparison to RNP4 group (p=0.0016). No statistically significant differences were observed in any other lineage, even in the different hCD34 subpopulations (Figure 33a).

PB reconstitution was evaluated using antibodies against hCD34 for HSPCs, hCD33 for myeloid cells, hCD19 for B cells and hCD3 for T cells. No statistically significant differences were observed in any cell lineage (Figure 33b).

Spleen multilineage reconstitution was evaluated using antibodies against hCD34 for HSPCs, hCD33 for myeloid cells, hCD19 for B cells and hCD3 for T cells. In this case, a mild decrease in human engraftment was observed in RNP4 group compared to control (p=0.0027) and mock mice groups (p=0.0079) (Figure 33c).

Finally, thymus multilineage reconstitution was evaluated using antibodies against hCD3 for T cells and the distinction between cytotoxic and helper T cells was made according to the presence of hCD8 and hCD4 antibodies, respectively. Statistically significant differences were observed in human engraftment level in mock recipients: the percentage of $hCD45^+$ cells was slightly decreased in comparison to control and RNP4 groups (p<0.0001). On the other hand, no additional differences were observed in any other lineage or in the T-cell subpopulations (Figure 33d).

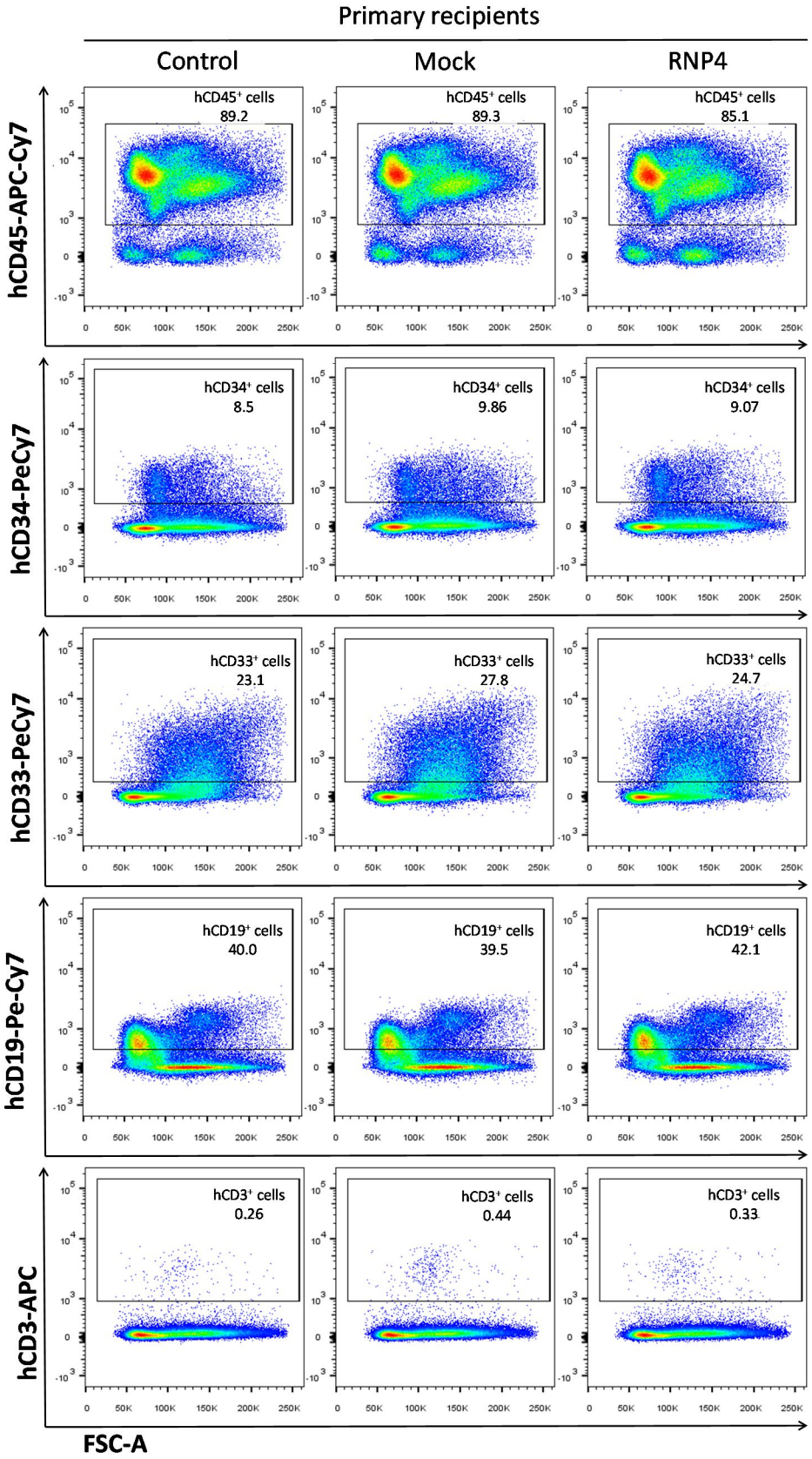
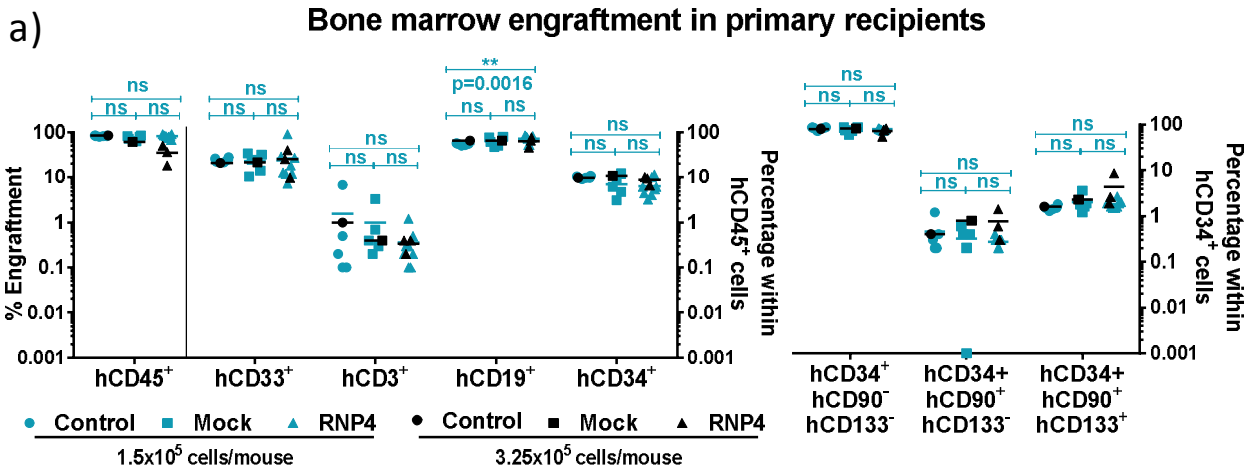
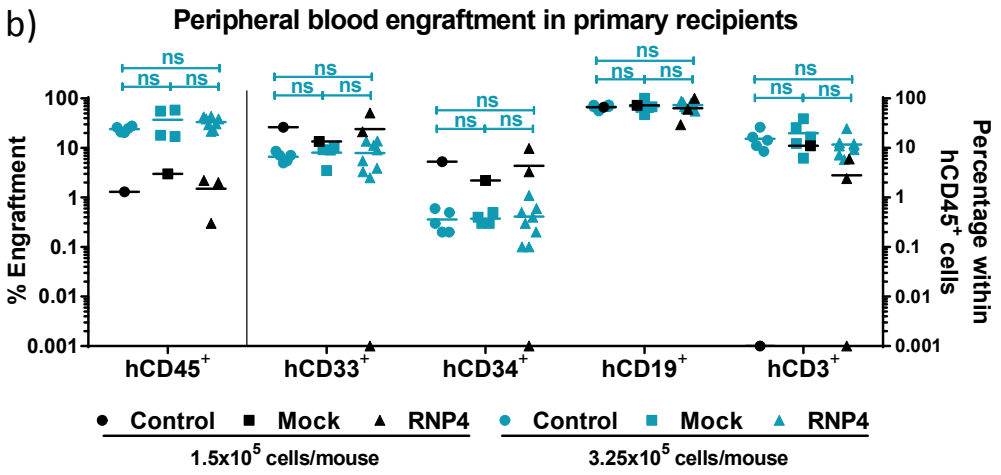
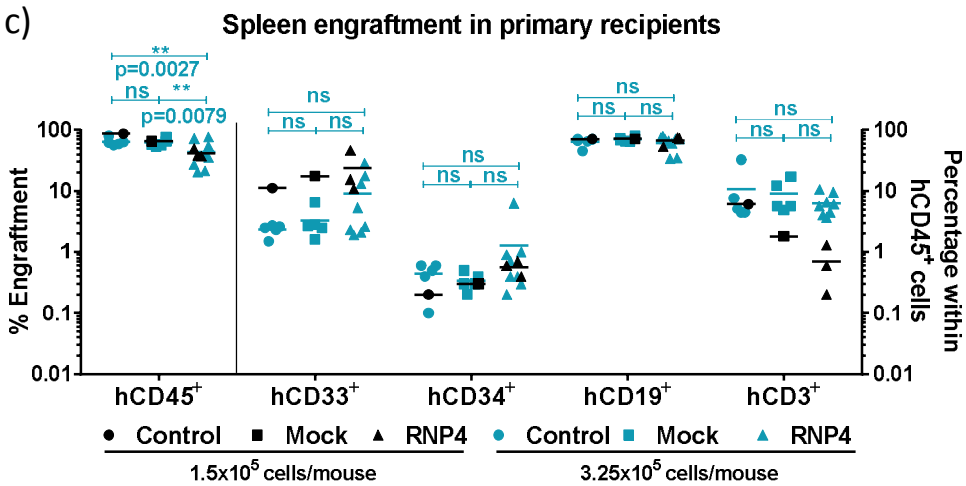


Figure 32.- Representative dot plot analysis of the BM from primary recipients transplanted with edited and non-edited hCD34⁺ cells (3.25x10⁵ cells/mouse). From left to right: mouse that received untreated cells (control); mouse receiving electroporated cells without nuclease (mock) and mouse receiving gene edited cells (RNP4). To investigate the multilineage reconstitution, an anti-hCD34 antibody was used for HSPCs, anti-hCD33 for myeloid cells, anti-hCD19 for B cells and anti-hCD3 for T cells.


a)
Bone marrow engraftment in primary recipients
% Engraftment
Percentage within hCD45+ cells
Percentage within hCD34+ cells
ns
p=0.0016
hCD45+
hCD33+
hCD3+
hCD19+
hCD34+
hCD34+ hCD90- hCD133-
hCD34+ hCD90+ hCD133-
hCD34+ hCD90+ hCD133+
Control
Mock
RNP4
1.5x10^5 cells/mouse
3.25x10^5 cells/mouse


b)
Peripheral blood engraftment in primary recipients
% Engraftment
Percentage within hCD45+ cells
ns
hCD45+
hCD33+
hCD34+
hCD19+
hCD3+
Control
Mock
RNP4
1.5x10^5 cells/mouse
3.25x10^5 cells/mouse


c)
Spleen engraftment in primary recipients
% Engraftment
Percentage within hCD45+ cells
p=0.0027
p=0.0079
ns
hCD45+
hCD33+
hCD34+
hCD19+
hCD3+
Control
Mock
RNP4
1.5x10^5 cells/mouse
3.25x10^5 cells/mouse

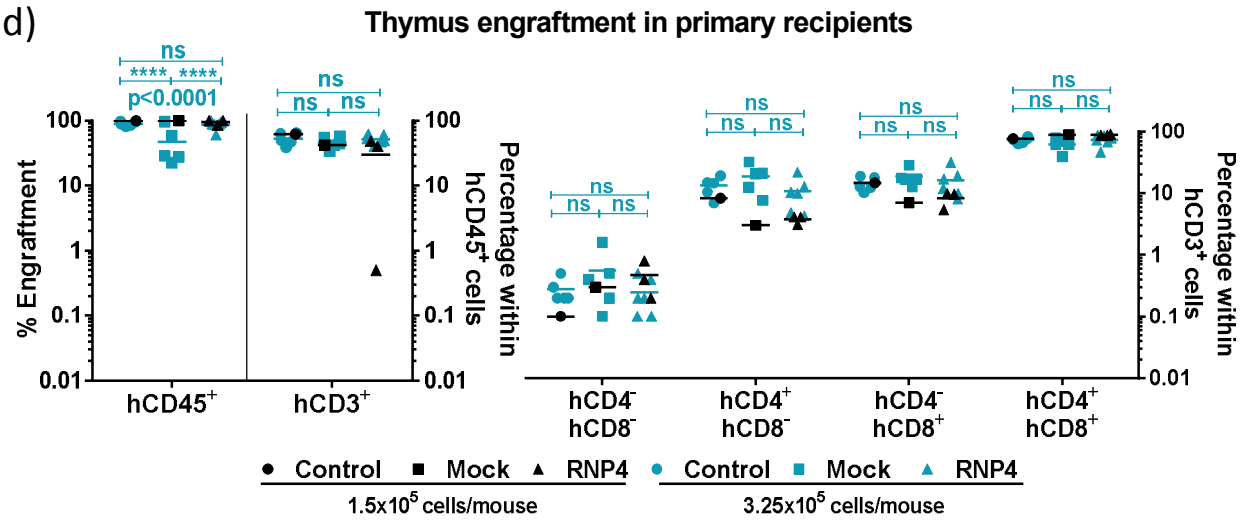


Figure 33.- Multilineage engraftment of human HSPCs in different hematopoietic organs in primary recipients. Three months after transplantation total BM (**a**), PB (**b**), spleen (**c**) and thymus (**d**) were analysed. Human hematopoietic engraftment was detected using an anti-hCD45 antibody. Myeloid cells were marked with an anti-hCD33 and B cells with an anti-hCD19. The HSC compartment was marked using anti-hCD34, anti-hCD90 and anti-hCD133. The antibody panel used for T cells was composed by an anti-hCD3 together with an anti-hCD4 and anti-hCD8 to distinguish between helper and cytotoxic cells, respectively. Mean values are represented with a horizontal line. A two-way ANOVA was performed followed by Tukey's post hoc test in all analyses. ns = not significant.

Taken together, these results demonstrate that the electroporation process did not impair the repopulation capacity of the HSPCs and their ability to differentiate into the different cell lineages.

To confirm that the long-term repopulating capacity was maintained after gene editing, total BM cells extracted from primary mice were transplanted into secondary recipients and the same groups were established. The follow-up of the human engraftment demonstrated that transplanted cells coming from the RNP4 mice group could efficiently engraft, achieving percentages of hCD45+ cells up to 70% (Figure 34c). Importantly, no significant differences were observed among the three groups established after a two-way ANOVA, demonstrating that the electroporation did not impair the long-term repopulation capacity.

