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TESIS DOCTORAL

Terapia génica y reprogramación celular en un modelo experimental de anemia de Fanconi con mutaciones en el gen *Fancd1-Brca2*

MEMORIA PARA OPTAR AL GRADO DE DOCTORA

PRESENTADA POR

Victoria Moleiro San Emeterio

Directores

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Memoria presentada por **VICTORIA MOLEIRO SAN EMETERIO** para optar al grado de Doctor con Mención Europea por la Universidad Complutense de Madrid.

Directores de Tesis:

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Dra. Susana Navarro Ordoñez, investigadora postdoctoral de la División de Terapias Innovadoras en el Sistema Hematopoyético del Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (CIEMAT) y **Dr. Juan Antonio Bueren Roncero**, Jefe de la División de Terapias Innovadoras en el Sistema Hematopoyético del Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (CIEMAT), certifican que la memoria adjunta titulada, "TERAPIA GÉNICA Y REPROGRAMACIÓN CELULAR EN UN MODELO EXPERIMENTAL DE ANEMIA DE FANCONI, CON MUTACIONES EN EL GEN *Fancd1/Brca2*" ha sido realizada por la licenciada Dña. Victoria Moleiro San Emeterio, bajo la dirección de los que suscriben, y cumple con las condiciones exigidas para optar al título de Doctor con Mención Europea por la Universidad Complutense de Madrid.

El presente trabajo de investigación ha sido realizado en la División de Terapias Innovadoras en el Sistema Hematopoyético del **CIEMAT**, el Centro de Investigaciones Biomédicas en Red de Enfermedades raras (**CIBERER**) del Instituto de Salud Carlos III y la Unidad Mixta de Terapias Avanzadas del CIEMAT y del Instituto de Investigación Sanitaria de la Fundación Jiménez Díaz.

3D	Three dimensional
DAPI	4',6-diamidino-2-phenylindole
8-oxo dG	8-hydroxy-2'-deoxyguanosine
ANC	Absolute neutrophil count
AML	Acute myeloid leukemia
ADA	Adenosine deaminase
ALD	Adrenoleukodystrophy
MAFs	Adult fibroblasts
AP	Alkaline phosphatase
AFP	Alpha feto protein
AF	Anemia de Fanconi
ATG*	Antithymocyte globulin
AGM	Aorta-gonada-mesonephro
A	Apoptosis
ATR	Ataxia telangiectasia and Rad3-related
ATM	Ataxia telangiectasia mutated
bp	base pairs
CFU-Ba	Basophil colony forming units
BM	Bone marrow
BMF	Bone marrow failure
BMP4	Bone morphogenetic protein 4
BRCA1	Breast cancer susceptibility 1
BRCA2	Breast cancer susceptibility 2
CA	Capsid
DNA-PKC	Catalytic subunit of DNA-PK
G0/G1 phase	Cell cycle phases after DNA synthesis
CDC42	Cell division control protein 42
CMH	Célula troncal hematopoyética
cGy	Centigray
CHK1	Checkpoint kinase 1
CHK2	Checkpoint kinase 2
CRISPR/CAS	Clustered Regularly Interspaced Short Palindromic Repeats
CAFC	Coblestone areas
CFCs	Colony forming cells
CLP	Common lymphoid progenitors
CGH	Comparative Genomic Hybridization
aCGH	Comparative Genomic Hybridization array
cDNA	Complementary DNA
c*Brca2*$^{\Delta 27/\Delta 27}$	Corrected *Brca2*$^{\Delta 27/\Delta 27}$
c*Fanca*$^{-/-}$	Corrected *Fanca*$^{-/-}$
Ct	Cycle threshold
CY	Cyclophosphamide
CK	Cytokines
C	Cytosine

Brca2$^{\Delta 27/\Delta 27}$	Deletion of exon 27, last exon of *Brca2* gene
DNA	Deoxyribonucleic acid
DEB	Diepoxybutane
DMSO	Dimethyl sulfoxide
CpG	DNA region with high C+G %
DSB	Double strand breaks
dsDNA	Double stranded DNA
EA	Early apoptosis
ELP	Early lymphoid progenitors
EBs	Embryoid bodies
ESC	Embryonic stem cell
ψ	Encapsidation signal
eGFP	Enhanced green fluorescent protein
CFU-Eo	Eosinophil colony forming units
CFU-E	Erythroid colony forming units
Epo	Erythropoietin
EU	European Community
FA	Fanconi anemia
FAN1	Fanconi-associated nuclease 1
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FISH	Fluorescence *in situ* Hybridization
FACS	Fluorescence-activated cell sorting
FOXO3A	Forkhead box O3
γ-RV	Gamma-retroviral vector
gDNA	genomic DNA
GVHD	Graft Versus Host Disease
GMLP	Granulocyte-monocyte lymphoid progenitors
CFU-GM	Granulo-macrophagic colony forming units
Gy	Grays
HGb	Haemoglobin
HSCT	Hematopoietic stem cell transplantation
HSC	Hematopoietic stem cells
H&E	Hematoxilin and eosin
ID complex	Heterodimer FANCD2-FANCI
MHF1	Histone Fold Protein 1
MHF2	Histone Fold Protein 2
MHF1	Histone-fold-containing protein complex 1
MHF2	Histone-fold-containing protein complex 2
HOXB4	Homeobox protein Hox-B4
HR	Homologous recombination
HDR	Homology directed repair
hBRCA2	Human Breast cancer susceptibility 2 protein
EF1α	Human elongation factor-1 alpha

hESC	Human ESC
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HLA	Human leukocyte antigen
iPSCs	Induced pluripotent stem cells
ICM	Inner cell mass
IN	Integrase
IDLV	Integration defective lentiviral vectors
Interference mRNAs	Interference messenger RNA
IFNy	Interferon gamma
IL-1b	Interleukin 1b
IL-6	Interleukin 6
IL-2R_	Interleukin-2R_
IL-3	Interleukin-3
IRES	Internal ribosome entry site
IFAR	International Fanconi Anemia Registry
ICL	Interstrand crosslinks
irr MEFs	Irradiated feeder cells
kb	kilobases
Klf4	Kruppel-like factor 4
LA	Late apoptosis
LV	Lentiviral
LIF	Leukemia inhibitory factor
LHX2 gene	LIM homeobox protein Lhx2
LSK	Lin-, sca1+, ckit+
Lin^-	Lineage negative
LTR	Long terminal repeats
LTC-IC	Long-term culture initiating cells
LT-HSCs	Long-term hematopoietic stem cells
CFU-Mast	Mastocyte colony forming units
MA	Matrix
CFU-Meg	Megacariocytic colony forming units
LDM	Metachromatic leukodystrophy
ATG	Methionine; translation initiation codon
MC	Methylcellulose
MAPK	Mitogen-Activated-Protein-Kinase
MMC	Mitomycin C
M	Mitotic cell cycle phase
MAFs	Mouse adult fibroblasts
MEFs	Mouse embryonic fibroblasts
mESC	Mouse embryonic stem cell
mES medium	Mouse embryonic stem cell medium
mFGF	Mouse fibroblast growth factor
mGapdh	Mouse Glyceraldehyde-3-phosphate dehydrogenase