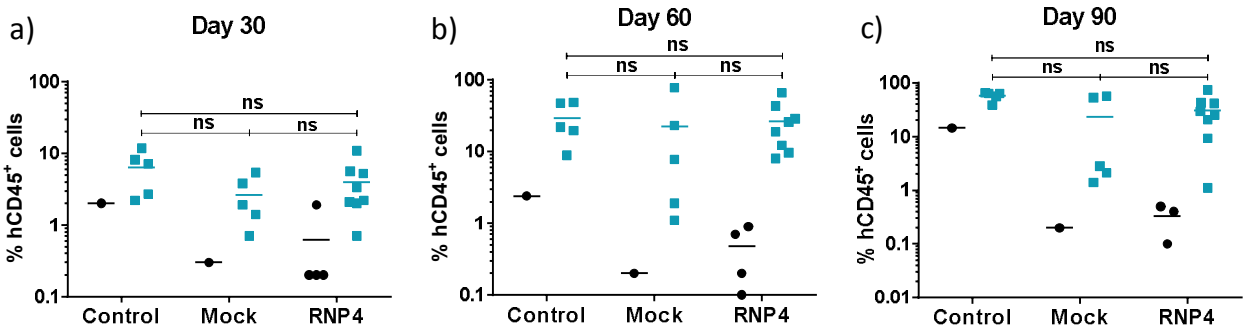


Figure 34.- Human engraftment at days 30, 60 and 90 in secondary recipients. Black circles: secondary recipients that received BM cells from those primary recipients transplanted with $1.5x10^5$ cells/mouse. Blue squares: secondary recipients that received bone marrow cells from those primary recipients transplanted with $3.25x10^5$ cells/mouse. A total of 28 mice were used. Mean values are represented with a horizontal line. A two-way ANOVA was performed followed by Tukey's post hoc test. ns = not significant. ns = not significant.

The multilineage reconstitution capacity of the transplanted cells was then analysed in the different hematopoietic organs with the same antibody panels used in primary recipients (Figure 35).

Turning to BM reconstitution, the percentage of hCD45$^+$ cells showed a slight decrease in mock and RNP4 groups in comparison to control mice ($p<0.0001$). Nevertheless, no statistically significant differences were found among the other cell lineages and, importantly, in the hCD34$^+$ cell compartment (Figure 36a).

In the case of PB reconstitution, no statistically significant differences were observed in any cell lineage (Figure 36b).

When the spleen was analysed, a smaller percentage of hCD45$^+$ cells was recorded in RNP4 group compared to control one ($p<0.0001$) and mock mice groups ($p=0.0279$). In addition, statistically significant differences were observed in the percentage of hCD19$^+$ population, which was slightly reduced in RNP4 compared to control mice ($p=0.0369$). Engraftment of the other cell populations showed no differences among the three groups of mice (Figure 36c).

Finally, concerning thymus multilineage reconstitution, statistically significant differences were only observed in the percentage of hCD45$^+$ cells, which was reduced in mock and RNP4 groups compared to control mice ($p=0.0075$ and $p=0.0002$, respectively). No differences were observed either in the T-cell engraftment or the T-cell subpopulations (Figure 36d).

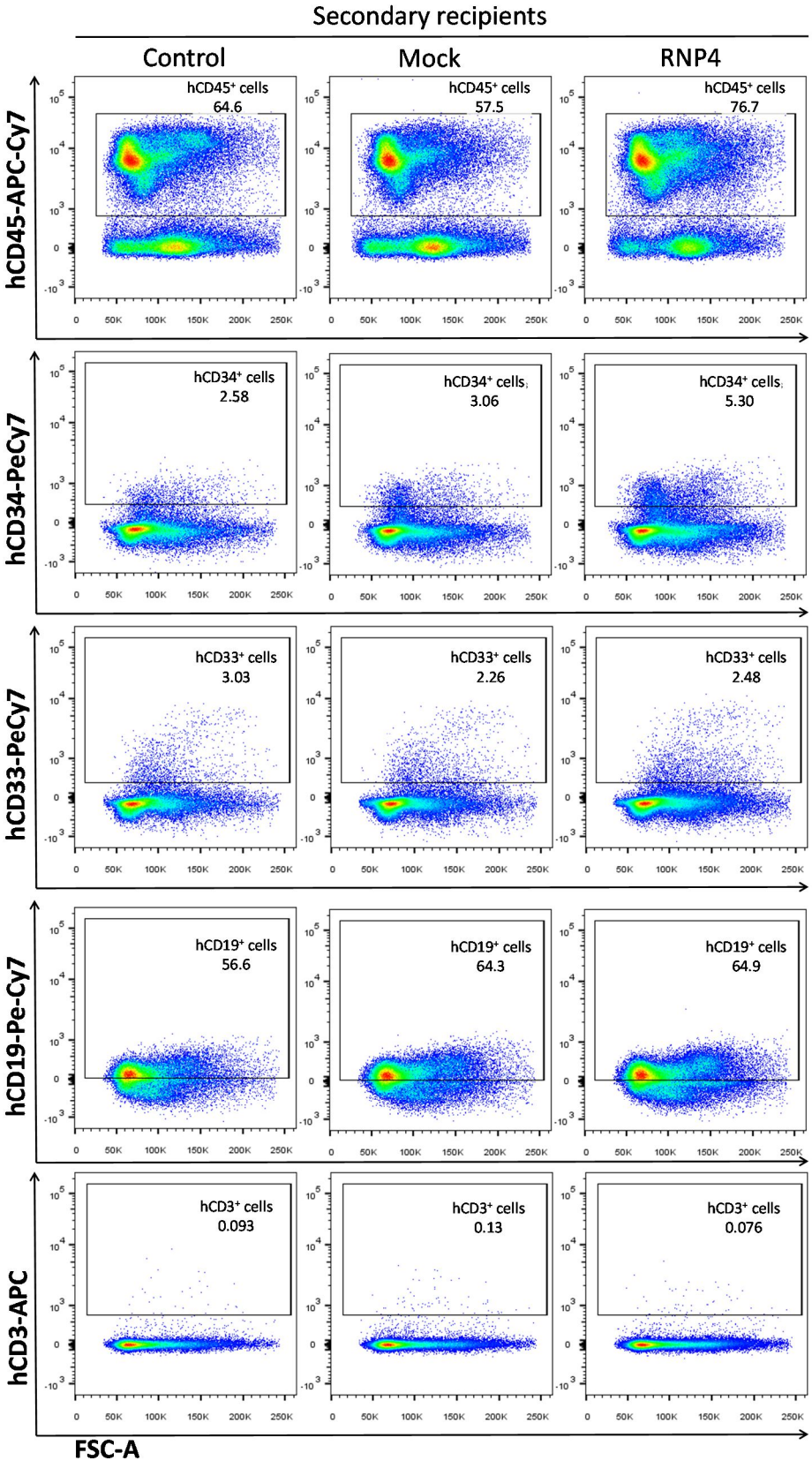
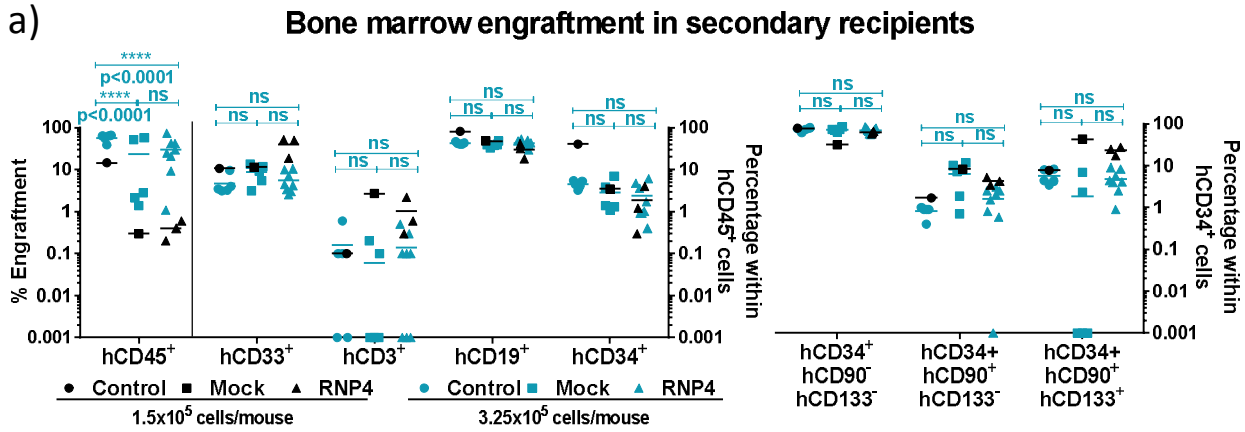
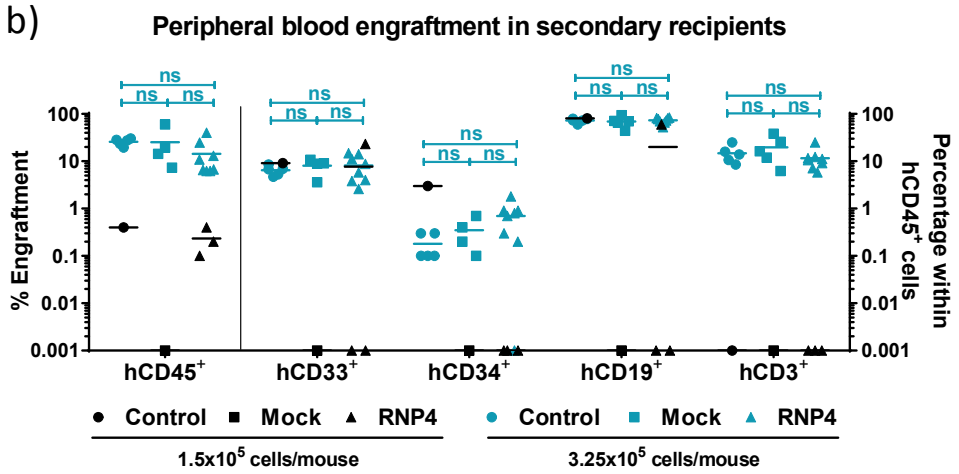
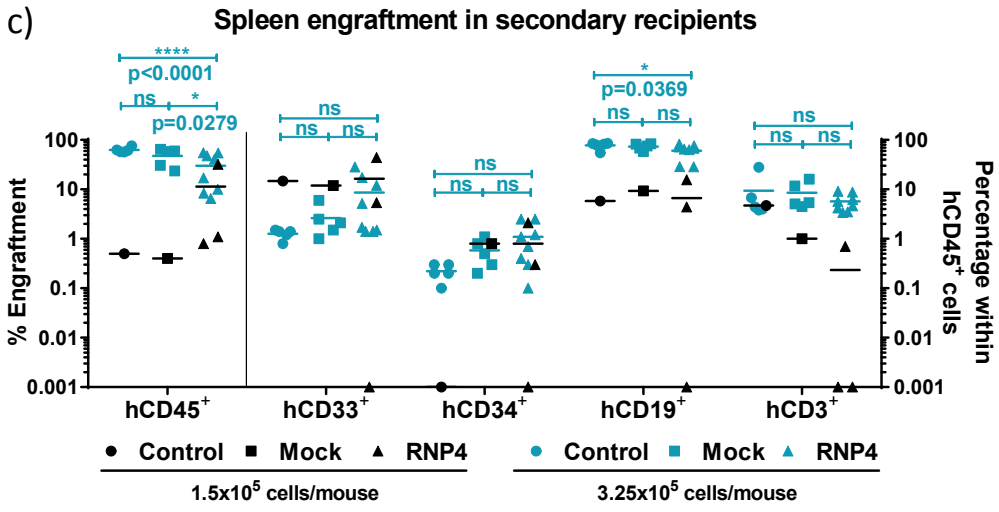


Figure 35.- Representative dot plot analysis of the BM from secondary recipients transplanted with hCD34⁺ cells obtained from the primary mice. From left to right: mouse that received untreated cells (control); mouse receiving electroporated cells without nuclease (mock) and mouse receiving gene edited cells (RNP4). The same panel of antibodies used to investigate the multilineage reconstitution in primary recipients were used in secondary ones.


a)
Bone marrow engraftment in secondary recipients
% Engraftment
Percentage within hCD45+ cells
Percentage within hCD34+ cells
hCD45+
hCD33+
hCD3+
hCD19+
hCD34+
hCD34+ hCD90- hCD133-
hCD34+ hCD90+ hCD133-
hCD34+ hCD90+ hCD133+
Control
Mock
RNP4
1.5x10^5 cells/mouse
3.25x10^5 cells/mouse
p<0.0001
ns


b)
Peripheral blood engraftment in secondary recipients
% Engraftment
Percentage within hCD45+ cells
hCD45+
hCD33+
hCD34+
hCD19+
hCD3+
Control
Mock
RNP4
1.5x10^5 cells/mouse
3.25x10^5 cells/mouse
ns


c)
Spleen engraftment in secondary recipients
% Engraftment
Percentage within hCD45+ cells
hCD45+
hCD33+
hCD34+
hCD19+
hCD3+
Control
Mock
RNP4
1.5x10^5 cells/mouse
3.25x10^5 cells/mouse
p<0.0001
p=0.0279
p=0.0369
ns

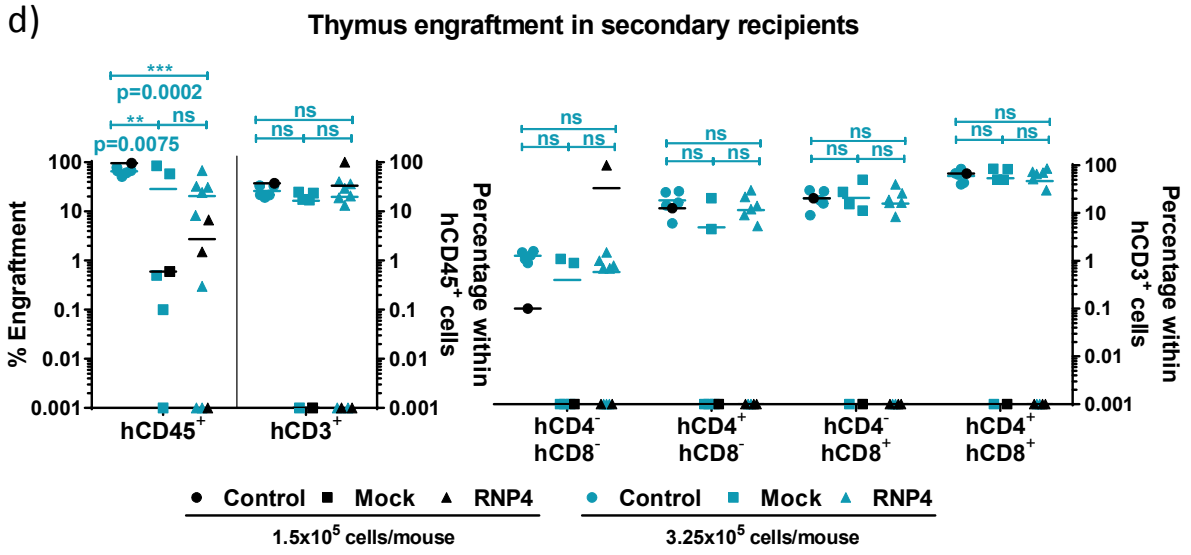


Figure 36.- Multilineage repopulation of human HSPCs in different hematopoietic organs from secondary recipients. Three months after transplantation total BM (**a**), PB (**b**), spleen (**c**) and thymus (**d**) were analysed. Human hematopoietic engraftment was detected using an anti-hCD45 antibody. Myeloid cells were marked with an anti-hCD33 and B cells with an anti-hCD19. The HSC compartment was marked using anti-hCD34, anti-hCD90 and anti-hCD133. The antibody panel used for T cells was composed by an anti-hCD3 together with an anti-hCD4 and anti-hCD8 to distinguish between helper and cytotoxic cells, respectively. Mean values are represented with a horizontal line. A two-way ANOVA was performed followed by Tukey's post hoc test in all analyses. ns = not significant.