LIF	Mouse Leukemia Inhibitory Factor
MRN	Mre11-Rad50-Nbs1 complex
MOI	Multiplicity of infection
MAPC	Multipotent adult progenitor cells
MPP	Multipotent progenitors
MLV	Murine leukemia virus
MSCV	Murine Stem Cell Virus
c-Myc	Myelocytomatosis oncogene T58A mutant
MDS	Myelodysplastic syndrome
NK	Natural killer
N	Necrosis
NBS	Nijmegen Breakage Syndrome
NSG	NOD.Cg-Prkdcscid IL2rgtm1Wjl mice
NHEJ	Non homologous end joining
NEAA	Non-essential aminoacids
FANCD2-S	Non-ubiquitinated form of FANCD2
ns	Not significant
NFKβ	Nuclear factor kappa-light-chain-enhancer of activated B cells
NC	Nucleo-capsid
NER	Nucleotide excision repair
OKSM	Oct3/4, Klf4, Sox2 and c-Myc
P/S	Penicillin-stremtomycin
PBS	Peripheral blood
P	Phosphate
PBS	Phosphate buffered saline
PBST	Phosphate-buffered Saline with Tween 20
PS	Phosphatidilserine
PGK	Phosphoglycerate kinase
γ-H2AX	Phosphorylated H2AX
PE	Phycoerythrin
PLT	Platelets
PEI	Polyethylenimine
PCR	Polymerase Chain Reaction
Oct3/4	POU domain, class 5, transcription factor 1
G2-phase	Pre-mitotic cell cycle phase
ROS	Reactive oxygen species
RT-qPCR	Real-Time Quantitative PCR
rhG-CSF	Rec.human granulocyte colony-stimulating factor
rhGM-CSF	Rec.human granulocyte-macrophage colony-stimulating factor
RFP	Red fluorescent protein
g	Relative centrifugal force
ORI	Replication origin
RT	Reverse transcriptase
RNA	Ribonucleic acid

SSC	Saline-sodium citrate buffer
F2A	Self-cleaving 2A peptide
SIN	Self-inactivating
SCID	Severe combined immunodeficiency
ST-HSCs	Short-term hematopoietic stem cells
SLAM	Signaling lymphocyte activation molecule
SSA	Single strand annealing
ssDNA	Single stranded DNA
SDS	Sodium dodecyl sulfate
SB	Southern Blot
SFFV	Spleen focus-forming virus
SCC	Squamous cell carcinoma
SSEA1	Stage-specific embryonic antigen-1
c-kit	Stem Cell Factor receptor
SDF-1	Stromal cell-derived factor 1α
S-phase	Synthesis cell cycle phase
TAI	Thoracoabdominal radiation
TAR	Thrombocytopenia Absent Radius
T	Thymine
TALE	Transcription activator-like effector
TSS	Transcription starting site
TG	Transgenes
TLS	Translesion synthesis
TNFα	Tumor necrosis factor alpha
FANCD2-L	Ubiquitinated form of FANCD2
UV	Ultra-violet radiation
UCB	Umbilical cord blood
U	Uracile
VEGF	Vascular endothelial growth factor
VCN	Vector copy number
VSV-G	Vesicular Stomatitis Virus´G-protein
WB	Western Blot
WBC	White blood cells
Wt	Wild type
WAS	Wiskott-Aldrich syndrome
Wpre	Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element
XPC	Xeroderma pigmentosum group C
XPF	Xeroderma pigmentosum group F
X-CGD	X-linked Chronic Granulomatosus Disease
X-SCID	X-linked Severe Combined Immunodeficiency

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INTRODUCCIÓN:

La anemia de Fanconi (AF) es una enfermedad compleja y muy heterogénea, que se describió por primera vez en 1927. La característica principal de esta enfermedad es el fallo de médula ósea. La AF se caracteriza también por anomalías en el desarrollo y por una alta predisposición a la aparición de tumores hematológicos, principalmente leucemia mieloide crónica, y también sólidos, particularmente carcinoma escamoso de cabeza y cuello y ano-genital (Howlett et al. (2005); (Kee and D'Andrea, 2012).

La AF está causada por mutaciones en cualquiera de los 16 genes AF descritos hasta la fecha, (*FANCA, B, C, D1/BRCA2, D2, E, F, G, I/BRIP1, J, L, M, N/PALB2, O/RAD51C, P/SLX4 y Q/XPF*), los cuales siguen un patrón de herencia autosómica recesiva, a excepción del gen *FANCB*, cuya herencia está ligada al sexo. Estos genes participan en la ruta AF/BRCA, la cual se divide en tres complejos: el complejo formado por las proteínas del "core" de AF; el complejo ID; y un tercer complejo que actúa por debajo de los dos complejos anteriores. Esta ruta está implicada en la regulación de la reparación del ADN, y es esencial para la reparación de los entrecruzamientos en las cadenas del DNA producidos por agentes entrecruzantes (Adam et al., 2003; Fanconi anaemia/Breast cancer, 1996; Kee and D'Andrea, 2010; Meetei et al., 2004; Meetei et al., 2005; Smogorzewska et al., 2007; Strathdee et al., 1992).

La severidad de la enfermedad es variable en función del gen AF mutado, así como también del tipo de mutación y del propio paciente. El gen que es responsable de la enfermedad en la mayor parte de los pacientes AF es *FANCA*, que participa en el primer complejo de la ruta. Mutaciones en genes como *FANCD1/BRCA2*, localizado por debajo de los dos primeros complejos de la ruta, implican una mayor severidad de la enfermedad, muy probablemente porque la proteína BRCA2 está implicada en la reparación del ADN mediante recombinación homóloga directa (HDR).

A nivel celular, las células afectadas por AF presentan un fenotipo característico. Así, estas células son hipersensibles a una variedad de agentes endógenos (aldehídos, oxígeno,

citoquinas inflamatorias), y también exógenos (agentes entrecruzantes del ADN, radiaciones ionizantes…). Existe controversia sobre si este fenotipo es consecuencia de la inestabilidad genómica de las células AF, de deficiencias en el sistema de reparación del ADN o de desequilibrios del metabolismo del oxígeno, aunque probablemente sea una combinación de todos estos mecanismos lo que determina el fenotipo de AF (Pagano et al., 2013); (Pallardo et al., 2010).

En la actualidad, el único tratamiento curativo para el fallo de médula ósea de pacientes AF, es el trasplante de células madre hematopoyéticas a partir de un donante compatible sano o portador de la enfermedad. En el caso de los pacientes AF, la complejidad de estos trasplantes es mayor a la que tiene lugar en otro tipo de pacientes, debido a la hipersensibilidad de las células y tejidos de los pacientes AF a los agentes utilizados para su acondicionamiento pretrasplante. Una opción terapéutica para pacientes con AF que no dispongan de un donante adecuado, sería la terapia génica (Rio et al., 2008). La principal población celular afectada en esta enfermedad es la de las células madre hematopoyéticas (CMHs). Por tanto, la introducción de una copia correcta del gen AF defectivo en las CMHs del paciente daría a estas células una ventaja proliferativa sobre las CMHs no transducidas. De este modo, la transducción de las CMHs del paciente con el vector terapéutico podría dar lugar a CMHs libres de enfermedad, y finalmente a células hematopoyéticas diferenciadas que repoblarían el sistema hematopoyético del paciente, resolviendo por tanto, el fallo de médula ósea.

El desarrollo de estrategias de terapia génica requiere cierto número de CMHs de partida para ser transducidas con el vector terapéutico. Se contempla que ésta será una de las limitaciones para que la terapia génica convencional pueda ser utilizada en un elevado número de pacientes con AF. Ello deriva de la observación de que una vez desarrollado el fallo de médula ósea, el número de CMHs presentes en la médula ósea es muy reducido, y en general de mala calidad.