In conclusion, as human hematopoietic cells were able to efficiently repopulate all the hematopoietic lineages in primary and also secondary recipients, these results demonstrate that the electroporation of the RNP complex did not have any harmful effect on the HSC long-term repopulation and multilineage differentiation capacities.

As the next step, the efficiency of NHEJ to target long-term (LT)-repopulating HSCs was analysed. To this end, NGS was conducted in human CD34+ cells prior to and after transplantation in primary and secondary recipients (Figure 37). Importantly, these results showed very similar indel rates not only in primary recipients as compared to the cell pool prior transplantation, but also in secondary recipients, strongly confirming that LT-repopulating HSCs were targeted by NHEJ-mediated editing at similar frequencies as compared to more mature progenitor cells.

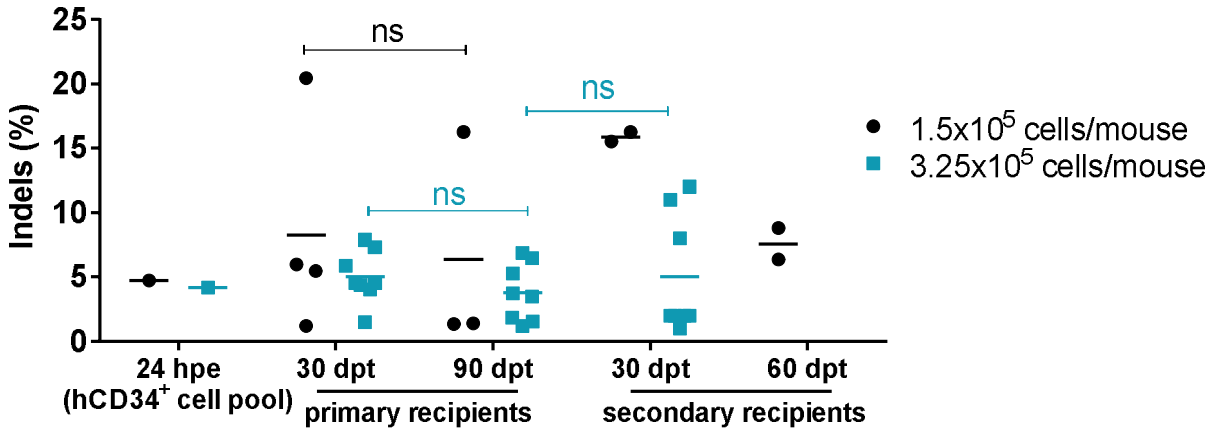


Figure 37.- NGS analyses showed similar indel rates before and after transplantation. The percentage of editing was measured in terms of the indels detected after a variant calling. Black circles: experiment in which primary recipients received $1.5x10^5$ cells/mouse. Blue squares: experiment in which primary recipients received $3.25x10^5$ cells/mouse. Mean values are represented with a horizontal line. No statistically significant differences were observed after a two-way ANOVA between the editing measured at days 30 and 90 in primary recipients and between the editing detected in primary recipients at 90 days and the one detected in secondary recipients at day 30. hpe= hours post-electroporation, dpt = days post-transplantation, ns = not significant.

Interestingly, when the contribution of the different indels detected by NGS was evaluated, an *in vivo* dynamic alternation of the editing events was observed. NGS analysis in the hCD34$^+$ cell pool after electroporation first evidenced the presence of more than 20 different indels with the predominant c.311delG (Figure 38, light blue) indel taking place next to the cutting site. The analysis of BM from three primary and two secondary recipients from mouse 3 (3.1 and 3.2) demonstrated that this indel took place in repopulating cells that reconstituted not only primary but also secondary recipients. Other indels, such as c.289_318del30 (dark blue) or c.304_311del8 (dark green), were only detected in primary recipient 2, but the bearing clones became more frequent in secondary recipients from primary recipient 3 (Figure 38). On the other hand, some indels such as c.311_314del4 (yellow) and c.315delG (pink), that were present in some primary recipients, disappeared in the secondary ones (Figure 38). Conversely, new, edited repopulating clones –not previously identified in any of the primary recipients– were detected when the BM of the secondary ones was analysed, as was the case of the c.283_324del42 (black colour in 3.2). In conclusion, these variations evidenced an *in vivo* clonal succession of edited HSCs over time, confirming that LT-repopulating HSCs had been targeted and modified by NHEJ after the RNP electroporation, offering the possibility of conducting *in vivo* tracking of edited clones in transplanted recipients.

Altogether, these *in vivo* experiments demonstrate that RNP electroporation is an efficient approach to target long-term repopulating HSCs, confirming the applicability of a NHEJ-mediated approach to conduct therapeutic gene editing in mutated HSCs, such as HSCs from patients with FA.

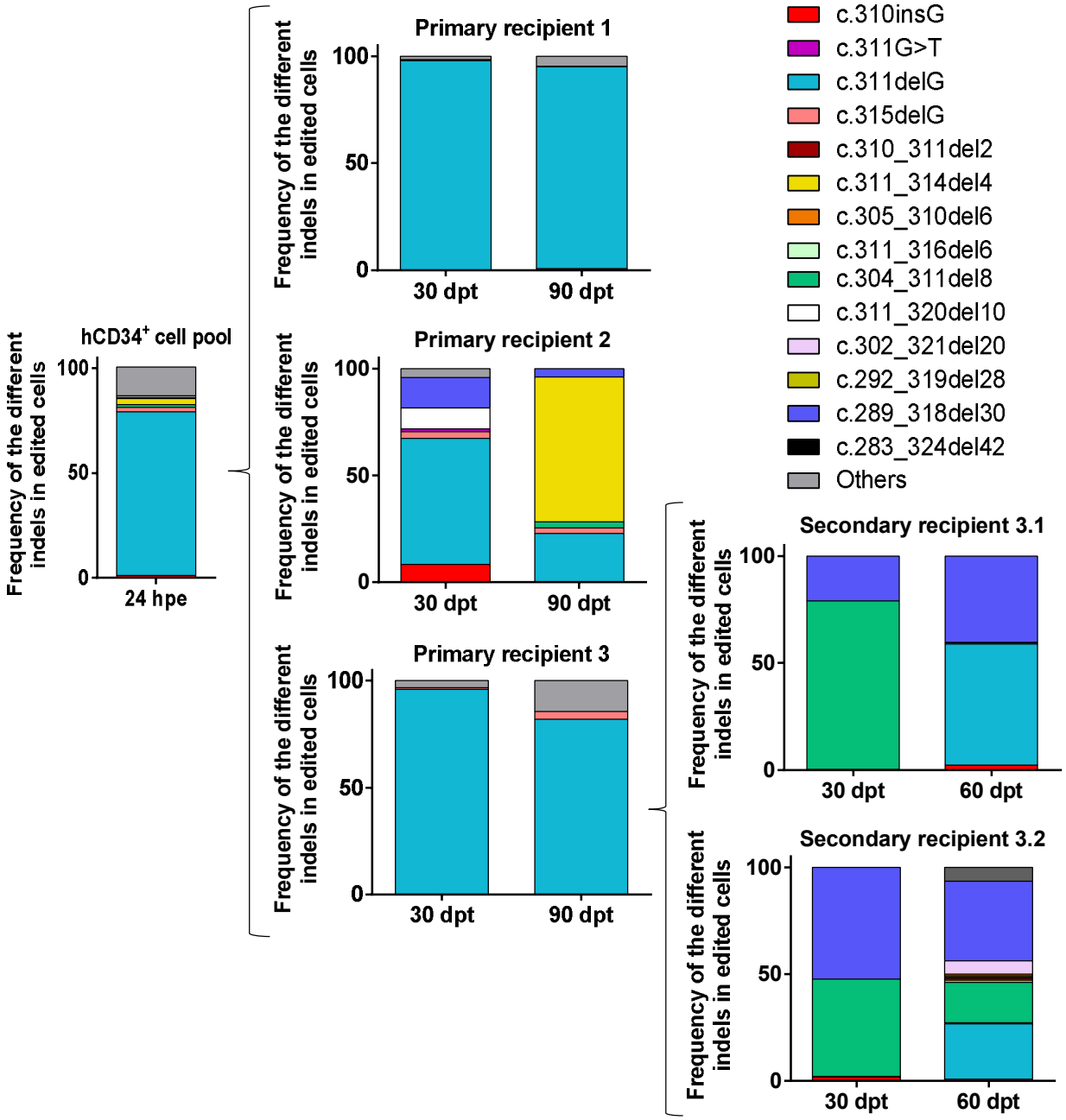


Figure 38.- *In vivo* clonal alternation of edited HSCs. Each colour represents an individual NHEJ-mediated editing event identified by NGS after performing a variant calling analysis. The graphs show the relative frequency of these events within edited cells in three primary recipients infused with RNP4-edited hCD34+ cells at 30 and 90 days post-transplantation, and in two secondary ones transplanted with cells coming from primary mouse 3 at days 30 and 60. "Others" (grey colour) = specific indels found in a sample analysis not shared with the others. hpe= hours post-electroporation, dpt = days post-transplantation.

4. Correction of HSPCs from FA-A Patients by NHEJ-mediated Gene Editing

After showing the feasibility of the NHEJ-based gene editing to correct LCLs from FA-A patients and to target LT-repopulating HSCs from HD, we moved to the real target of our gene editing approach: human HSPCs from FA-A patients. Four different samples from three FA-A patients carrying the homozygous c.295C>T mutation were treated by RNP4 electroporation.

Strikingly, editing conducted in BM hCD34$^+$ cells from patient FA-807 led to a much higher percentage of potentially therapeutic events (7.98%) than the one observed in LCLs, most of them (7.84%) corresponding to the same 30-bp deletion (c.289_318del30) previously demonstrated to be therapeutic, while 15.35% were non-therapeutic (Figure 39).

Figure 39.- NHEJ-mediated gene editing in BM hCD34$^+$ cells from patient FA-807. The percentage of editing is measured in terms of the indels detected after a variant calling. Sequences obtained 5 days after electroporation were classified as in previous figures: those that were not edited (unedited alleles –grey–); sequences suffering a DSB that was repaired by NHEJ but did not restore the ORF (other indels –red–), and those in which a NHEJ-repair event that compensated the mutation, restoring the ORF occurred (therapeutic indels –green–). Guide gGM4 recognition sequence and cutting site are signalled with an orange line and a triangle, respectively. PAM sequence is underlined in black. The c.295C>T mutation is signalled in red. Codons are represented by alternating bold and normal upper-case letters, and intronic sequence is showed in lower-case letters. The symbol "-" stands for deletion.

Twenty-four hours after electroporation, colony forming cell (CFC) assays were conducted both in the absence and in the presence of MMC in order to investigate the restoration of the FA pathway in edited HSPCs. Sanger sequencing showed that 50% of the individual colonies carried at least one potentially corrective indel. These therapeutic events were mainly represented by the 30-bp deletion –41.6% of total indels– (Table 10, upper panel). This deletion showed a 5.9-fold increase from the analysis conducted in hCD34$^+$ cells 5 days post-electroporation (7.84%), demonstrating the strong *in vitro* proliferative advantage conferred to the bearing cells. Moreover, sequencing of the individual colonies resistant to 3 and 10 nM MMC showed a progressive selection of corrected cells: 85.5% and 100%, respectively.

Importantly, all of them carried the therapeutic 30-bp deletion (Table 10, middle and bottom panel), thus confirming not only the proliferative advantage, but also the functionality of the corrected alleles in HSPCs.

Table 10.- Sanger sequencing in BM FA-807 hematopoietic CFCs in the absence and presence of MMC.

FA-807 BM CD34⁺ CFCs (0 nM MMC)				
Allele	**Editing**	**Sequence**	**Frequency**	**Phenotype**
1	c.288_338del51	ttaag**GC**TC----------A**GCC**GG	3.8% (1/26)	Healthy 50% (13/26)
2	NE	ttaag**GCTCTGCTTTGTAGGATCAAGCCTCAAGGCTGGGGGTTCCCGTGGGTATTCTCTCAGCCGG		
1	c.289_318del30	ttaag**GC**TCT----------**GTTCCCGTGGGTATTCTCTCAGCCGG**	30.8% (8/26)	
2	NE	ttaag**GCTCTGCTTTGTAGGATCAAGCCTCAAGGCTGGGGGTTCCCGTGGGTATTCTCTCAGCCGG		
1	c.289_318del30	ttaag**GC**TCT----------**GTTCCCGTGGGTATTCTCTCAGCCGG**	11.5% (3/26)	
2	c.304_311del8	ttaag**GCTCTGCTTTGTAGGATCAA**--------G**CTGGGGGTTCCCGTGGGTATTCTCTCAGCCGG**		
1	c.289_318del30	ttaag**GC**TCT----------**GTTCCCGTGGGTATTCTCTCAGCCGG**	3.8% (1/26)	
2	c.286_320del35	ttaag**GC**----------**TCCCGTGGGTATTCTCTCAGCCGG**		
1	c.304_317del14	ttaag**GCTCTGCTTTGTAGGATCAA**----------G**GTTCCCGTGGGTATTCTCTCAGCCGG**	3.8% (1/26)	FA 50% (13/26)
2	NE	ttaag**GCTCTGCTTTGTAGGATCAAGCCTCAAGGCTGGGGGTTCCCGTGGGTATTCTCTCAGCCGG**		
1	c.311delG	ttaag**GCTCTGCTTTGTAGGATCAAGCCTCAA**-G**CTGGGGGTTCCCGTGGGTATTCTCTCAGCCGG**	7.7% (2/26)	
2	NE	ttaag**GCTCTGCTTTGTAGGATCAAGCCTCAAGGCTGGGGGTTCCCGTGGGTATTCTCTCAGCCGG**		
1	c.310delA	ttaag**GCTCTGCTTTGTAGGATCAAGCCTCA**-GG**CTGGGGGTTCCCGTGGGTATTCTCTCAGCCGG**	3.8% (1/26)	
2	NE	ttaag**GCTCTGCTTTGTAGGATCAAGCCTCAAGGCTGGGGGTTCCCGTGGGTATTCTCTCAGCCGG**		
1	NE	ttaag**GCTCTGCTTTGTAGGATCAAGCCTCAAGGCTGGGGGTTCCCGTGGGTATTCTCTCAGCCGG**	34.6% (9/26)	
2	NE	ttaag**GCTCTGCTTTGTAGGATCAAGCCTCAAGGCTGGGGGTTCCCGTGGGTATTCTCTCAGCCGG**		

FA-807 BM CD34⁺ CFCs (3 nM MMC)				
Allele	**Editing**	**Sequence**	**Frequency**	**Phenotype**
1	c.289_318del30	ttaag**GC**TCT----------**GTTCCCGTGGGTATTCTCTCAGCCGG**	14.3% (1/7)	Healthy 85.5% (6/7)
2	NE	ttaag**GCTCTGCTTTGTAGGATCAAGCCTCAAGGCTGGGGGTTCCCGTGGGTATTCTCTCAGCCGG**		
1	c.289_318del30	ttaag**GC**TCT----------**GTTCCCGTGGGTATTCTCTCAGCCGG**	42.9% (3/7)	
2	c.304_311del8	ttaag**GCTCTGCTTTGTAGGATCAA**--------G**CTGGGGGTTCCCGTGGGTATTCTCTCAGCCGG**		
1	c.289_318del30	ttaag**GC**TCT----------**GTTCCCGTGGGTATTCTCTCAGCCGG**	14.3% (1/7)	
2	c.311delG	ttaag**GCTCTGCTTTGTAGGATCAAGCCTCAA**-G**CTGGGGGTTCCCGTGGGTATTCTCTCAGCCGG**		
1	c.289_318del30	ttaag**GC**TCT----------**GTTCCCGTGGGTATTCTCTCAGCCGG**	14.3% (1/7)	
2	c.310_311del2	ttaag**GCTCTGCTTTGTAGGATCAAGCCTCA**--G**CTGGGGGTTCCCGTGGGTATTCTCTCAGCCGG**		
1	NE	ttaag**GCTCTGCTTTGTAGGATCAAGCCTCAAGGCTGGGGGTTCCCGTGGGTATTCTCTCAGCCGG**	14.3% (1/7)	FA 14.3% (1/7)
2	NE	ttaag**GCTCTGCTTTGTAGGATCAAGCCTCAAGGCTGGGGGTTCCCGTGGGTATTCTCTCAGCCGG**		

FA-807 BM CD34⁺ CFCs (10 nM MMC)				
Allele	**Editing**	**Sequence**	**Frequency**	**Phenotype**
1	c.289_318del30	ttaag**GC**TCT----------**GTTCCCGTGGGTATTCTCTCAGCCGG**	33.3% (2/6)	Healthy 100% (6/6)
2	NE	ttaag**GCTCTGCTTTGTAGGATCAAGCCTCAAGGCTGGGGGTTCCCGTGGGTATTCTCTCAGCCGG**		
1	c.289_318del30	ttaag**GC**TCT----------**GTTCCCGTGGGTATTCTCTCAGCCGG**	33.3% (2/6)	
2	c.304_311del8	ttaag**GCTCTGCTTTGTAGGATCAA**--------G**CTGGGGGTTCCCGTGGGTATTCTCTCAGCCGG**		
1	c.289_318del30	ttaag**GC**TCT----------**GTTCCCGTGGGTATTCTCTCAGCCGG**	16.7% (1/6)	
2	c.311delG	ttaag**GCTCTGCTTTGTAGGATCAAGCCTCAA**-G**CTGGGGGTTCCCGTGGGTATTCTCTCAGCCGG**		
1	c.289_318del30	ttaag**GC**TCT----------**GTTCCCGTGGGTATTCTCTCAGCCGG**	16.7% (1/6)	
2	c.302_321del20	ttaag**GCTCTGCTTTGCAGGATC**----------**CCCGTGGGTATTCTCTCAGCCGG**		

The frequency and phenotype of the different allele combination detected in the CFCs are indicated. NE = not edited. The c.295C>T mutation is signalled in red. Corrective NHEJ-repair events are marked in green while non-corrective ones are shown in red. Codons are represented by alternating bold and normal upper-case letters, and intronic sequence is showed in lower-case letters. The symbol "-" stands for deletion.

We then moved to mobilized peripheral blood (mPB) hCD34⁺ cells from FA patients, the main source of HSCs used for gene therapy trials nowadays. When mPB hCD34⁺ cells from patient FA-807 were analysed by NGS at day 5 after editing, 2.65% potentially corrective indels was observed. Interestingly, most of them (2.64%) corresponded once again to the therapeutic 30-bp deletion (Figure 40).

Figure 40.- NHEJ-mediated gene editing in mPB hCD34⁺ cells from patient FA-807. The percentage of editing is measured in terms of the indels detected after a variant calling. Sequences obtained at day 5 post-electroporation were classified as in previous figures. Guide gGM4 recognition sequence and cutting site are signalled with an orange line and triangle, respectively. The c.295C>T mutation is signalled in red. PAM sequence is underlined in black. Codons are represented by alternating bold and normal upper-case letters, and intronic sequence is showed in lower-case letters. The symbol "-" stands for deletion.

A CFC assay was performed and individual colonies were analysed by Sanger sequencing. Notably, sequencing revealed that 25% of the isolated colonies carried a therapeutic NHEJ-repair event and, in all cases, this event was the 30-bp deletion, whose frequency experienced a 9.5-fold increase with respect to cells analysed 5 days post-electroporation by NGS (Table 11).

Table 11.- Sanger sequencing in mPB FA-807 hematopoietic colonies 14 days after electroporation.

FA-807 mPB CD34⁺ CFCs (0 nM MMC)				
Allele	**Editing**	**Sequence**	**Frequency**	**Phenotype**
1	c.289_318del30	ttaag**GC**TCT----------**GTT**CCC**GTG**GGT**ATT**CTC**TCA**GCC**GG**	25% (1/4)	Healthy 25% (1/4)
2	NE	ttaag**GC**TCT**GCT**TTG**T**AG**CAA**GCC**TCA**AGG**CTG**GGG**GTT**CCC**GTG**GGT**ATT**CTC**TCA**GCC**GG**		
1	c.304_311del8	ttaag**GC**TCT**GCT**TTG**T**AG**CAA**--------G**CTG**GGG**GTT**CCC**GTG**GGT**ATT**CTC**TCA**GCC**GG**	25% (1/4)	FA 75% (3/4)
2	NE	ttaag**GC**TCT**GCT**TTG**T**AG**CAA**GCC**TCA**AGG**CTG**GGG**GTT**CCC**GTG**GGT**ATT**CTC**TCA**GCC**GG**		
1	NE	ttaag**GC**TCT**GCT**TTG**T**AG**CAA**GCC**TCA**AGG**CTG**GGG**GTT**CCC**GTG**GGT**ATT**CTC**TCA**GCC**GG**	50% (2/4)	
2	NE	ttaag**GC**TCT**GCT**TTG**T**AG**CAA**GCC**TCA**AGG**CTG**GGG**GTT**CCC**GTG**GGT**ATT**CTC**TCA**GCC**GG**		

The frequency and phenotype of the different allele combination detected in the CFCs are indicated. The c.295C>T mutation is signalled in red. Corrective NHEJ-repair events are marked in green while non-corrective ones are shown in red. Codons are represented by alternating bold and normal upper-case letters, and intronic sequence is showed in lower-case letters. The symbol "-" stands for deletion.

A similar experiment was conducted in mPB hCD34⁺ cells from patient FA-739. Five days after RNP4 electroporation, deep sequencing data revealed an indel rate of 12.3%. However, only a small proportion (0.45%) corresponded to therapeutic NHEJ-repair events (Figure 41a). Interestingly, when a new NGS was conducted at day 14 after electroporation, the proportion of total therapeutic indels increased to 2.33% (5-fold increase), while the non-therapeutic ones were slightly reduced to 9.78% (Figure 41b). Moreover, among the different potentially therapeutic NHEJ-repair events found, the same 30-bp deletion was detected at a low frequency (0.03%) at day 5 (Figure 41c). However, its frequency experienced a 55.3-fold increase, becoming the most frequent therapeutic indel (1.66%) within only 9 days of *in vitro* culture (Figure 41d). These data confirmed the marked proliferative advantage conferred by the c.289_318del30 deletion in FA-A mPB-CD34⁺ cells.

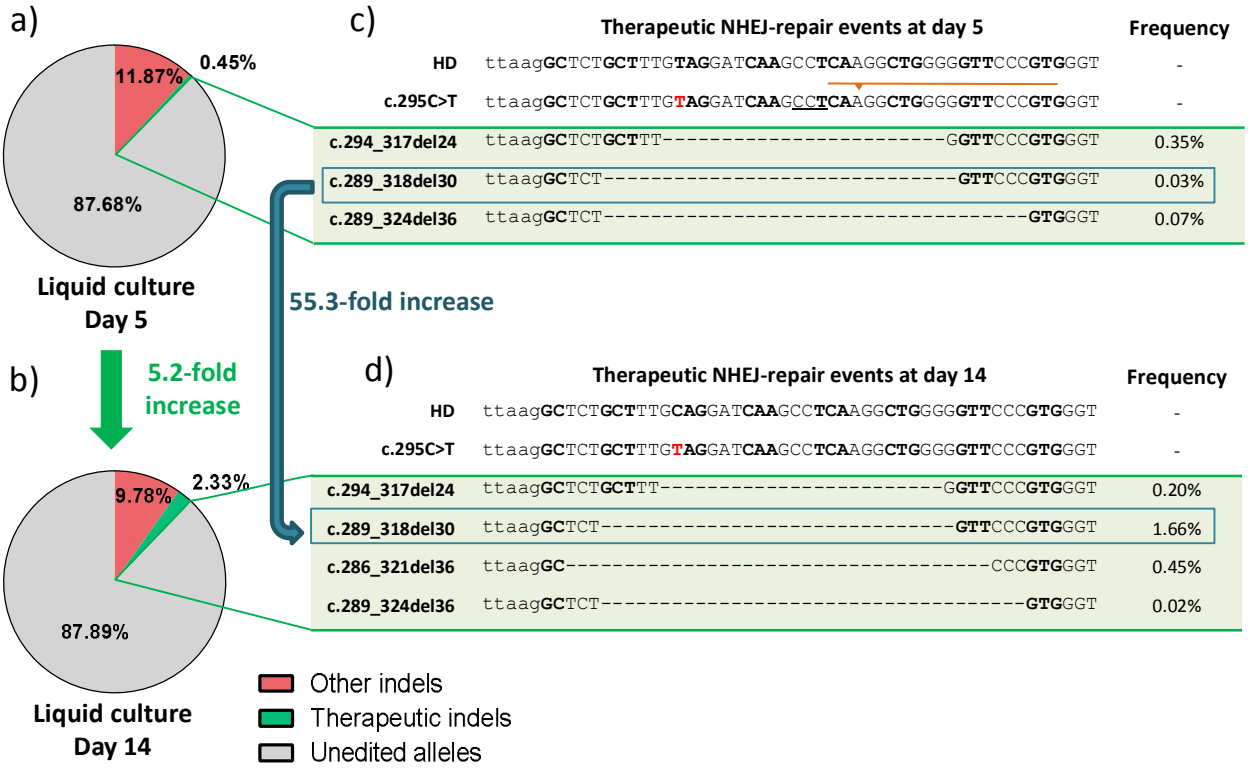


Figure 41.- NHEJ-mediated gene editing in mPB hCD34⁺ cells from patient FA-739 at 5 and 14 days after electroporation. The percentage of editing is measured in terms of the indels detected after a variant calling. Sequences obtained at 5 (**a**) and 14 (**b**) days were classified as in previous figures. The therapeutic NHEJ-repair events found at 5 (**c**) and 14 (**d**) days after electroporation and their frequencies are listed in the adjacent tables. Guide gGM4 recognition sequence and cutting site are signalled with an orange line and triangle, respectively. PAM sequence is underlined in black. The c.295C>T mutation is signalled in red. Codons are represented by alternating bold and normal upper-case letters, and intronic sequence is showed in lower-case letters. The symbol "-" stands for deletion.

In order to confirm the safety of the approach, the top-five *in silico* predicted off-target loci were analysed in FA-A hCD34⁺ cells by NGS. As a result, no unspecific nuclease activity was detected in any of them (Table 12), as the frequency of variations registered at these loci by NGS was always below the detection limit of the technique (0.01%).

Table 12.- NGS analyses of the top-five *in silico* predicted off-target loci for gGM4.

Locus	Sequence (5'-3')	Score	Mismatches	FA-739 (mPB CD34+)	FA-807 (mPB CD34+)	FA -807 (BM CD34+)
On target	CACGGGAACCCCCAGCCTTG	-	-	12.32%	16.84%	23.33%
OT1	CACGGGAGCCCCCAGCCTTG	100.0	1MMs [8]	<0.01%	<0.01%	<0.01%
OT2	CTCGGGTACTCCCAGCCTTG	1.7	3MMs [2:7:10]	<0.01%	<0.01%	<0.01%
OT3	CCCAGCAACCCCCAGCCTTG	1.5	3MMs [2:4:6]	<0.01%	<0.01%	<0.01%
OT4	CAGGAGAACCCCCAGCCTTC	1.4	3MMs [3:5:20]	<0.01%	<0.01%	<0.01%
OT5	CTCCTGAGCCCCCAGCCTTG	1.4	4MMs [2:4:5:8]	<0.01%	<0.01%	<0.01%

The probability score calculated by the *CRISPR Design* tool is shown. The mismatches are signalled in red and the number and position is indicated in brackets. The percentage of indels detected is represented for each sample.

Once we demonstrated the efficiency and safety of NHEJ-mediated gene editing in total hCD34$^+$ cells and their clonogenic progenitor cells, we finally investigated the possibility of editing and transplanting mPB HSPCs from a new FA-A patient harbouring the c.295C>T mutation (FA-655). Twenty-four hours after electroporation of the RNP4, cells were plated in methylcellulose to conduct a clonogenic assay and 1.75x10^5 cells were transplanted into one NSG mouse. Although 10% of the 56 CFCs analysed displayed NHEJ-repair events, no corrective ones could be detected (Table 13), indicating that the initial frequency of therapeutic indels in electroporated CFCs had been lower than 1.8% (1/56).

Table 13.- NHEJ-repair events identified by Sanger sequencing in gene edited CFCs from patient FA-655.

FA-655 mPB CD34$^+$ CFCs (0 nM MMC)				
Allele	Editing	Sequence	Percentage	Phenotype
1	NE	ttaagGCTCTGCTTTGTAGGATCAAGCCTCAAGGCTGGGGGTTCCCGTGGGTATTCTCTCAGCCG	75% (42/56)	FA 100% (56/56)
2	NE	ttaagGCTCTGCTTTGTAGGATCAAGCCTCAAGGCTGGGGGTTCCCGTGGGTATTCTCTCAGCCG		
1	NE	ttaagGCTCTGCTTTGTAGGATCAAGCCTCAA*GGCTGGGGGTTCCCGTGGGTATTCTCTCAGCC	7.1% (4/56)	
2	c.310insG	ttaagGCTCTGCTTTGTAGGATCAAGCCTCAAGGGCTGGGGGTTCCCGTGGGTATTCTCTCAGCC		
1	NE	ttaagGCTCTGCTTTGTAGGATCAAGCCTCAAGGCTGGGGGTTCCCGTGGGTATTCTCTCAGCCG	8.9% (5/56)	
2	c.315delG	ttaagGCTCTGCTTTGTAGGATCAAGCCTCAA*GGCT-GGGGTTCCCGTGGGTATTCTCTCAGCC		
1	NE	ttaagGCTCTGCTTTGTAGGATCAAGCCTCAAGGCTGGGGGTTCCCGTGGGTATTCTCTCAGCCG	1.8% (1/56)	
2	c.313_316del4	ttaagGCTCTGCTTTGTAGGATCAAGCCTCAAGG----GGGTTCCCGTGGGTATTCTCTCAGCCG		
1	NE	ttaagGCTCTGCTTTGTAGGATCAAGCCTCAAGGCTGGGGGTTCCCGTGGGTATTCTCTCAGCCG	1.8% (1/56)	
2	c.311_316del7	ttaagGCTCTGCTTTGTAGGATCAAGCCTCA-------GGGTTCCCGTGGGTATTCTCTCAGCCG		
1	NE	ttaagGCTCTGCTTTGTAGGATCAAGCCTCAAGGCTGGGGGTTCCCGTGGGTATTCTCTCAGCCG	1.8% (1/56)	
2	c.311_320del11	ttaagGCTCTGCTTTGTAGGATCAAGCCTCA-----------TCCCGTGGGTATTCTCTCAGCCG		
1	NE	ttaagGCTCTGCTTTGTAGGATCAAGCCTCAAGGCTG***GGGGTTCCCGTGGGTATTCTCTCAG	1.8% (1/56)	
2	c.315ins3	ttaagGCTCTGCTTTGTAGGATCAAGCCTCAAGGCTGATCGGGGTTCCCGTGGGTATTCTCTCAG		
1	c.315delG	ttaagGCTCTGCTTTGTAGGATCAAGCCTCAAGGCT-GGGGTTCCCGTGGGTATTCTCTCAGCCG	1.8% (1/56)	
2	c.298_339del43	ttaagGCTCTGCTTTGTA----------GCCG		

The c.295C>T mutation is signalled in red. Codons are represented by alternating bold and normal upper-case letters, and intronic sequence is showed in lower-case letters. Non-corrective NHEJ-repair events are shown in red. NE = not edited. The symbol "-" stands for deletion.