Como alternativa a la utilización de CMHs para la terapia génica de la AF, en el año 2006 se abrió un amplio horizonte de oportunidades gracias al desarrollo de la tecnología de reprogramación celular. Con ello era posible obtener células con pluripotencia inducida

(iPSCs) a partir de células adultas diferenciadas mediante la transducción de unos pocos genes (Yamanaka and Takahashi, 2006). Esta tecnología constituye una fuente de células muy prometedora en enfermedades que cursan con fallo de médula ósea, puesto que facilitaría la generación de un elevado número de CMHs autólogas corregidas genéticamente, que se podrían utilizar para la reinfusión del paciente. En este sentido, en un trabajo previo desarrollado por nuestro laboratorio, en colaboración con el Centro de Medicina Regenerativa de Barcelona y la Universidad Autónoma de Barcelona, se demostró la posibilidad de obtener *in vitro* progenitores hematopoyéticos libres de enfermedad procedentes fibroblastos reprogramados de la piel de pacientes con mutaciones en los genes *FANCA* y *FANCD2* (Raya et al., 2009).

A diferencia de lo que ocurre con estos genes, *BRCA2 es* uno de los genes AF localizados aguas abajo en la ruta AF/BRCA, y cuya deficiencia da lugar a un fenotipo más severo de la enfermedad. En el año 2000 se generó un modelo de ratón con mutaciones hipomórficas en el gen *Brca2/Fancd1*. Este modelo, caracterizado en nuestro laboratorio (Navarro et al., 2006), es uno de los que mejor reproduce el fenotipo hematopoyético característico de la FA, y ha constituido el modelo experimental con el que hemos desarrollado una gran parte del trabajo que se presenta.

JUSTIFICACIÓN Y OBJETIVOS:

Puesto que hasta la realización de nuestro trabajo se desconocía cuál era la relevancia del gen *Brca2* en reprogramación celular, el primer objetivo de esta Tesis fue investigar comparativamente la relevancia de dicho gen, frente a *Fanca,* en el proceso de reprogramación celular. Para ello utilizamos células adultas y embrionarias procedentes de los modelos de ratón *Brca2*$^{\Delta 27/\Delta 27}$ y *Fanca*$^{-/-}$. En segundo lugar nos propusimos desarrollar un protocolo combinado de terapia génica y reprogramación celular para la generación de iPSCs corregidas genéticamente, las cuales serían sometidas a un proceso de diferenciación hematopoyética, con objeto de generar una fuente abundante de CMHs libres de la enfermedad. Por último nos propusimos explorar la posibilidad de utilizar estas células diferenciadas para corregir los defectos hematopoyéticos que caracterizan a

los ratones *Brca2*$^{\Delta 27/\Delta 27}$ mediante trasplante de las mismas en ratones afectados por la enfermedad.

RESUMEN DE RESULTADOS:

La generación de iPSCs mediante la transducción de células *Fanca*$^{-/-}$ y *Brca2*$^{\Delta 27/\Delta 27}$ con vectores gamma retrovirales portadores de cuatro factores de reprogramación mostró que los genes *Fanca* y *Brca2* juegan un papel importante en el proceso de reprogramación de fibroblastos adultos. Sin embargo, *Brca2* mostró un papel mucho más crítico que *Fanca* en tal proceso, puesto que la deficiencia de *Brca2* no permitió la activación de los genes endógenos de pluripotencia, ni por tanto, la obtención de células con características de iPSCs.

La corrección del defecto genético en fibroblastos embrionarios de ratones *Brca2*$^{\Delta 27/\Delta 27}$, seguido de su transducción con un vector lentiviral de reprogramación policistrónico y escindible permitió la reprogramación eficaz de células sin mutaciones en genes AF o de células con mutaciones en el gen *Fanca*, pero no de células *Brca2*$^{\Delta 27/\Delta 27}$.

El análisis de formación de focos de reparación de la proteína RAD51 después de la transducción con el vector de reprogramación de células sin mutaciones en genes AF o con mutaciones en el gen *Fanca*, demostró la activación del proceso de HDR durante el proceso de reprogramación celular. Sin embargo, en células *Brca2*$^{\Delta 27/\Delta 27}$ la formación de focos de RAD51 no se vio aumentada durante la reprogramación celular. Estos resultados explican el papel crítico que juega tanto la proteína BRCA2 como el proceso de HDR durante la reprogramación celular.

Puesto que durante la reprogramación de células *Brca2*$^{\Delta 27/\Delta 27}$ se produjo un incremento en la apoptosis temprana y tardía de estas células, proponemos que la inducción de tales efectos serían responsables, al menos en parte, de la muy baja eficiencia de reprogramación que caracteriza a estas células.

Las caracterización molecular y funcional de iPSCs procedentes de fibroblastos embrionarios de genotipo *Brca2*$^{\Delta 27/\Delta 27}$ corregidos genéticamente permitió observar que estas células cumplían la mayor parte de los criterios para ser considerados "*bona fide*-

iPSCs", aunque no fueron capaces de generar teratomas tras su implantación subcutánea en ratones inmunodeficientes. La escisión completa del casete de reprogramación permitió, sin embargo, la generación de teratomas con células diferenciadas a las tres capas embrionarias y en consecuencia la obtención de células iPSC completamente reprogramadas *("bona fide*-iPSCs*")*. Las iPSCs de genotipo *Brca2*$^{\Delta 27/\Delta 27}$ corregidas genéticamente expresaban el ARNm de *BRCA2*, eran capaces de traslocar la proteína RAD51 a los *foci* de reparación del ADN y además eran resistentes a concentraciones de mitomicina C que resultaban altamente tóxicas en células AF.

La caracterización de la ploidía y cariotipo de estas iPSCs mostró que a pase 17, únicamente dos clones mantenían un contenido de ADN diploide y un cariotipo normal. Tras la escisión del vector de reprogramación, un subclón que fue seleccionado por mantener un cariotipo normal también mostró inestabilidad genética a la vista de las alteraciones observadas en estudios de hibridación genómica comparada. El origen de estas anomalías no pudo ser determinado, ya que pudo deberse tanto al proceso de reprogramación, como al mantenimiento en cultivo durante largos periodos o al proceso de escisión del provirus reprogramador.

Finalmente, la diferenciación hematopoyética de iPSCs de genotipo *Brca2*$^{\Delta 27/\Delta 27}$ corregidas genéticamente mediante diferentes aproximaciones experimentales descritas en la literatura, permitió la producción de células que expresaban marcadores característicos de células hematopoyéticas. No obstante, esta diferenciación resultó modesta, y el trasplante de estas células en ratones *Brca2*$^{\Delta 27/\Delta 27}$ no consiguió reconstituir su sistema hematopoyético.

CONCLUSIONES DERIVADAS DE ESTE TRABAJO:

A la vista del trabajo realizado podemos decir que la ruta FA/BRCA juega un papel importante en el proceso de reprogramación celular, siendo BRCA2 una proteína crítica para la generación de células con pluripotencia inducida. Nuestros resultados también demostraron que durante la reprogramación celular se produce la activación de

reparación la del ADN mediante recombinación homóloga directa, lo que se midió mediante el reclutamiento de la proteína RAD51 a los *foci* de reparación del ADN en respuesta a la reprogramación.

El incremento en la apoptosis detectado durante los primeros estadios de la reprogramación celular podría ser responsable de la limitada reprogramación en células *Brca2*$^{\Delta 27/\Delta 27}$. La complementación génica de las células *Brca2*$^{\Delta 27/\Delta 27}$ con el vector portador del gen terapéutico, restauró la capacidad de éstas para reclutar la proteína RAD51 a los *foci* y permitió la reprogramación de estas células.