However, NGS analysis of the BM aspiration conducted 30 days post-transplantation in the mouse not only demonstrated a marked increase in the indel rate (17.31%), but also the presence of corrected alleles: 8.28% contained the therapeutic c.289_318del30 indel that removed the premature stop codon preserving the ORF (Figure 42). These results demonstrate for the first time the *in vivo* proliferative advantage of FA-A HSPCs corrected by NHEJ.

Figure 42.- ***In vivo*** **expansion of NHEJ-corrected FA-655 patient HSPCs.** The percentage of editing was measured as indels detected after a variant calling. Sequences obtained at 30 days post-transplantation were classified as in previous figures. The therapeutic NHEJ-repair events found and their frequencies are listed in the adjacent table. Guide gGM4 recognition sequence and cutting site are signalled with an orange line and a triangle, respectively. The c.295C>T mutation is signalled in red. PAM sequence is underlined in black. Codons are represented by alternating bold and normal upper-case letters, and intronic sequence is showed in lower-case letters. The symbol "-" stands for deletion.

Taken together, these results demonstrate for the first time the possibility of phenotypically correcting HSPCs from FA-A patients by a NHEJ-mediated gene editing approach using RNP. Corrected cells exhibited a remarkable *in vitro* and *in vivo* expansion due to the proliferative advantage conferred by the corrected alleles, as well as the reversion of the characteristic MMC hypersensitivity observed in FA cells.

5. Paving the Way for the *in Vivo* Correction of HSPCs from FA-A Patients by NHEJ-mediated Gene Editing

Trying to explore the possibility of conducting *in vivo* gene editing in HSPCs from FA patients, we focused on adeno-associated viral vectors (AAVs). To this end, two different serotypes previously described to transduce hCD34⁺ cells were tested: AAV2 and AAV6 (119, 253, 254).

To evaluate the efficacy of both serotypes to transduce HSPCs, self-complementary (sc) and single-stranded (ss) constructs carrying the enhanced green fluorescence protein (eGFP) as a marker gene were used. Human CD34⁺ cells from HD UCBs were transduced with these four constructs at different multiplicities of infection (MOIs).

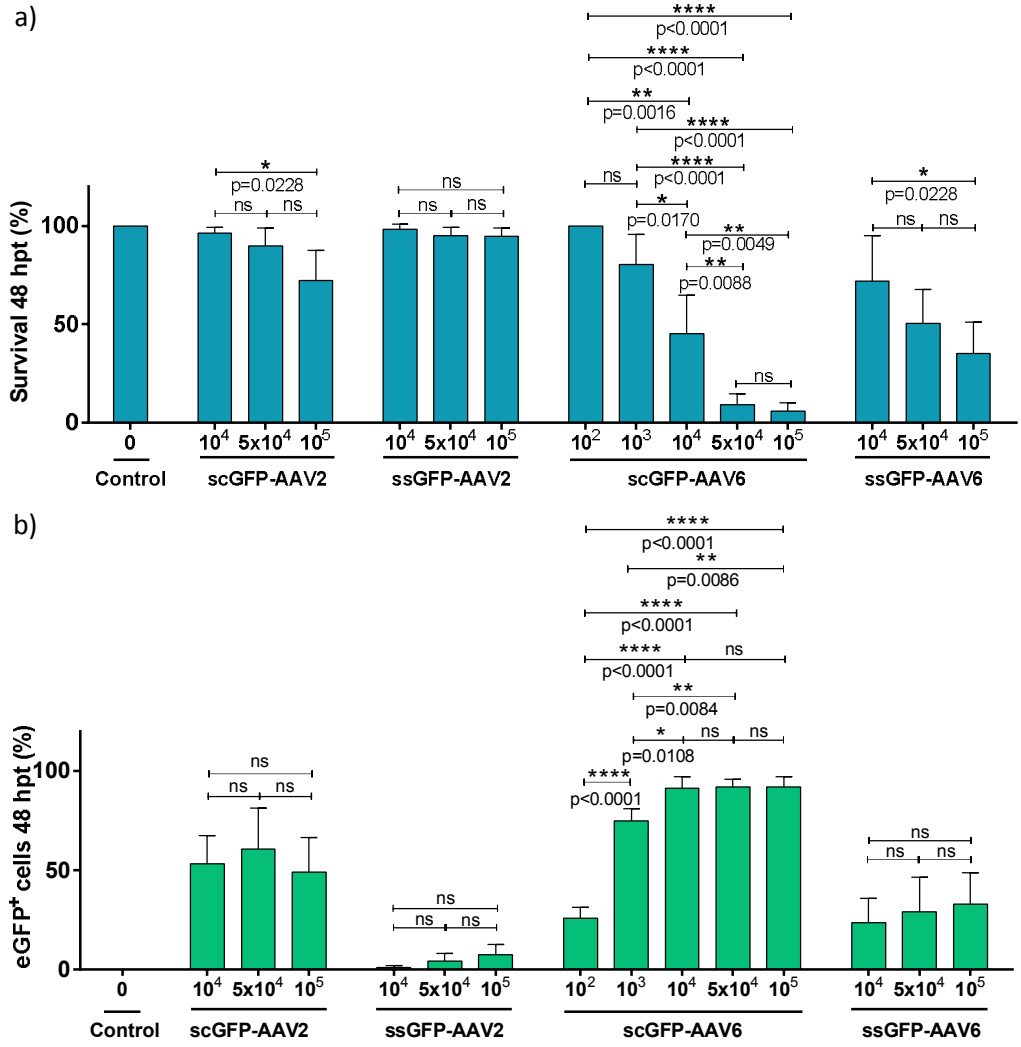


Figure 43.- Transduction efficacy of the AAV vectors in fresh hCD34⁺ cells. In order to identify the best construct and transduction condition for HSPCs, survival (**a**) and percentage of eGFP⁺ cells (**b**) were measured by flow cytometry 48 hours post-transduction (hpt). Survival was evaluated in terms of the percentage of DAPI negative cells. Bars represent mean ± SD of three different analyses. A two-way ANOVA was performed followed by Tukey's post hoc test.

Although AAV6 showed to be more toxic than AAV2, the results obtained showed that the transduction efficiency was always significantly higher when serotype 6 was used either in self-complementary or single-stranded conformation (Figure 43), so the cell viability could be improved by reducing the MOI used without compromising the transduction efficacy. Therefore, AAV6 was selected for the development of our gene editing vectors.

Unfortunately, in spite of the superior performance of the self-complementary construct over the single-stranded ones, the size of the Cas9 nuclease gene did not allow its packaging under a self-complementary conformation. Considering that a single-stranded conformation had to be used to deliver the Cas9, we aimed at improving the transduction efficacy obtained with this vector. As dimethyl prostaglandin E2 (dmPGE2) had recently been described to increase the lentiviral vector (LV) transduction in HSPCs (292), we investigated its efficiency in improving AAV-mediated transduction with the aim of reducing the MOI used.

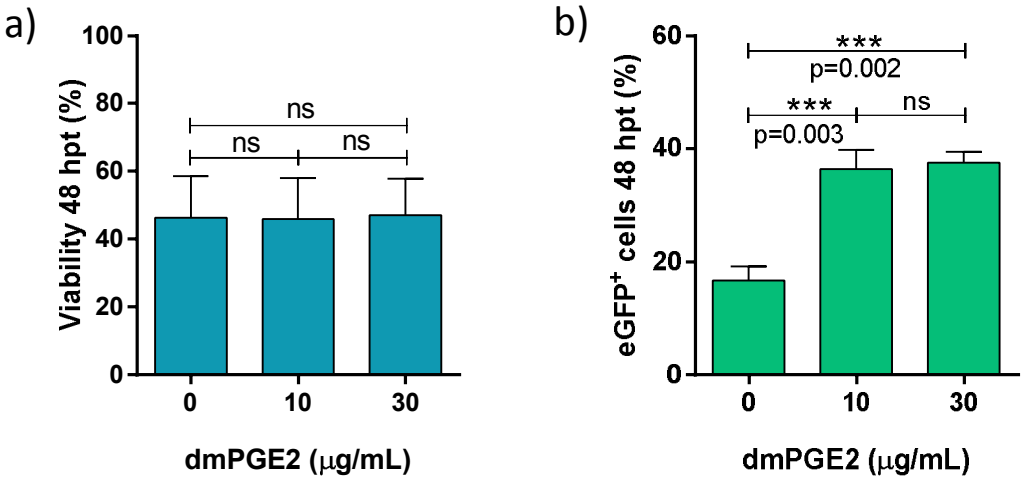


Figure 44.- Effect of dmPGE2 in the ssGFP-AAV6 transduction. Human CD34⁺ cells were transduced with the ssGFP-AAV6 at MOI 10^5 in the presence of 0, 10 and 30 μg/mL dmPGE2. Cell viability (**a**) and transduction efficacy (**b**) were assessed 48 hours post-transduction (hpt) by flow cytometry in terms of the percentage of DAPI⁺ and GFP⁺ cells, respectively. Bars represent mean ± SD of five different analyses. A two-way ANOVA was performed followed by Tukey's post hoc test.

As a result, dmPGE2 mediated a 2-fold increment in the transduction ability of the ssGFP-AAV6 vector without compromising the viability of hCD34⁺ cells (Figure 44). As no statistically significant differences were obtained between 10 and 30 μg/mL dmPGE2, and considering that the 10 μg/mL dose had already been tested in hCD34⁺ cells without any detrimental effect over the repopulating ability (292), this dose was chosen for further experiments.

As the next step, we evaluated the *in vivo* transduction efficacy of the hCD45+ cells with AAV6. To this end, immunodeficient NSG mice previously engrafted with HD hCD34+ cells from UCBs were inoculated with a scGFP-AAV6 using two different administration routes: intravenous and intrabone. In these tests, a scGFP-AAV6 was used due to its higher transduction efficiency. Forty-eight hours post-infusion, the percentage of GFP+ cells within human and mouse hematopoietic cells (CD45) was analysed by flow cytometry in total BM. As a result, both administration routes showed a similar transduction efficacy: 1.1 ± 0.9% for the tail vein administration and 1.1 ± 1.1% in the case of the intrafemoral injection (Figure 45).

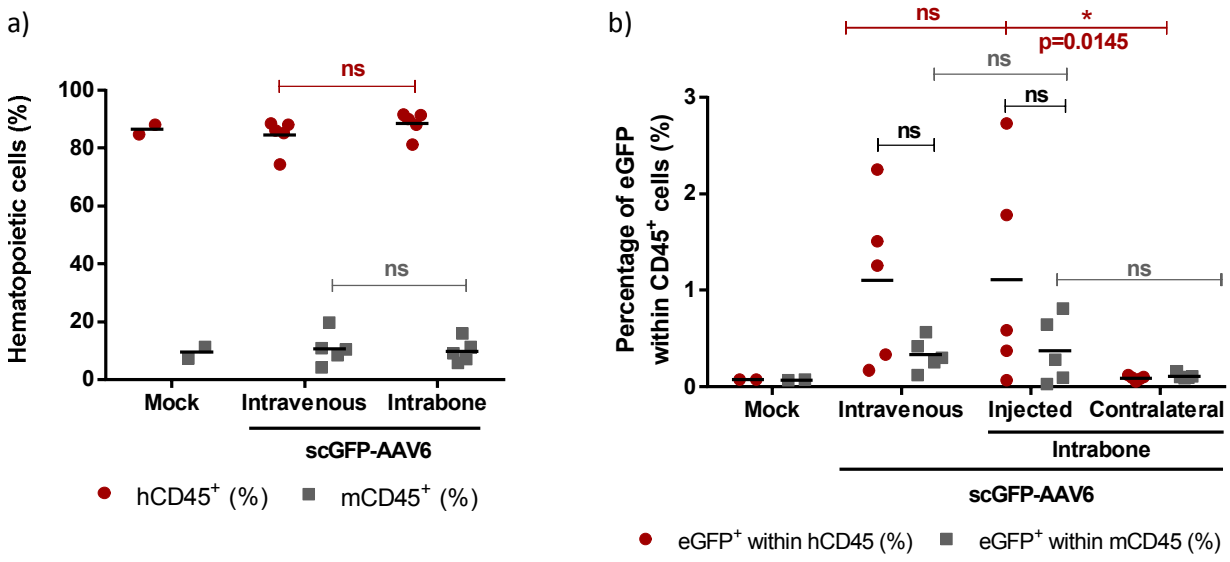


Figure 45.- *In vivo* transduction of hematopoietic cells using AAV6. (**a**) Human and murine engraftment was evaluated in the BM by flow cytometry using CD45 specific antibodies. (**b**) Efficacy of transduction was investigated by the presence of eGFP+ cells within human or murine CD45+ cells. Mean values are represented with a horizontal line. A two-way ANOVA was performed followed by Tukey's post hoc test.

Although there are no statistically significant differences in the transduction ability showed by the intravenous and intrafemoral administration, the intrafemoral route was selected for the infusion of the AAV construct carrying the CRISPR/Cas9 system as the volume of vector needed is 10 times lower.

Finally, with the aim of finding the most efficient approach to conduct an AAV-mediated gene editing in HSPCs, two different strategies were conducted:

1. A two-component system by which the *S. pyogenes* Cas9 is packaged in a ssAAV6 (ssSpCas9-AAV6) and the sgRNA in a scAAV6 (scgGM10-AAV6).
2. An all-in-one ssAAV6 system in which the two CRISPR/Cas9 components are packaged using the smaller *S. aureus* Cas9 (ssgGM2SaCas9-AAV6).

In vitro testing of the two different systems demonstrated that only the all-in-one construct carrying the *S. aureus* Cas9 allowed a significant gene editing efficiency with a mean indel percentage of 16.2 ± 6.6% (Figure 46), which was similar to the mean values obtained with RNP.