La transducción de fibroblastos *Brca2*$^{\Delta 27/\Delta 27}$ corregidos genéticamente con cuatro vectores retrovirales portadores de los genes de reprogramación no permitió la generación de iPSCs que cumplieran todos los criterios de reprogramación. Sin embargo, la utilización de un vector lentiviral de reprogramación policistrónico, seguido de la completa escisión del provirus reprogramador permitió generar iPSCs *bona fide* sin fenotipo FA.

Finalmente, los protocolos de diferenciación hematopoyética *in vitro* basados en la transducción con HOXB4, seguido de la generación de cuerpos embrionarios y co-cultivo sobre estromas hematopoyéticos permitió una diferenciación modesta de iPSC c*Brca2*$^{\Delta 27/\Delta 27}$ hacia el sistema hematopoyético. Estas células no fueron capaces de injertar en ratones *Brca2*$^{\Delta 27/\Delta 27}$ irradiados, lo que indica una generación deficiente de células hematopoyéticas repobladoras en estos cultivos.

INTRODUCTION:

Fanconi anemia (FA) is a complex and heterogeneous disease that was described for the first time in 1927. It is characterized by developmental abnormalities, cancer predisposition and bone marrow failure (BMF), being the BMF the most frequent cause of death of these patients.

FA is caused by mutations in any of the 16 FA genes described so far (*FANC-A, B, C, D1/BRCA2, D2, E, F, G, I/BRIP1, J, L, M, N/PALB2, O/RAD51C, P/SLX4, Q/XPF)*. All of them except *FANCB* follow an autosomal recessive inheritance (Adam et al., 2003; Fanconi anaemia/Breast cancer, 1996; Meetei et al., 2004; Meetei et al., 2005; Smogorzewska et al., 2007; Strathdee et al., 1992). These genes participate in the FA/BRCA pathway, that regulates several DNA repair pathways and is essential to resolve DNA interstrand-crosslinks (ICLs) produced by ICL agents (Kee and D'Andrea, 2010). This pathway is composed of three different complexes, the “core complex”, the “I-D complex” and “downstream proteins”.

The severity of the disease varies depending on the mutation. *FANCA* is the most frequently mutated FA gene, and participates in the first complex of the pathway. Mutations in genes such as *FANCD1/BRCA2*, localized in the third complex, result in a stronger severity of the disease, most probably because BRCA2 directly participates in the homology directed DNA repair (HDR).

At a cellular level, FA cells have a characteristic response to certain endogenous (aldehydes, oxygen, inflammatory cytokines) and/or exogenous (ICL-agents, ionizing radiation…) agents. There is controversy about what causes the FA phenotype. While some authors support the idea that the phenotype of these cells is consequence of a defective ICL-DNA damage repair system that leads to the accumulation of genomic instability and damaged DNA, others relate the FA phenotype to an increased oxidative stress and imbalances in the oxygen metabolism. It is not clear yet what causes the FA

phenotype but most probably a combination of both mechanisms will be responsible for it.

There are several palliative treatments for the hematopoietic affectation of FA patients, mainly based on the use of androgens and growth factors. The only current curative treatment for the BMF of FA patients is the hematopoietic stem cell transplantation (HSCT) from a compatible donor. This approach is limited to FA patients with an HLA-compatible donor and has particular difficulties compared to transplants in other patients due to sensitivity of FA cells to conditioning regimes used for HSCT.

Gene therapy is one of the most promising new therapeutic approaches to overcome the BMF in FA in patients without an HLA-identical donor (Rio et al., 2008). The insertion of a correct copy of the defective gene in the affected cells (HSCs) by gene therapy would give them a proliferative advantage over the defective cells. Thus, the transduction of a small number of HSCs with the therapeutic vector could give rise to disease-free HSCs, and thus to the whole progeny of differentiated cells that would restore the hematopoietic system (Qasim et al., 2007). The development of gene therapy strategies requires a minimum number of cells to be transduced with the therapeutic vector. This is one of the handicaps of conventional FA gene therapy, because once the BMF has been developed, the number and quality of HSCs in the bone marrow is markedly reduced. For this reason, the possibility of having an unlimited source of HSCs to perform gene therapy in BMF diseases would be of marked relevance.
In 2006, a new horizon to the field of gene cell therapy opened was opened thanks to the development of the cell reprogramming technology (Yamanaka and Takahashi, 2006). This technology would allow the generation of high numbers of gene-corrected autologous iPSC-derived HSCs to infuse them into patients suffering from BMF syndromes.

In this respect, a previous work carried out by our laboratory, in collaboration with the Centre of Regenerative Medicine in Barcelona and with the Autonomous University of Bacelona, demonstrated the possibility of generating disease-free hematopoietic

progenitors from reprogrammed skin fibroblasts obtained from patients with mutations in *FANCA* and *FANCD2* (Raya et al., 2009).

In contrast to proteins encoded by these genes, BRCA2 is localized downstream in the FA/BRCA pathway, and its deficiency gives rise to a disease with a more severe phenotype compared to patients with mutations in other FA genes. In 2002 (McAllister et al., 2002a), K. McAllister developed a mouse model with hypomorphic mutations in *Brca2/Fanc1* gene. This model was characterized in our laboratory (Navarro et al., 2006) and is one of the models that best reproduces the hematopoietic phenotype characteristic of FA. This experimental model (*Brca2*$^{\Delta 27/\Delta 27}$) has been used along the present work.

OBJECTIVES:

Until our work was developed, the relevance of *Brca2* in cell reprogramming was unknown. For this reason, the first objective of this thesis was to investigate the relevance of *Brca2*, compared with *Fanca,* in the cell reprogramming process required to generate iPSCs. For this purpose, cells from *Fanca*$^{-/-}$ and *Brca2*$^{\Delta 27/\Delta 27}$ mouse models were used. Our next purpose consisted on the development of a combined gene therapy and cellular reprogramming protocol that would result in the generation of gene-corrected *Brca2*$^{\Delta 27/\Delta 27}$ iPSCs. These cells would be then differentiated towards the hematopoietic system aiming to generate large numbers of disease-free HSCs. Lastly, we explored the possibility of using these cells to correct the hematopoietic defects that characterize *Brca2*$^{\Delta 27/\Delta 27}$ mice.

RESULTS:

The reprogramming of uncorrected and gene-corrected *Fanca*$^{-/-}$ and *Brca2*$^{\Delta 27/\Delta 27}$ fibroblasts gamma-retroviral vectors individually harbouring the *Oct-4, Sox2, Klf-4* and *c-myc* showed that *Fanca* and *Brca2* genes play important roles in cell reprogramming. *Brca2* was, however, markedly more critical in cell reprogramming as no activation of the endogenous pluripotency genes could be reproducibly detected in *Brca2*$^{\Delta 27/\Delta 27}$cells.

Additionally, our data showed that reprogramming by this method of gene-corrected *Brca2*$^{\Delta 27/\Delta 27}$ fibroblasts did not generate "bona fide" iPSCs.

On the other hand, reprogramming of gene-corrected *Brca2*$^{\Delta 27/\Delta 27}$ fibroblasts with a polycistronic lentiviral vector, significantly increased the reprogramming efficiency compared with the results obtained with individual gamma-retroviral vectors.

The analysis of RAD51 foci formation in wild-type cells and also in *Fanca*$^{-/-}$ cells during the cell reprogramming process demonstrated the activation of homology directed repair (HRD) in both cell types. However, this mechanism was not activated during reprogramming of *Brca2*$^{\Delta 27/\Delta 27}$ cells. Additionally, apoptosis studies in *Brca2*$^{\Delta 27/\Delta 27}$ cells revealed an increase in early and late apoptosis in these cells during the reprogramming process, accounting for the extremely low efficiency of reprogramming of these cells.

Gene-corrected c*Brca2*$^{\Delta 27/\Delta 27}$ iPSCs fulfilled most of the criteria to be *"bona fide"* iPSCs, but they were not able to form teratomas. The excision of the reprogramming cassette, facilitated however, the generation of teratomas and, in consequence the generation of "bona fide" iPSCs. These corrected *Brca2*$^{\Delta 27/\Delta 27}$ iPSCs expressed *hBRCA2* mRNA, were capable of translocating RAD51 to DNA repair *foci,* and were resistant to MMC concentrations that resulted toxic in FA cells.

The characterization of ploidy and karyotype of corrected c*Brca2*$^{\Delta 27/\Delta 27}$ iPSCs revealed that by passage 17 only two clones had a diploid DNA content and normal karyotype. After the excision of the reprogramming vector, only one sub-clone conserved a normal karyotype, but it also showed genomic instability as deduced from comparative genomic hybridization studies.

Finally, the hematopoietic differentiation of corrected c*Brca2*$^{\Delta 27/\Delta 27}$ iPSCs allowed the production of cells that expressed markers characteristic of hematopoietic system. This hematopoietic differentiation was, however, very modest, and the transplantation of the iPSC-derived cells did not engraft into *Brca2*$^{\Delta 27/\Delta 27}$ mice.

CONCLUSIONS:

The FANCA/BRCA pathway plays an important role in the process of cell reprogramming, being *Brca2* critical for the generation of iPSCs. Furthermore, the process of cell reprogramming activates the homology directed DNA repair, measured by the recruitment of RAD51 to DNA repair *foci*, in response to cell reprogramming. It was observed that increased apoptosis during the first stages of cell reprogramming could account for the limited reprogramming of *Brca2*$^{\Delta 27/\Delta 27}$cells.

The complementation of *Brca2*$^{\Delta 27/\Delta 27}$ cells restored the capacity of these cells to recruit RAD51 to DNA repair *foci* and allowed the reprogramming of these cells. The transduction of genetically-corrected *Brca2*$^{\Delta 27/\Delta 27}$ fibroblasts with four gamma-retroviral vectors was unable to generate *bona fide* iPSCs. On the other hand, the reprogramming of genetically corrected *Brca2*$^{\Delta 27/\Delta 27}$ firboblasts with a polycistronic lentiviral reprogramming vector restored the FA/BRCA pathway and generated *bona fide* iPSCs after the complete excision of the reprogramming cassette.

In vitro hematopoietic differentiation protocols based on iPSC transduction with *HOXB4*, followed by embryo body generation and co-cultures over hematopoietic stromas, modestly differentiated c*Brca2*$^{\Delta 27/\Delta 27}$ iPSCs towards the hematopoietic system and these cells were not able to engraft into irradiated *Brca2*$^{\Delta 27/\Delta 27}$ mice, indicating a deficient generation of hematopoietic repopulating cells in these cultures.

Taken together, the results presented in this manuscript show the critical role of BRCA2 in cell reprogramming and reveal the current advances and limitations of cell reprogramming approaches to be used in hematopoietic cell therapy.

A. FANCONI ANEMIA

1. GENERAL CHARACTERISTICS OF FANCONI ANEMIA

Fanconi anemia (FA) was first described in 1927 by a Swiss pediatrician named Guido Fanconi. It is a highly heterogeneous genomic instability syndrome characterized by developmental abnormalities, bone marrow failure (BMF) and cancer predisposition. BMF appears in virtually all Fanconi anemia patients and in about 50% of these patients it appears during the first decade of life.

FA patients are particularly sensitive to suffer from myelodysplastic syndrome (MDS), acute myeloid leukemia (AML) and squamous cell carcinoma (SCC) in head, neck and oral cavities due to the genomic instability of the cells (Howlett et al. (2005). It has recently been reported that specific chromosomal abnormalities are frequently observed in FA patients with MDS or AML (e.g., gain of 1q23-32, 3q26), suggesting that these abnormalities can be used as predictive markers (Kee and D'Andrea, 2012).

FA is caused by mutations in any of the 16 genes described so far (*FANC-A, B, C, D1/BRCA2, D2, E, F, G, I/BRIP1, J, L, M, N/PALB2, O/RAD51C, P/SLX4, Q/XPF*), from which 16 complementation groups have been identified (Table 1). All of them follow an autosomal recessive inheritance except *FANCB*, whose inheritance is linked to the X chromosome (Adam et al., 2003; Fanconi anaemia/Breast cancer, 1996; Meetei et al., 2004; Meetei et al., 2005; Smogorzewska et al., 2007; Strathdee et al., 1992). The genetic products of these genes function in a pathway known as the FA/BRCA pathway, a common DNA repair pathway that cooperates with other DNA repair proteins to resolve DNA interstrand-crosslinks (ICLs) during replication (Kee and D'Andrea, 2010).

At a cellular level, FA affected cells present a high percentage of chromosomal aberrations and hypersensitivity to DNA interstrand-crosslinking agents that can be caused by endogenous sources, such as aldehydes generated as products of cellular metabolism or exogenous agents, such as cysplatin, mitomycin C (MMC), diepoxybutane (DEB), etc. ICLs are the most deleterious

DNA lesions that block DNA replication and transcription (Howlett et al., 2005; Kee and D'Andrea, 2010).

FANCONI ANEMIA GENES					
COMPLEX	GENE	LOCUS	PROTEIN FUNCTION		PROTEIN COMPLEX FUNCTION
Core Complex	*FANCA*	16q24.3	Necessary for the stability of the core complex and the mono-ubiquitination of *FANCD2/FANCI*	Scaffold for FA core complex	ICL repair
	FANCB	Xp22.31		Scaffold for FA core complex	
	FANCC	9q22.3		Scaffold for FA core complex	
	FANCE	6p21.3		Scaffold for FA core complex	
	FANCF	11p15		Scaffold for FA core complex	
	FANCG	9p13		Scaffold for FA core complex	
	FANCL	2p16.1		E3 Ubiquitin ligase	
	FANCM	14q21.3		Scaffold for FA core complex Branch migration/ATPase act.	
ID Complex	*FANCD2*	P25.3	Binds DNA structures		
	FANCI	15q25-26	Participates in the mono-ubiquitination of *FANCD2*		
Downstream proteins	*FANCJ/ BRIP1*	17q22.3	Homologous recombination/activation FA pathway/5´-3´helicase		
	FAND1/BRCA2	13q12.3	Homologous recombination/ loading of RAD51 onto the DNA		
	FANCN/PALB2	16p12.1	Homologous recombination/ promotion of *BRCA2* function		
	FANCO/RAD51C	17q22	Homologous recombination/Interacts with RAD51 and paralogs		
	FANCP/SLX4	16p13.3	Coordination of XPF-ERCC1, MUS81-EME1 and SLX1 NUCLEASES		
	FANCQ/XPF	16p24.3	Homologous recombination/ ICL repair		

Table 1. Fanconi anemia genes. The table shows the Fanconi anemia genes and corresponding proteins, with their position in the FA pathway. The core complex is the one located at the beginning of the pathway. This complex activates the ID-complex. The ID-complex is composed by two genes, *FANCI* and *FANCD2* and when ubiquitinated it becomes active. Downstream proteins form the third complex have an essential role in DNA repair by homology directed repair (HDR).

2. FANCONI ANEMIA/BRCA PATHWAY

As previously commented, FA proteins function in the FA/BRCA pathway, a complex pathway that regulates the repair of DNA interstrand-crosslinks (ICLs). The proteins that form this pathway are classified in three groups depending on their localization; the “core complex”, the “ID-Complex” or “downstream proteins”.