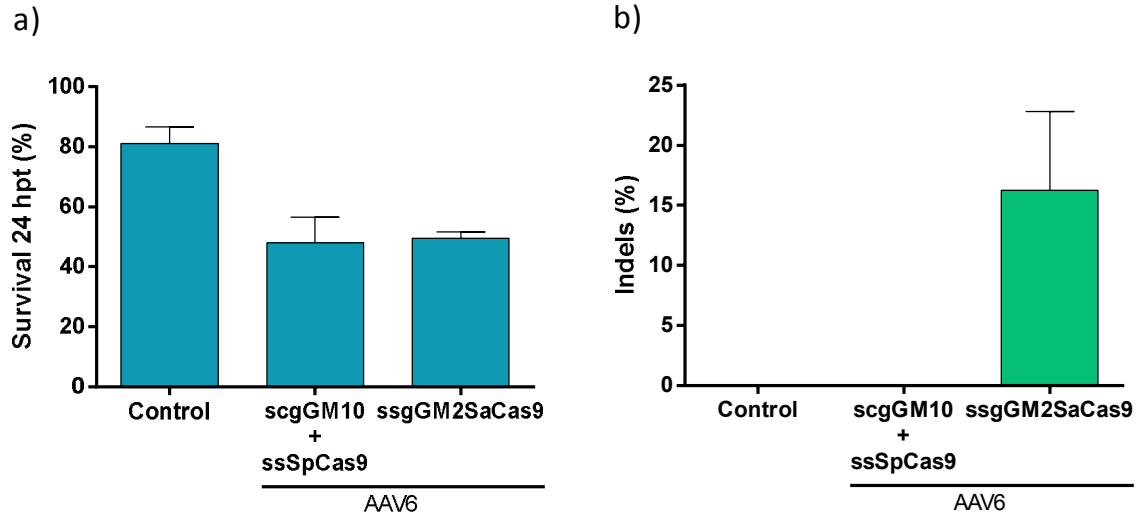


Figure 46.- Editing efficacy of the different AAV-mediated CRISPR/Cas9 delivery strategies. Human CD34$^+$ cells were transduced using 10 µg/mL dmPGE2 with the ssgGM2SaCas9-AAV6 (MOI 10^5) or ssSpCas9-AAV6 (MOI 10^5) in combination with scgGM10-AAV6 (MOI 10^3). (**a**) Cell viability was assessed 24 hours post-transduction (hpt) by flow cytometry in terms of the percentage of DAPI$^+$ cells. (**b**) Editing efficacy was measured in terms of the percentage of indels detected by *Surveyor assay* 5 days after transduction. Bars represent mean ± SD of two different experiments.

Taking these results into account, future *in vivo* experiments will focus on AAV-mediated gene editing in HSCs using the all-in-one construct carrying the *S. aureus* Cas9.

"No queda sino batirse."

— Arturo Pérez-Reverte, El Capitán Alatriste.

Discussion

1. NHEJ-mediated Gene Editing: Mimicking Somatic Mosaicism in Fanconi Anemia

Fanconi anemia (FA) is nowadays considered a good candidate for gene therapy. One of the main reasons that support this statement is the somatic mosaicism phenomenon reported in some FA patients. The proliferative advantage acquired by the hematopoietic stem and progenitor cells (HSPCs) that reverse the pathogenic mutation can result in the recovery of the hematological parameters in the patient, constituting an example of a natural gene therapy process. Interestingly, the evidence of peripheral blood (PB) cell count restoration as a consequence of the reversion of a reduced number of multipotent HSCs constitute an extraordinarily relevant observation. This distinguishes FA from several other monogenic diseases already treated by gene therapy, such as X-linked severe combined immunodeficiency (X-SCID) or adenosine deaminase deficiency (ADA) (192). In this context, our laboratory has demonstrated that a similar proliferative advantage can be obtained *in vivo* in immunodeficient mice transplanted with FA-A patient HSPCs after an *ex vivo* gene therapy protocol using lentiviral vectors (LVs) to deliver a healthy copy of the *FANCA* gene (221). Moreover, the preliminary results obtained in the ongoing clinical trial conducted by the Spanish team (NCT03157804) indicate that this repopulating process is already taking place in the patients, even when a relative low number of corrected cells are infused without any conditioning regimen (222). These observations suggest that gene therapy may constitute a real therapeutic alternative for FA patients.

Nowadays, the use of programmable nucleases constitutes a new step in the development of more efficient and safer gene therapy approaches, as these molecules markedly increase the frequency of homology-directed repair (HDR) even in patient HSPCs suffering from different hematological disorders, thus allowing the targeted integration of the transgene. That is the case in the sickle-cell disease (SCD) (8, 75, 76, 123), X-SCID (101, 236) or chronic granulomatous disease (CGD) (70, 71), although the efficiency varies depending on the nuclease used, the donor delivery system and the disease.

Considering the proven advantages of these nucleases, our group has also developed homologous recombination (HR)-mediated gene targeting approaches in FA cells. Initially fibroblasts (218) and then HSPCs (77) from FA-A patients were phenotypically corrected by the specific integration of a healthy copy of the *FANCA* gene in the *AAVS1* "safe harbour" locus.

This process was promoted by zinc-finger nucleases (ZFN) that efficiently cleaved this locus after the delivery of the donor therapeutic construct in a LV. Up to now, no other groups have achieved gene editing correction of FA HSPCs. Gene editing studies conducted by Jakub Tolar's group have also shown the feasibility of correcting FA cells by HDR, but in fibroblasts. However, a selection step was always needed to be able to isolate corrected cells, suggesting a very low basal recombination efficacy. Firstly, the authors conducted the replacement of a *FANCC* mutation in transformed patient fibroblasts, obtaining 88% integration but only after puromycin selection (201). Similarly, they corrected FA-I patient fibroblasts previously reprogrammed into induced pluripotent stem cells (iPSCs), achieving 70% correction after exposure to MMC (200). More recently, gene targeting using a single-stranded oligonucleotide donor (ssODN) was used to replace a *FANCD1* mutation in primary fibroblasts, obtaining up to 26% integration, once again, after selection (249).

Considering the difficulties involved in inducing HDR in quiescent cells and especially in FA, we pursued the development of a simpler and more efficient strategy. To this end, we focused on the alternative double-strand break (DSB) repair pathway: non-homologous end-joining (NHEJ). Since NHEJ is an error-prone DSB repair mechanism, it has been mainly used to knock out specific genes by the generation of insertion and deletion (indels) events that disrupt the targeted gene. However, in certain cases, NHEJ-mediated gene editing has been used as a therapeutic strategy. That is the case in Duchenne Muscular Dystrophy (DMD), where the removal of the pathogenic mutation by exon skipping partially corrected dystrophin function (203). Taking this fact into account, the mutations were targeted by different engineered nucleases to induce frame recovery as a consequence of the indels introduced by NHEJ after the DSB (169, 202, 204). A similar approach has also been tested in keratinocytes (58) and epidermal stem cells (176) from recessive dystrophic epidermolysis bullosa patients. The targeting of a frameshift mutation in *COL7A1* exon 80 using TALENs gave rise to the recovery of the open reading frame (ORF) and the restoration of a functional collagen VII protein capable of regenerating a phenotypically normal human skin in immunodeficient mice. Despite all previous studies, NHEJ-based editing approaches have never been applied either to correct pathogenic mutations in proteins without repetitive motifs or in primitive HSCs.

As NHEJ is the preferred DNA repair mechanism in long-term (LT)-HSCs (22, 173, 180), and given that its function has been reported to be enhanced in FA cells (81, 205, 217), in the current

study we hypothesized that the direct removal of FA-associated mutations induced by NHEJ might constitute a more efficient gene editing approach than HDR in FA HSCs. In this case, the inaccuracy of the NHEJ when repairing a DSB could be positively exploited to generate indels that may remove/compensate the original pathogenic mutation, restoring gene function and leading to the progressive expansion of phenotypically corrected HSCs, which mimics the natural compensatory mutation events responsible for the reversion described in FA mosaic patients (108, 172, 220, 272).

To test this hypothesis, two different *FANCA* mutations were targeted using CRISPR/Cas9 nucleases in FA-A patient derived LCLs. The versatility of these nucleases (215) permitted the easy design of different sgRNAs to assess the feasibility of our approach. The first mutation consists of a homozygous insertion of a Guanine in exon 36 (c.3558insG), leading to a 28-animo acid frameshift that ends in a premature stop codon (p.R1187EfsX28). This mutation was chosen as a proof of principle, because previous studies reported that the spontaneous reversion of this mutation resulted in the hematological improvement of some patients (46), suggesting that the protein domain encoded by exon 36 allows slight sequence variations without compromising its functionality. This observation was of great interest because FANCA 3D structure is unknown, as it has not been crystallized yet. In theory, any (3N+2) bp insertion, (3N+1) bp deletion, or a combination of both events that compensates the frameshift would be sufficient. Accordingly, the next-generation sequencing (NGS) analysis of the gINS11-edited cell pool conducted 5 days after electroporation demonstrated that although the targeting efficacy of the construct was moderate (9.94%), potentially therapeutic NHEJ-repair events occurred with a high frequency: nearly 77% of the total indels (7.62% from the total 9.94% indel rate) recovered the ORF (Figure 13a). Interestingly, there was one event whose frequency was remarkably higher compared to the other registered indels: the deletion of the Guanine immediately adjacent to the Cas9 cutting site (c.3579delG). Although small deletions surrounding a DSB were the most frequent NHEJ-repair events generated (41), further analysis revealed that additional reasons underlie this high frequency. When a new NGS analysis was conducted after 30 days of *in vitro* culture, the frequency of potentially therapeutic NHEJ-repair events increased to 16.45%, but this was mainly due to c.3579delG indel, which was the only detectable therapeutic event whose frequency increased over time. The 2.5-fold expansion experienced by this deletion −while the other events disappeared− clearly suggested that it was the only functional editing event which

conferred proliferative advantage to the bearing cells, demonstrating that the recovery of the ORF was necessary but not enough. FANCA protein BLAST analysis showed that exon 36 encodes for a highly conserved domain. However, c.3558insG mutation takes place at the beginning of a 21-bp region of non-conserved nucleotides and gINS11 construct cuts close to the end of this region (Figure 14). Indels that affected nucleotides upstream of the mutation (those that were induced by gINS8 construct) and indels that affected nucleotides downstream of the gINS11 cutting site, disrupted conserved domains that may play important structural/functional roles. As a consequence, the deletion of the Guanine next to the cutting site was the only NHEJ-repair event that restored the protein ORF without affecting this conserved domain.

To further evaluate the extent of the NHEJ-mediated gene editing approach proposed, a more challenging mutation was also targeted. It consists of the substitution of Cytosine 295 by a Thymine (c.295C>T) that results in a premature stop codon in *FANCA* exon 4 (p.Q99X). In contrast to the previous one, this mutation must be removed to eliminate the premature stop codon while maintaining the ORF. As a consequence, the two sgRNAs with the closest target sites to the mutation were chosen among the best-rated sgRNAs found by the design software. However, the proximity of the mutation to the 5'-end of the exon limited the options, so only sgRNAs targeting loci downstream of the mutation were selected. According to these restrictions, the deletions that were required to remove the mutation were at least 15- and 18-bp long (for gGM4 and gGM10 construct, respectively). Consequently, the initial frequencies of potentially therapeutic NHEJ-repair events were extremely low (0.29% and 0.41%, respectively). As previously observed, not all the putatively corrective NHEJ-repair events initially obtained were truly therapeutic. Strikingly, however, in this case the only events whose frequencies increased over the incubation time were 33- and 30-bp deletions obtained with gGM4 and gGM10, respectively. Although FANCA protein BLAST analysis demonstrated that exon 4 is not conserved among vertebrates (especially due to the length variability showed by this region, Figure 23), there must be an important conformation restriction in human FANCA that primed the expansion of two of the largest therapeutic deletions obtained over the smaller ones that also removed the stop codon and maintained the ORF. Despite the size of the therapeutic deletion being higher than the minimum needed, a functional FANCA protein was re-expressed, as demonstrated by the restoration of the FA pathway functionality and the

reversion of both the MMC hypersensitivity and the elevated ROS production characteristic of FA cells.

These results demonstrate for the first time the possibility to correct FA-associated mutations by a NHEJ-mediated gene editing approach that mimics the spontaneous reversions experienced by FA mosaic patients. Furthermore, we tested that this strategy is efficient even when targeting challenging mutations such as c.295C>T, where the probability of spontaneous reversion is very low. Additionally, the feasibility of correcting this mutation is of great clinical relevance because it is the most frequent mutation in FA patients from Spain: approximately 30% bear this mutation in homozygosis (40). Therefore, a great number of patients could benefit from the same sgRNA, already tested and proved to generate therapeutic events. Although the strategy proposed involves the design of specific and efficient sgRNAs for each mutation, this is far from being a limitation: the versatility and sublimity of the CRISPR/Cas9 system greatly facilitates the generation and testing of different sgRNAs in an inexpensive and simple way.

The only mutations that cannot be corrected by NHEJ are those directly affecting a critical structural domain. In those cases, nothing but a precise restoration of the wild-type (WT) sequence could correct the phenotype of the cell. But this is not usually known in advance, as no FANCA structural information is available. Only when a complete protein is still present in the patient (small in-frame deletions or missense mutations) can we suspect that a crucial domain has been affected. In the rest of the cases, we are not able to anticipate if the introduction of an indel will render a functional protein, even when removing/compensating the mutation. However, the study of the conservation degree of the protein demonstrated to be informative. As a non-targeted strategy that relies on a random repair process, the efficacy of this approach strongly depends on the nature of the mutation, the region where it is placed and how far the CRISPR/Cas9 nucleases cut from the mutations. Nevertheless, thanks to the recessive nature of the FA disease, the recovery of just a single allele has demonstrated to be enough to trigger a phenotypic reversion. Even in those cases of challenging mutations, such as the c.295C>T substitution, the remarkable proliferative advantage acquired by those cells containing the appropriate NHEJ-repair event compensated the initial low frequency at which this therapeutic indel took place. Therefore, other diseases where corrected cells have also shown proliferative advantage, such as X-SCID, ADA deficiency and β-thalassemia (193), would also benefit from this approach. Moreover, the simplicity of the strategy makes this

approach of great clinical interest, as it significantly reduces the extensive *ex vivo* manipulation of the cells that the gene targeting strategies mediated by HDR require: only the delivery of the nuclease is needed. This manipulation would be critical when treating FA HSPCs due to its exacerbated hypersensitivity to *in vitro* culture and because their long-term (LT)-repopulation capacity could be seriously compromised. Interestingly, recent evidences published by Richardson *et al.* (217) demonstrated that, in contrast to NHEJ, the HDR mechanism is hampered in FA cells, further supporting the convenience of developing gene editing approaches based on NHEJ. Remarkably, Sürün and colleagues have very recently come up with a similar conclusion, proposing NHEJ as an alternative to take into account to simplify therapeutic gene editing approaches in hematopoietic cell lines (260). They have demonstrated that the NHEJ could remove mutations associated with X-linked CGD from myeloid leukemia *CYBB*-null cells transduced with a lentiviral vector (LV) carrying a modified copy of *CYBB* gene with patient-specific mutations, thus recovering the WT phenotype and further supporting our hypothesis.

Although the guides we used were always chosen according to the lowest theoretical off-target rate, concerns about the potential, real unspecificity of the system made us wonder whether similar results could be obtained with more specific engineered nucleases (206). To this end, a pair of TALEN monomers were designed by Dr. Claudio Mussolino to target the c.295C>T mutation and compared with our sgRNAs. These gene editing tools were chosen due to their higher specificity compared to other programmable nucleases (59). Moreover, one of the monomers designed directly bound to the locus containing the mutation, so it was expected that this TALEN could discriminate between a mutated (on-target) and a non-mutated allele (off-target). However, the nuclease demonstrated to be incapable of making this distinction, as it rendered similar targeting efficacies when the healthy donor (HD) and the FA-55 LCL were electroporated. As they did not reduce the toxicity of the electroporation, and even complicate the delivery into HSPCs in a clinical setting due to their repetitive structure (141), we decided to carry on with the more versatile CRISPR/Cas9 system.