2.1 PATHWAYS INVOLVED IN DNA DOUBLE STRAND BREAKS REPAIR AND DNA INTERSTRAND-CROSSLINKS (ICLs)

When there is a lesion in the DNA or under conditions of replicative stress, the replication fork stalls after finding a defective template and starts the replication mechanism dependent on homology directed repair (HDR) that has to be extremely well coordinated to maintain the

integrity of the genome (Howlett et al., 2005). The ability to sense and respond to these events is critical for cell survival (Smogorzewska et al., 2007).

Non homologous end joining (NHEJ) and HDR are the two major mechanisms by which double strand breaks (DSB) are repaired in mammalian cells (Xia et al., 2001). When there is a DSB during the S-phase of the cell cycle, NHEJ and HDR DNA repair pathways compete to overcome the damage. In contrast, when the DSB takes place in other phases of the cell cycle, the system tends to repair the DSB by NHEJ (Figure 1).

The ICL damage leads to a DSB in one sister chromatid of the DNA whose repair requires the coordination of multiple DNA repair processes needed to cut out the ICL. The only error-free mechanism by which DSB derived from an ICL can be repaired is HDR. It goes through previous steps that involve nucleotide excision repair (NER) and translesion synthesis (TLS) ending up in the HDR process that is the one that finally resolves the DSB (Figure 2).

- Nonhomologous end joining -NHEJ-

This is an efficient and rapid DSB repair process that does not require a homologous template. It is the preferred pathway in mammals in most of the phases of the cell cycle except in the S phase when it competes with other DNA repair mechanisms. NHEJ is error-prone, because it works ligating two broken DSB ends without a template and often the sequence changes at the site where the DNA broke (Kee and D'Andrea, 2012). This mechanism starts by the high affinity binding of the heterodimer KU70/KU80 to the ends of the broken DNA to stabilize it. Then, this complex recruits DNA-PKc that is the catalytic subunit of a DNA dependent kinase to process the DNA ends by phosphorylation. Finally the DNA ligase IV XRCC4 is the enzyme that ligates the two DNA ends to complete the repair of the damaged DNA (Figure 1).

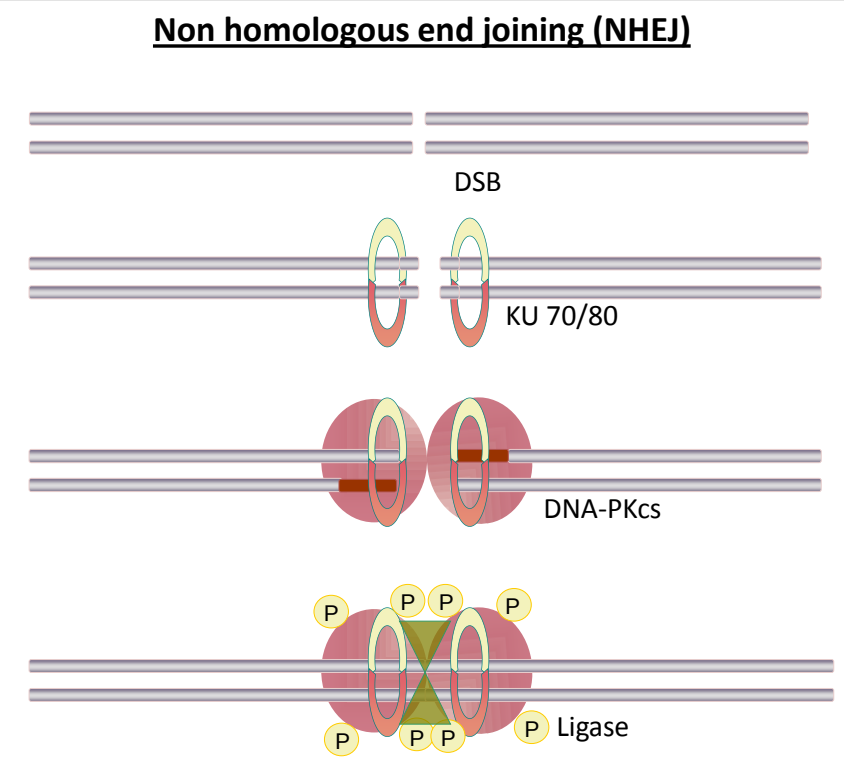


Figure 1. NHEJ repair pathway and the main proteins involved in the function of the pathway. When a DSB is repaired by this mechanism, KU70/KU80 heterodimer protein strongly binds at both sides of the broken DNA. Then the ends of broken DNA are phosphorylated by the enzymatic activity of the DNA-PKC to be finally ligated by DNA ligase IV XRCC4.

- Nucleotide excision repair (NER)

The nucleotide excision repair is the preferred DNA repair mechanism of lesions caused by ultra-violet radiation (UV) in cells that are actively dividing. Briefly, this DNA repair pathway consists of recognizing both sides of the DNA lesion. Then, an incision in the DNA occurs and the damaged DNA strand is degraded by an endonuclease and the remaining fragment is eliminated by an exonuclease enzyme (de Laat et al., 1999).

- Translesion synthesis (TLS)

After ICL unhooking, the lesion in the DNA needs to be bypassed by a specialized low fidelity translesion synthesis polymerase that is recruited to the lesion by a post-translational modification of a polymerase processivity factor activated after the cell replication arrest. This polymerase extends the master strand, that is then used as a template for HDR, to restore the replication fork and make it functional again because it is able to restore distorted bases and also mismathches in base pairs (Chang and Cimprich, 2009). TLS is a damage tolerance process, essential in ICL repair because it prevents a prolonged replication fork stall that would result in DSB and ensures that the cells replicate, although without correcting the DNA lesion but avoiding the replication fork to stall.

- Homology directed repair (HDR)

The homology directed DNA repair mechanism is used to repair DSB originated by ICL. It uses a homologous DNA strand as a template to repair the DNA with high fidelity, generating as few mutations as possible. After exposure to agents that damage DNA during S-phase, the cellular stress response mechanism starts, first arresting the cell cycle to allow its repair and then the FA pathway starts functioning, orchestrated by ATR and ATM kinases that by phosphorylation, activate key proteins of the pathway required to repair the DNA (Collins et al., 2009). The damaged strand is capped by the protein 53BP1 that, if not degraded, the lesion undergoes NHEJ repair. If BRCA1 (involvement of FA pathway) and CTIP work properly they degrade 53BP1, directing the DNA repair to HDR. Then the MRN complex binds to the DNA resection part of the strand. This resected DNA requires to be stabilized by RPA protein and is at this moment when RAD51 moves the DNA strand to the sister chromatid and the HDR process starts. Once the homologous sequence is found, homologous recombination (HR) starts by the generation of the so called “Holliday junction” that is in the end resolved (Wu and Hickson, 2003). This is how the FA pathway promotes HDR suppressing NHEJ in order to prevent inappropriate DNA repair (Figure 2).

An alternative DNA repair mediated by HDR is the single strand annealing (SSA) in which one strand is shifted and the annealing of the other single strand end is then filled and ligated without any cross-over.