2. NHEJ-based Gene Editing in Human Hematopoietic Stem and Progenitor Cells

Despite the efficacy and simplicity that the NHEJ-mediated gene editing demonstrated in LCLs, the greatest challenge was setting up efficient editing conditions in HSPCs. The components of the CRISPR/Cas9 system were delivered into purified umbilical cord blood (UCB) HSPCs from HDs under different conformations and using different transfection methods. However, the only approach that mediated an efficient gene editing without compromising the cell viability was the electroporation of the *in vitro* transcribed (IVT) sgRNA previously assembled to the Cas9 protein, conforming a ribonucleoprotein (RNP) complex. These results were consistent with the initial observations reported by Liang *et al*. (160) and, in fact, this approach is currently the most frequently used to target HSPCs with the CRISPR/Cas9 system (17, 75).

The experiments conducted in HD hCD34$^+$ cells confirmed that RNP electroporation was not only effective but also did not induce toxicity in HSCs, as treated cells repopulated the hematopoiesis in primary and secondary immunodeficient recipient mice with efficacies similar to control, unedited hCD34$^+$ cells (Figure 31-Figure 36). Although in some organs slight differences were observed among groups for a certain hematopoietic subpopulation (Figure 33a,c,d and Figure 36a,c,d), these differences were not present in all the hematopoietic organs analysed. Moreover, in spite of these differences, the presence of human cells from all the hematopoietic lineages was clearly detected in all organs over the three months that both the primary and secondary recipients were monitored (Figure 33 and Figure 36). Taken together, these results demonstrated that the repopulation capacity of the LT-HSCs remained unaltered after the RNP treatment, in contrast to previous results which evidenced a marked impairment in the repopulation ability of hCD34$^+$ cells electroporated with the CRISPR/Cas9 system delivered as plasmid and mRNA (28), further supporting the use of the RNP to conduct the HSPC gene editing. Additionally, the maintenance of the HSPC repopulating properties after RNP-mediated gene editing has also been demonstrated in different studies (15, 17, 76).

Notably, NGS analysis of the electroporated cells prior to and after transplantation showed that the indel rates remained constant over time, even in secondary recipients (Figure 37), strongly suggesting that the RNP had edited LT-HSCs. Moreover, these results also demonstrated that the NHEJ-mediated gene editing efficacy in engrafted cells was comparable

to that obtained in the bulk of hCD34$^+$ cells. This observation clearly differs from previous results obtained by different groups when targeting HSPCs using HDR-based approaches, where the editing events detected in LT-repopulating cells were significantly lower than those reported in more mature progenitors. This behaviour was observed by Genovese *et al.*, who conducted the targeted integration of the *IL2RG* exons 5 to 8 together with a GFP cassette into the endogenous *IL2RG* locus of HD HSPCs. Although nearly 18% of GFP$^+$ cells were obtained in the bulk of hCD34$^+$ cells *in vitro*, no more than 10% of human cells expressed this reporter protein in the transplanted mice (101). Similarly, Hoban *et al.* conducted the site-specific correction of the sickle mutation in hCD34$^+$ cells. In this case, only 0.21% of the human cells that repopulated the hematopoiesis of NSG mice contained the insertion of the donor template, in contrast to the 10.5% reported *in vitro* (123). Similar results were obtained by Dever and coworkers targeting the *HBB* locus using CRISPR/Cas9 nucleases but again, only 3% of human engrafted cells were edited, a value that was significantly lower than the 16% detected in the bulk hCD34$^+$ population prior to transplantation (75). Analogous results were also observed when the donor template was a ssODN (76), whose integration –in contrast to double-stranded DNA– is not RAD51-dependant (217). The explanation of the increased NHEJ efficacy compared to HR probably relies on the intrinsic HSC quiescence (22, 180), which hampers the integration of the donor sequences by HDR in these primitive precursor cells. These observations reinforce the relevance of NHEJ-mediated editing for the correction of hematopoietic diseases.

Additionally, the monitoring of the different editing events identified in the hCD34$^+$ cell pool prior to transplantation and in several primary and secondary recipients demonstrated an *in vivo* clonal succession of certain edited clones (Figure 38), similar to the alternation previously described observed in conventional gene therapy approaches using LVs (2, 25, 27, 43, 265). This observation shows for the first time the possibility of using editing labels as signatures of HSCs capable of conducting *in vivo* hematopoietic repopulation, and confirms that an oligoclonal reconstitution has been reached after a NHEJ-mediated gene editing approach

3. Correction of HSPCs from FA-A Patients by NHEJ-mediated Gene Editing

After confirming the feasibility of targeting LT-repopulating HSCs by a NHEJ-based gene editing approach and verifying that the repopulation ability of these cells was not compromised after the editing process, we aimed at the correction of HSPCs from FA patients carrying the homozygous c.295C>T mutation. First, hree samples from two different patients (FA-807 and FA-739) were treated by RNP electroporation. In all cases, NGS analyses were conducted 5 days after electroporation to measure the gene editing rate and characterise the different NHEJ-repair events that took place in these cells. Strikingly, the same 30-bp deletion previously shown to be therapeutic in FA-55 LCL (c.289_318del30) was always present among the potentially corrective NHEJ events (Figure 39-Figure 41). This recurrence further demonstrated the existence of conformational requirements that –even within this short 5-day period of time– primed the selection of this deletion over smaller ones that, in theory, should also correct the mutation. Interestingly, the total indel rate together with the frequency of therapeutic NHEJ-repair events were remarkably higher (7.98% from a total 23.33% editing rate) in the edited hCD34$^+$ cells purified from FA-807 total bone marrow (BM) sample (Figure 39), compared to the data obtained in mobilized peripheral blood (mPB) hCD34$^+$ cells (2.65% from a total 16.84% in FA-807 and 0.45% from 12.32% in FA-739) (Figure 40 andFigure 41). Unfortunately, only one BM sample could be analysed, as mPB is the most common source of hCD34$^+$ cells in clinic, so this result should be only considered as a preliminary observation.

Sequencing analysis of the isolated colonies obtained from the colony forming cell (CFC) assays conducted in FA-807 BM and mPB samples showed that 50% and 25% of them respectively contained this c.289_318del30 indel (Table 10 andTable 11). This striking observation demonstrates the natural selection of this therapeutic event due to the proliferative, or at least, survival advantage conferred in the progenitor cells. These results are very similar to the 21-51% correction previously obtained by LV-mediated *FANCA* gene addition (221), and notably increase the 10% correction obtained by HDR-mediated gene targeting of *FANCA* HR cassette into the *AAVS1* locus (77). Importantly, sequencing analysis of colonies derived from hCD34$^+$ cells edited by NHEJ after MMC treatment showed that 100% of the clones analysed contained the therapeutic c.289_318del30 deletion, confirming the restoration of the FA pathway in FA HSPCs. A possible explanation regarding these differences could be where the

correction is taking place. Although in all cases gene editing results in the re-expression of a functional FANCA protein, NHEJ restores the gene expression under its endogenous promoter, while gene targeting in the *AAVS1* locus and LV-mediated gene addition introduce an exogenous copy of the *FANCA* gene whose expression is not under its native regulators' control. A similar result was demonstrated by De Ravin *et al.* when hCD34$^+$ cells from CGD patients were corrected by the targeted integration of the therapeutic cassette into the *AAVS1* locus and transplanted into NSG mice. Only a 3.7% of human cells transplanted showed phenotypic restoration, even after using a synthetic promoter to obtain robust functional correction, highlighting the difficulties in driving an adequate high-level expression of the therapeutic protein in HSPCs from the *AAVS1* locus (71). These results reinforce the hypothesis that NHEJ-mediated gene editing generates a more physiological correction of the *FANCA* gene.

The proliferative advantage observed was not only confirmed in the CFC assays but also after *in vitro* culture of the edited cells. In this respect, NGS analysis of the cell pool of edited FA-739 mPB CD34$^+$ cells 14 days after electroporation also showed a remarkable 55.3-fold increase of the c.289_318del30 frequency after only 9 days of *in vitro* culture, demonstrating an unprecedented proliferative advantage (Figure 41), even higher than the one reported in edited FA-55 LCLs. Interestingly, despite the low correction probability of the challenging c.295C>T mutation, the initial proportion of therapeutic NHEJ-repair events among the total indels generated in these cells was also higher in edited FA HSPCs (ranging from 3.6% to 32.2%) than in LCLs (0.9% and 1.3%). This observation further supports the idea that natural selection is acting immediately after the editing, and the restoration of the cell functions is probably playing a more crucial role in the survival of primary cells than in an immortalised cell line. Moreover, NHEJ efficacy is higher in these quiescent cells in comparison to proliferative LCLs where HR is also occurring.

In order to further confirm the safety of this approach, the specificity of the RNP was assessed. Due to the scarcity of the samples available, the top-five *in silico* predicted off-target loci were selected to be analysed by NGS in these three samples. Variant calling analysis showed that the frequency of the variations registered at these loci was below the detection limit of the technique (0.01%, Table 12) (limit in which indels generated by an unspecific activity or by the polymerase inaccuracy during the amplification PCR cannot be distinguished) (258), suggesting

the absence of off-target activity in these top-five loci, even for the most probable off-target locus (OT1, 100.0% score). As we could not conduct unbiased analyses, the absence of unspecific activity cannot be categorically concluded. Nevertheless, the results obtained in the NGS analyses of the top-five *in silico* predicted off-target loci from these three samples strongly suggested the high specificity of the sgRNA used in our studies and, therefore, the safety of the approach.

Interestingly, recently published results have evidenced that CRISPR/Cas9 targeting induces p53-mediated DNA damage response in edited cells (111, 127). Considering that this response is already exacerbated in FA (48, 49, 159), the p53 status should be additionally evaluated in our NHEJ-edited FA HSPCs in order to prevent any toxic effect due to uncontrolled p53 activity.

As our next goal, we aimed at evaluating the engraftment capacity of FA patient HSPCs corrected by gene editing. Sanger sequencing showed that 10% of isolated colonies contained edited alleles, but none of them carried a potentially therapeutic indel (Table 13). Nevertheless, when BM cells from the only mouse that could be transplanted with edited FA hCD34$^+$ cells were analysed, an editing rate of 17.31% was determined. Strikingly, 47.8% of these indels corresponded to the only therapeutic NHEJ-repair event present: c.289_318del30 (Figure 42). This result further confirms the functionality of the new FANCA protein generated, demonstrating the remarkable *in vivo* proliferative advantage also conferred to those cells bearing the 30-bp therapeutic deletion, whose frequency increased from less than 1.8% (no corrected CFCs in a total of 56 colonies analysed) to 8.28% within 15 days. These results are consistent with previous data achieved in our laboratory by LV-mediated gene therapy (221) and evidenced that the lower efficiency when correcting the challenging *FANCA* c.295C>T mutation is compensated by the high proliferative advantage of the FA HSCs harbouring therapeutic indels, not only *in vitro* in liquid cultures, but also *in vivo* after transplantation. Taking this fact into account, if higher editing rates were obtained, the initial frequency of the therapeutic event could be increased, thus boosting the proliferative advantage and the efficacy of the strategy.

Nowadays targeting efficacies as high as 95% can be achieved thanks to the recently described chemical modifications of sgRNAs (15, 35), and to the development of Cas9 protein variants with enhanced activity and fidelity (147). In addition, new Cas9 nucleases capable of recognizing protospacer adjacent motifs (PAMs) of different sequences (7, 148) and length (197)

have also been engineered. NHEJ-mediated gene editing will significantly benefit from these expanded abilities, as they offer new opportunities to target those genomic regions that were out of range the canonical *S. pyogenes* Cas9. These modified nucleases are of great interest because –as they expand the targeting restrictions– the probability of obtaining a sgRNA that binds the exact desired position increases, offering the possibility to induce precise deletions using pairs of CRISPR/Cas9 nickases (227, 286) without compromising the safety of the approach, as only when both nickases cut does the deletion takes place.

In our studies, the NHEJ-mediated gene editing strategy proposed for FA HSPCs has not only demonstrated to be more efficient, but also simpler than HR-mediated gene targeting, as it significantly reduces the *in vitro* manipulation of the cells, something even more critical in the context of FA. The HDR approach previously developed in our lab required a 24-hour pre-stimulation of the HSCs to facilitate LV transduction and to increase the frequency of the HDR. After this 16-24-hour process, cells were electroporated with ZFNs and finally transplanted into immunodeficient mice 24 hours later. In contrast, as NHEJ-based editing does not require a donor template, the protocol can be reduced to only 24 hours. Although in our experiments with cryopreserved FA HSPCs a 24-hour pre-stimulation was also used, the transplantation experiments conducted in HD hCD34^{+} cells from UCB indicated that efficient editing can be achieved even in the absence of pre-stimulation when fresh cells are used. Since NHEJ takes place during the whole cell cycle, the cycling of the HSPCs might not be necessary, in contrast to HDR. In a clinical context, patient HSPCs could be electroporated immediately after hCD34 immunoselection and reinfused 24 hours later, shortening the protocol to only one day of *in vitro* culture. As previously highlighted, this simplification is of great interest in the FA context as it was demonstrated that the reduction of *ex vivo* HSPC handling prevents the impairment of the LT-repopulation capacity of the HSCs (221). Moreover, the use of NHEJ would also preserve the endogenous regulation of the gene, resulting in more physiologic and appropriately regulated gene expression (86) than the introduction of a healthy copy of the gene in a "safe harbour" locus. In addition, the current availability of clinical grade electroporation systems (244) further supports the consolidation of NHEJ-based gene editing as a real therapeutic alternative accessible to patients.

Taking all these facts into account, the results shown in this doctoral thesis demonstrate for the first time the possibility to phenotypically correct FA HSPCs by a donor-free gene editing

approach based on the NHEJ repair pathway, thus leading to a marked proliferative advantage of corrected cells both *in vitro* and *in vivo*. This new strategy paves the way for the development of a new therapeutic alternative for the future treatment of monogenic blood disorders affecting HSCs, especially in those cases where the corrected cells display proliferative advantage, such as FA.

4. Paving the Way for the *in Vivo* NHEJ-mediated Gene Editing of FA HSCs

After demonstrating that NHEJ-mediated gene editing can efficiently correct FA patient HSPCs, we wanted to go one step further and investigate the feasibility of applying this strategy *in vivo*. As previously mentioned, the reduced reservoir of HSCs in FA patients in advanced stages of the disease hampers the collection of clinically relevant numbers of progenitors (138). Moreover, the high sensitivity of FA HSPCs to *in vitro* manipulation, together with the significant loss of HSPCs associated with the immunoselection of $hCD34^+$ cells, markedly reduce the number of HSPCs available for therapy and compromise their LT-repopulation capacity. For these reasons, the development of an *in vivo* gene editing approach that avoids the collection and the *ex vivo* manipulation of these invaluable cells is of great interest, particularly for those pathologies in which the number of HSCs is already reduced, such as FA.

Currently, adeno-associated viral vectors (AAVs) are the most widely used delivery platform for *in vivo* gene therapy approaches due to their low immunogenicity and their non-integrative nature. In addition, the simplicity of engineering, not only their genome, but also the capsid has contributed to their consolidation as the preferred vectors for *in vivo* gene therapy. These particles have already been used in more than 160 clinical trials (121) and even been commercialised as gene therapy products: *Glybera,* which delivers a healthy copy of the lipoprotein lipase gene to treat lipoprotein lipase deficiency (37), was the first AAV-based gene therapy treatment approved by the European Medicines Agency (288). Later, *Luxturna*, which delivers a healthy copy of the *RPE65* gene to treat congenital blindness (23), was the first AAV-drug approved by the Food and Drug Administration to conduct classical addition gene therapy (121). Furthermore, gene editing strategies based on the disruptive ability of the NHEJ-

repair pathway have already been tested *in vivo* using AAVs to deliver the nucleases. That is the case of DMD, where systemic *in vivo* administration of CRISPR/Cas9 nucleases into DMD mice partially restored the reading frame of the dystrophin gene, resulting in the functional improvement of the skeletal and cardiac muscle of treated animals (6, 194, 263). Similar strategies have also been successfully applied in Huntington's disease (177), Leber congenital amaurosis subtype 10 (227) and in an autosomal dominant form of retinitis pigmentosa (289). Taking these facts into account, we aimed at evaluating the potential of AAVs to mediate the delivery of CRISPR/Cas9 nucleases into HSPCs to conduct our NHEJ-based gene editing approach in FA.