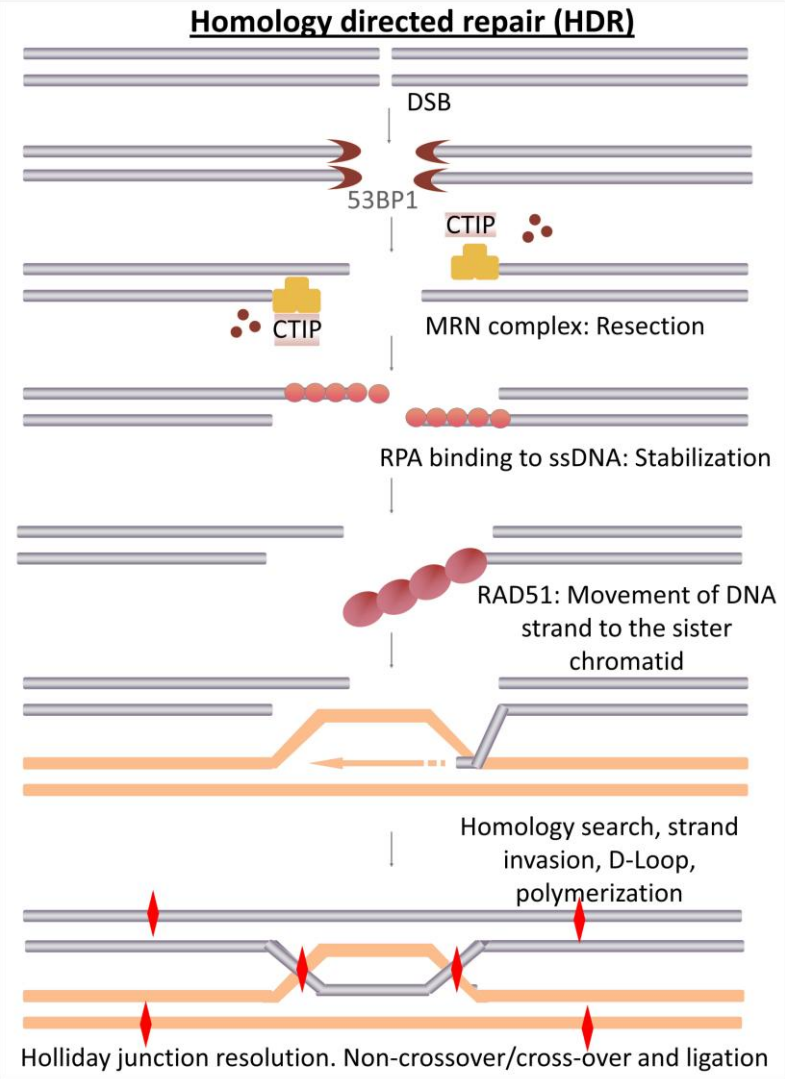


Figure 2. Homology directed repair pathway (HDR). The first step consists of the capping of the damaged DNA strand by the protein 53BP1 followed by its degradation by BRCA1 in collaboration with CTIP. The next step is the resection of the damaged fragment carried out by the MRN complex. The DNA resulting from the resection is stabilized by RPA protein and is moved by RAD51 to the sister chromatid to recombine the DNA and finish the HDR DNA repair.

2.2 FANCONI ANEMIA/BRCA PATHWAY DESCRIPTION AND FUNCTIONING

The FA/BRCA pathway is an early responder of interstrand-crosslink derived (ICL) damage, as it works at the initial steps of the ICL repair process, recruiting the endonuclease XPF and its partner ERCC1 (important role in the initial steps) to perform the incision on the damaged DNA strand, flanking the region where the ICL is located. This incision on the DNA creates a DSB that has to be repaired by HDR, using the other DNA strand, that was previously repaired by the low fidelity translesion synthesis mechanism (TLS), as a template (Wang et al., 2013).

FA cells that were damaged by DNA crosslinking agents accumulate chromosomal breaks, radial and fused chromosomes, due to their inability to repair the damage caused by the ICL. This

genomic unstable cellular phenotype vary depending on the affected FA protein, being mutations in some proteins located downstream in the FA pathway the ones that show a stronger incapacity to respond to ICL-DNA damage because some of these downstream proteins are directly involved in the HDR process (Kee and D'Andrea, 2010).

-Core complex

The proteins located in the FA core complex (FANC-A, B, C, E, F, G, L, M), are essential for the activation of the FA pathway. FANCM, together with the associated protein FAAP24, recognize the ICL in the DNA (Tischkowitz and Dokal, 2004). The association of FANCM to the chromatin occurs thanks to the action of the proteins MHF1 and MHF2 (Histone Fold Protein 1 and 2) and the DNA damage signalling mediated by ATR (ataxia telangiectasia and Rad3-related) (Hirsch et al., 2004). Once the lesion is recognized, the rest of the core complex proteins associate together. If FANCM is not functional, the mismatch repair complex MutS has a redundant activity and is able to recognize DNA ICLs and start the activation of the FA pathway (Wagner et al., 2004) (Figure 3).

FANCL is the FA core complex protein that has an intrinsic E3 ubiquitin ligase activity necessary to carry out the activation of the ID-complex (FANCD2-FANCI) with the E2 conjugating enzyme UBE2T (Kennedy and D'Andrea, 2006; Wang, 2007), once FANCM is phosphorylated, to indicate that there is DNA damage. This ubiquitin ligase activity activates the mono–ubiquitination, a post-translational modification in which an ubiquitin (76 a.a) covalently binds, by an internal amide linkage to residues of lysine, in a manner dependent on each other (Ishiai et al., 2008), on lysine 561 of the FANCD2 protein and on lysine 523 in the FANCI (Kee and D'Andrea, 2010). This makes these two highly conserved proteins, FANCD2 and FANCI, to heterodimerize, giving rise to the ID-complex. As said, several associated proteins, FAAP24, FAAP100, FAAP20, MHF1, MHF2, are critical for the correct activation of FANCD2 (Kee and D'Andrea, 2010).

Apart from FA-associated proteins, there are other DNA repair factors with important roles in the FA pathway in response to DNA damage, such as DNA damage checkpoint proteins (ATR, ATM, CHK1, CHK2, γ-H2AX), that after DNA damage, arrest the cell cycle in a G2/M phase (Moldovan and D'Andrea, 2009; Wang, 2007). Most of the FA proteins are substrates of ATR or of its effector CHK1 like FANCD2, FANCI, FANCA or FANCE (Collins et al., 2009; Wang, 2007) (Zhang et al., 2005) (Figure 3).

The activation of the core complex is mediated by a previous ATR-dependent phosphorylation process that serves as a substrate for the ligase. This process has not been fully elucidated yet but it has been proposed that the phosphorylation of FANCI on multiple sites mediated by ATR, functions as a switch to turn the FA pathway on, by promoting the mono-ubiquitination of FANCD2 and as a consequence its own mono-ubiquitination, being the mono-ubiquitination of FANCI dispensable for the function of the FA pathway (Ishiai et al., 2008).

- **ID-complex**

The ID-complex, when activated, translocates and loads onto chromatin, localizing at the nuclear repair *foci* with the DNA repair proteins BRCA1 and RAD51 (Smogorzewska et al., 2007).

Mutations in any of the FA genes located in the core complex, except FANCM (with redundant activity found in other proteins) give rise to a deficient mono-ubiquitination of FANCD2 and FANCI being this one the most important event in the regulation of the FA pathway (Kee and D'Andrea, 2012; Kottemann and Smogorzewska, 2013) (Figure 3).