The low packaging capacity of the AAV capsid initially required the split of the CRISPR/Cas9 system into two different AAVs. Therefore, the Cas9 nuclease was necessarily introduced under a single-stranded conformation (ssAAV), while the sgRNA could be packaged under either a ssAAV or a self-complementary one (scAAV) –an artificially-generated conformation that promotes the direct expression of the transgene, avoiding the synthesis of the complementary DNA strand: the first step in an AAV infection– (279). Previous studies demonstrated that AAV6, among the different AAV serotypes, best mediates the HSPC transduction (253, 254). In our studies, the transduction efficacy of an AAV6 carrying the eGFP reporter gene under the two different DNA conformations was tested in HD hCD34$^+$ cells and compared with AAV2, which has been previously described to target HSPCs (239). Our results clearly demonstrated the higher efficacy of the AAV6 serotype over AAV2, which is consistent with previously reported data (162). In addition, our scGFP-AAV6 construct was able to mediate nearly 100% transduction of hCD34$^+$ cells at MOIs that were 2-fold lower than those used by Ling and colleagues. Therefore, AAV6 was selected to mediate the *in vivo* delivery of our CRISPR/Cas9 system.

As the Cas9 had to be be packaged in a single-stranded conformation, we tried to improve the transduction yield using dimethyl prostaglandin E2 (dmPGE2). Although the high transduction efficacy of the self-complementary construct could not be reached, the use of dmPGE2 during ssGFP-AAV6 infection demonstrated that this molecule increases the transduction of hCD34$^+$ cells nearly 2-fold, as was initially described with LVs (292).

In order to test the *in vivo* transduction ability of the AAV6 and the most efficient administration route, the scGFP construct was delivered into immunodeficient mice previously

transplanted with HD hCD34$^+$ cells from UCB via tail vein or by intrafemoral perfusion. Although similar transduction efficacies were obtained in both cases, intrafemoral perfusion was the route selected for the delivery of our all-in-one AAV6 construct, since the dose of vector is ten times lower compared to intravenous doses (20 μL versus 200 μL, respectively). Moreover, although targeting other tissues would not be a problem –as every cell type is affected in FA– the delivery of the vector in a confined HSPC-enriched environment could maximize the editing of these clinically relevant cells. In this context, one important concern about the *in vivo* modification of HSPCs using AAVs is the off-target risk due to persistent Cas9 activity favoured by the continued presence of the episomal AAV. Furthermore, as two studies have demonstrated that immune responses either against Cas9 (60) and sgRNA (281) can take place, great efforts are being made to prevent these potential risks. One example is the development of self-inactivating Cas9 nucleases that act in a hit-and-run fashion (177).

In our final set of experiments, AAV-mediated CRISPR/Cas9 gene editing in hCD34$^+$ cells was evaluated following two different strategies. Transduction using the Cas9 nuclease packaged into an ssAAV6 and the sgRNA in a scAAV6 did not generate detectable indels. Only when the smaller Cas9 nuclease from *Staphylococcus aureus* that allowed the simultaneous packaging of the sgRNA and the nuclease into a single ssAAV construct (213) was used, efficient gene editing comparable to that obtained with the RNP was achieved (Figure 46). These results demonstrate for the first time the feasibility of conducting the HSPC genome modification by CRISPR/Cas9 nucleases delivered by an AAV6 *in vitro*.

Currently, we are investigating the *in vivo* gene editing capacity of the all-in-one AAV6 construct. If HSPC-specific delivery of an efficient CRISPR/Cas9 system could be achieved *in vivo*, the remarkable proliferative advantage demonstrated by the corrected FA HSPCs would open the possibility of considering *in vivo* NHEJ-mediated gene editing as a new, and perhaps ideal gene therapy approach for the treatment of bone marrow failure in FA patients.

The most exciting phrase to hear in science,

the one that heralds new discoveries,

is not "Eureka!" but "That's funny …"

— Isaac Asimov.

Conclusions

DNA repair mediated by non-homologous end-joining (NHEJ) constitutes the preferential mechanism for the resolution of double-strand breaks in quiescent cells, such as hematopoietic stem and progenitor cells, the main target in Fanconi anemia gene therapy approaches. Moreover, this pathway has been also described to be enhanced in Fanconi anemia cells. Although the insertions and deletions generated by NHEJ are normally used to induce gene disruption, we propose that NHEJ can be therapeutically exploited to remove/compensate Fanconi anemia associated mutations, mimicking the reversion events reported to trigger hematological improvement in Fanconi anemia mosaic patients. In this doctoral thesis, the feasibility of correcting mutations associated to Fanconi anemia subtype A by NHEJ-mediated gene editing after CRISPR/Cas9 targeting has been investigated. The main conclusions obtained in this study are the following:

1

Therapeutic NHEJ-repair events can be generated by designed CRISPR/Cas9 nucleases in lymphoblastic cell lines from Fanconi anemia subtype A patients bearing different mutations in the *FANCA* gene.

2

Edited lymphoblastic cell lines from Fanconi anemia subtype A patients carrying therapeutic NHEJ-repair events show a remarkable *in vitro* proliferative advantage and recover the expression of a functional FANCA protein. This leads to the correction of the FA cell phenotype, which is demonstrated by the restoration of the FANCD2 foci formation, the reversion of the mitomycin C hypersensitivity and the reduction of reactive oxygen species production.

3

The electroporation of designed CRISPR/Cas9 ribonucleoprotein complex in healthy donor human $CD34^+$ cells mediates the editing of hematopoietic stem and progenitor cells without compromising their *in vivo* pluripotent repopulation ability. Moreover, in contrast to editing approaches based on homology-directed repair, the NHEJ-mediated gene editing targets with similar efficacy the more committed progenitor cells and the long-term repopulating hematopoietic stem cells.

4

NHEJ-mediated gene editing in CD34⁺ cells from Fanconi anemia subtype A patients induces their phenotypic correction, demonstrated by a remarkable *in vitro* and *in vivo* proliferative advantage and the reversion of their mitomycin C hypersensitivity.

5

The absence of off-target activity in the top-five *in silico* predicted loci in the Fanconi anemia patients' CD34⁺ cells edited with the ribonucleoprotein complex, suggests the high specificity of the sgRNA designed in this study and the safety of the proposed approach.

6

The *in vitro* delivery of the CRISPR/Cas9 system by serotype 6 adeno-associated viral vectors in healthy donor hematopoietic stem and progenitor cells results in efficacies of NHEJ-based gene editing comparable to those obtained by the ribonucleoprotein complex electroporation, opening the possibility of using these vectors for the future *in vivo* gene editing of Fanconi anemia patients' hematopoietic stem and progenitor cells.

Altogether, the results obtained in this doctoral thesis demonstrate for the first time that NHEJ-mediated gene editing can be used to correct the phenotype of hematopoietic stem and progenitor cells from Fanconi anemia patients, paving the way for the development of simple and efficient editing approaches for the treatment of monogenic disorders affecting hematopoietic stem cells, especially of diseases such as Fanconi anemia, where corrected cells display proliferative advantage.

La unión de extremos no homólogos (NHEJ, de sus siglas en inglés) es el mecanismo preferente de reparación de roturas de doble hebra en el ADN de células quiescentes, como las células madre hematopoyéticas, la principal diana para la terapia génica en la anemia de Fanconi. Además, se ha descrito que esta ruta se encuentra favorecida en células Fanconi. Aunque las inserciones y deleciones generadas por NHEJ se utilizan normalmente para inducir disrupción génica, proponemos que el NHEJ puede ser aprovechado de forma terapéutica para eliminar o compensar mutaciones asociadas a la anemia de Fanconi, emulando los eventos de reversión responsables de la mejora hematológica observada en los pacientes mosaico con anemia de Fanconi. En esta tesis doctoral, se ha evaluado la posibilidad de corregir mutaciones causantes de la anemia de Fanconi subtipo A mediante NHEJ inducida por el corte de nucleasas tipo CRISPR/Cas9. Las principales conclusiones obtenidas en este trabajo son las siguientes:

1

La reparación por NHEJ del corte inducido por nucleasas tipo CRISPR/Cas9 en líneas celulares linfoblastoides de pacientes Fanconi del subtipo A con distintas mutaciones en el gen *FANCA* puede generar la aparición de inserciones y/o deleciones terapéuticas.

2

Las líneas celulares linfoblastoides de pacientes Fanconi tipo A editadas que portan eventos de reparación terapéuticos generados por NHEJ muestran una ventaja proliferativa muy significativa *in vitro* y recuperan la expresión de una proteína FANCA funcional. Esta reexpresión da lugar a la corrección del fenotipo Fanconi, demostrado por la recuperación de la formación de focos de la proteína FANCD2, la reversión de la hipersensibilidad a mitomicina C y la reducción de la producción de especies reactivas de oxígeno.

3

La introducción por electroporación de las nucleasas CRISPR/Cas9 diseñadas en forma de complejo ribonucleoproteico en células $CD34^+$ sanas permite la edición de las células madre hematopoyéticas sin comprometer su capacidad de reconstitución. Además, a diferencia de la edición génica basada en recombinación homóloga, la modificación mediada por NHEJ permite editar con igual eficacia tanto células progenitoras más comprometidas como células madre hematopoyéticas con capacidad de reconstitución a largo plazo.

4

La edición génica de células $CD34^+$ de pacientes Fanconi del subtipo A mediada por NHEJ da lugar a su corrección fenotípica, demostrada por una significativa ventaja proliferativa tanto *in vitro* como *in vivo*, así como por la reversión de la hipersensibilidad a mitomicina C.

5

La ausencia de edición inespecífica en los 5 loci más probables en todas las muestras de células $CD34^+$ de pacientes Fanconi editadas con el complejo ribonucleoproteico sugiere la alta especificidad del ARN guía diseñado en este estudio, así como la seguridad de la estrategia propuesta.

6

La introducción *in vitro* del sistema CRISPR/Cas9 en células madre hematopoyéticas sanas por medio de un vector viral adenoasociado de serotipo 6 da lugar a eficacias de edición génica basada en NHEJ comparables a las obtenidas por electroporación del complejo ribonucleoproteico, ofreciendo la posibilidad de llevar a cabo en un futuro la edición génica *in vivo* de células madre hematopoyéticas de pacientes Fanconi a través de estos vectores.

En resumen, los resultados obtenidos en esta tesis doctoral demuestran por primera vez la posibilidad de corregir el fenotipo de células madre hematopoyéticas de pacientes Fanconi por medio de la edición génica basada en NHEJ, ofreciendo las bases para el desarrollo de estrategias de edición génica simples y eficaces que permitan el tratamiento de enfermedades monogénicas que afectan a las células madre hematopoyéticas, en especial en aquellos casos donde las células corregidas presentan ventaja proliferativa, tal y como ocurre en la anemia de Fanconi.

Retirado en la paz de estos desiertos,
con pocos, pero doctos libros juntos,
vivo en conversación con los difuntos,
y escucho con mis ojos a los muertos.
Si no siempre entendidos, siempre abiertos,
o enmiendan, o fecundan mis asuntos;
y en músicos callados contrapuntos
al sueño de la vida hablan despiertos.
— Francisco de Quevedo, Sonetos.

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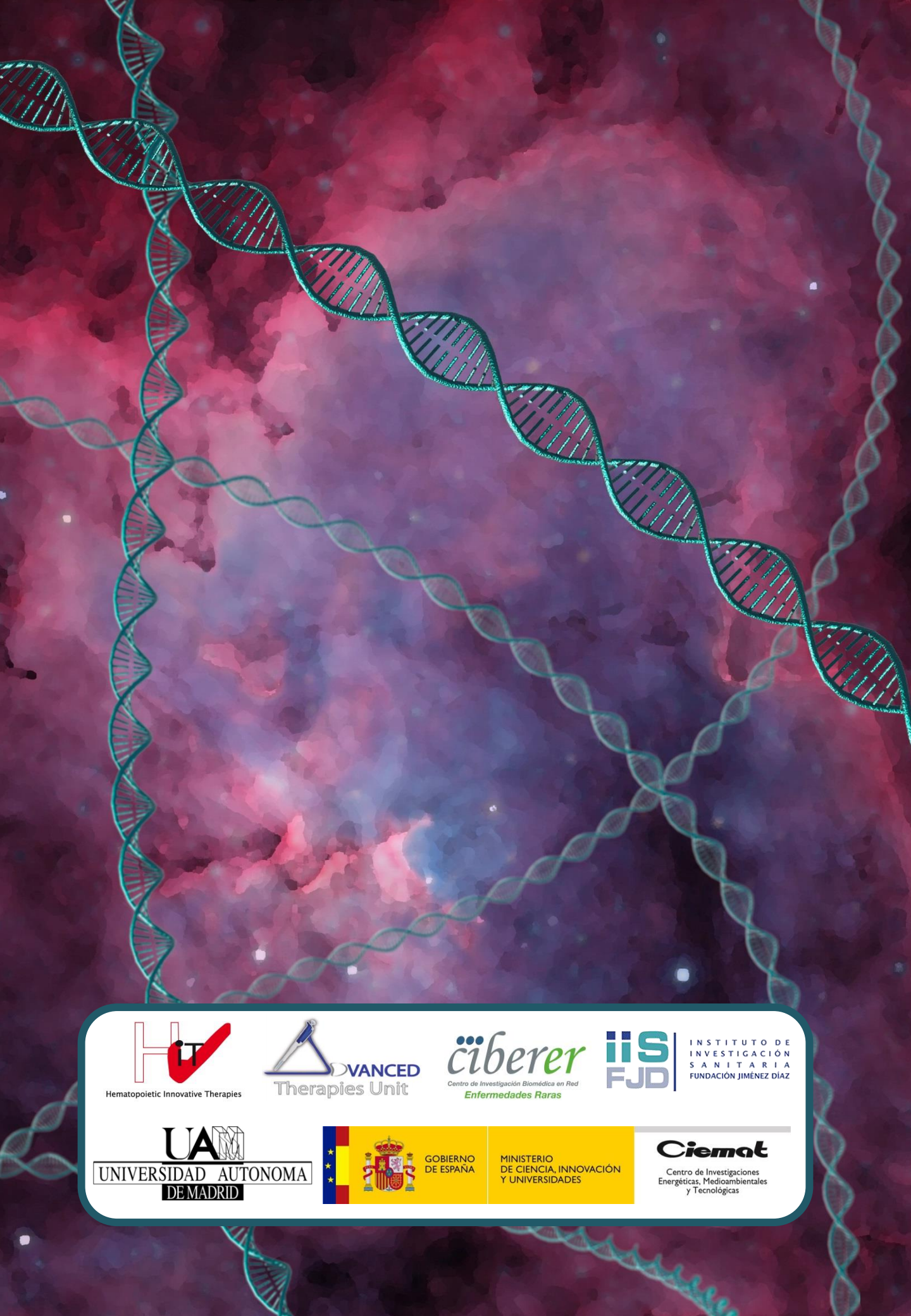
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