- **Downstream proteins**

The activation of the heterodimer FANCD2-FANCI moves FANCD1/BRCA2 protein into chromatin complexes and it also interacts with FANCJ/BRIP1, FANCN/PALB2 and BRCA1 facilitating the assembly of DNA damage-inducible RAD51 nuclear *foci* (Howlett et al., 2005). Another recently identified associated protein, FAN1, (Fanconi-associated nuclease 1) binds to activated FANCD2 and provides the necessary nuclease activity during ICL repair, probably restructuring the injured DNA (Kee and D'Andrea, 2010). The up-regulation of mono-ubiquitinated FANCD2 during S-phase and its localization to RAD51 *foci* suggests that the FA pathway is required in the DNA replication process (Howlett et al., 2005) and represents a good model for studying DNA repair and DNA damage response, specially after ICL generation (Kee and D'Andrea, 2010) (Figure 3).

The multiple DNA repair processes involved in the repair of ICL-damaged DNA are catalyzed by different structure specific nucleases that are recruited once FANCD2 is mono-ubiquitinated, when the DNA needs to be repaired. They are regulated by the highly conserved FA downstream protein FANCP/SLX4 that serves as a scaffold. This protein interacts with three different nucleases at independent places that do not overlap, regulating them in the DNA repair process. At the N-terminal region, SLX4 interacts with the nuclease complex XPF/ERCC1, at a central region it binds to MUS81-EME1 complex and at the C-terminal it has interaction with the SLX1 nuclease. This

protein also interacts with the mismatch repair recognition complex MSH2-MSH3 and with other compounds of the telomere protecting complex TRF-RAP1 (Kottemann and Smogorzewska, 2013).

The interaction of SLX4 with both MUS81-EME1 and XPF-ERCC1 promotes the cross-over that generates a DSB in the DNA that is repaired by the TLS mechanism by the action of DNA polymerases that copy damaged DNA to restore the DNA strand. Finally, the DSB generated is repaired by HDR, process in which the proteins located downstream in the FA pathway play an important role. HDR also functions in multiple processes outside of the FA pathway. These proteins help RAD51 loading into the DNA repair *foci* and also participate in the resolution of the recombination intermediates. Four of these proteins (FANCD1/BRCA2, FANCN/PALB2, FANCJ/BRIP1, FANCO/RAD51C) (Kottemann and Smogorzewska, 2013) also confer susceptibility to breast and ovarian cancer when mutated in one copy, due to the involvement of these proteins in HDR repair. The last step in the DNA repair after the generation of an ICL is the repair of the formed adducts, that are restored by the already described NER and the nicks in the DNA are filled by DNA polymerases.

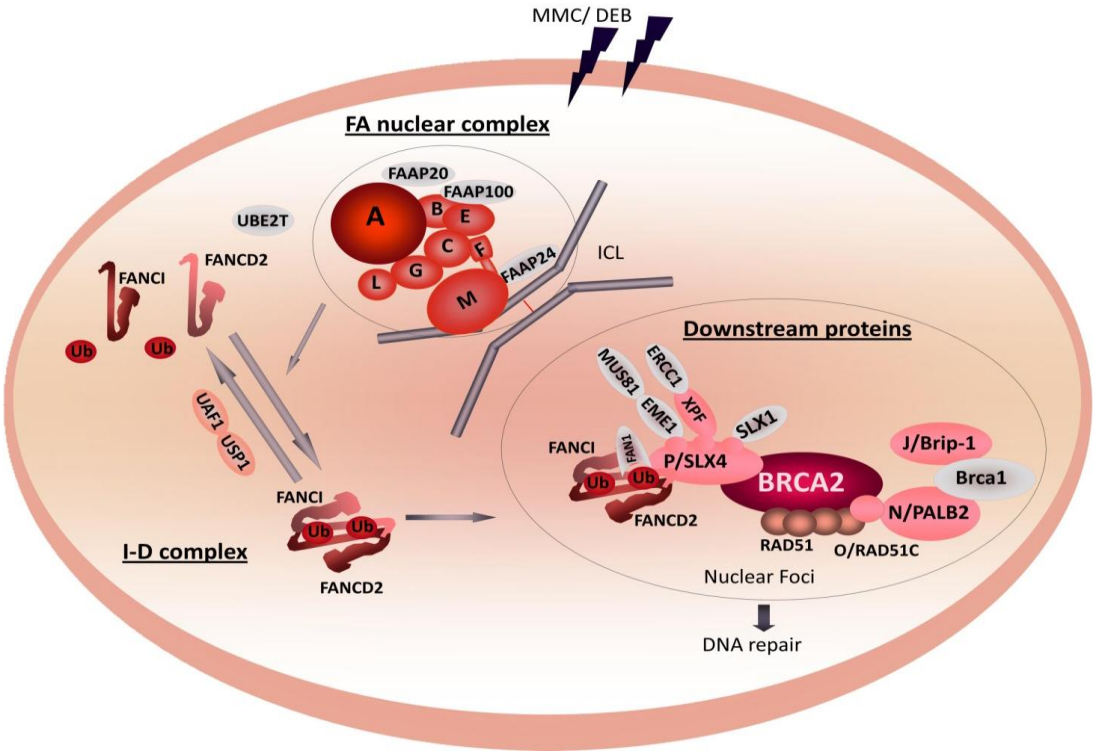


Figure 3. FA pathway and the proteins involved in its functioning to repair DNA after ICL damage. The FA proteins forming the nuclear complex are represented in red colour (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL and FANCM). The accessory proteins FAAP20, FAAP24 and FAAP100 together with the protein UBE2T are represented in grey. The proteins FANCI and FAND2 that form the ID-complex, when ubiquitinated have a saxophone shape as is represented in the figure. In the complex formed by downstream proteins of the FA pathway the FA proteins are represented in light and dark pink (FANCD1/BRCA2, FANCJ/BRIP-1, FANCN/PALB2, FANCO/RAD51C, FANCP/Slx4 and FANCQ/XPF) and proteins involved but not considered as FA proteins are represented in grey.

This pathway must not always be active, so, there is a de-ubiquinating complex with enzymatic activity, named USP1/UAF1 that deactivates the FA pathway removing the ubiquitin both from FANCD2 and FANCI (Smogorzewska et al., 2007). This deubiquitination is required for ICL repair as it has been reported that the loss of USP1 is associated with ICL sensitivity (Wang and Gautier, 2010) (Figure 3).

There is an important link between FA pathway and BRCA pathway because it is well known that BRCA2 plays an essential role in HDR as it interacts with BRCA1 and with RAD51, a central effector in HDR, and promotes its loading to the ssDNA coated by RPA, the protein that stabilizes this ssDNA for its repair (D'Andrea, 2013).

3.PHENOTYPE OF FANCONI ANEMIA CELLS

FA cells have a characteristic response to certain endogenous and/or exogenous agents that determines their phenotype. There is controversy about what causes the FA phenotype. While some authors support the idea that the phenotype of these cells is consequence of a defective ICL-DNA damage repair system that leads to the accumulation of genomic instability and damaged DNA, others relate the FA phenotype to an increased oxidative stress and imbalances in the oxygen metabolism. It is not clear yet what causes the FA phenotype but it is probable that a combination of both mechanisms is responsible for it (Pagano et al., 2013), (Pallardo et al., 2010) (Figure 4).

3.1 OXIDATIVE STRESS

FA proteins are known to work in cooperation between them, although their individual role in the cell has not been deeply studied, there are evidences suggesting that some of the FA proteins (FANCA, FANCC, FANCG, FANCD2 and FANCJ) participate in redox and detoxification metabolism through the interaction with enzymes involved in the sense and response to the damage caused by oxygen stress, such as cytochrome p-450 or proteins involved in the metabolism of hydrogen peroxide. For this reason it is believed that the deficiency in any of these proteins could be responsible for the increase in the level of reactive oxygen species (ROS) in FA cells (Sejas et al., 2007; Zhang et al., 2007).

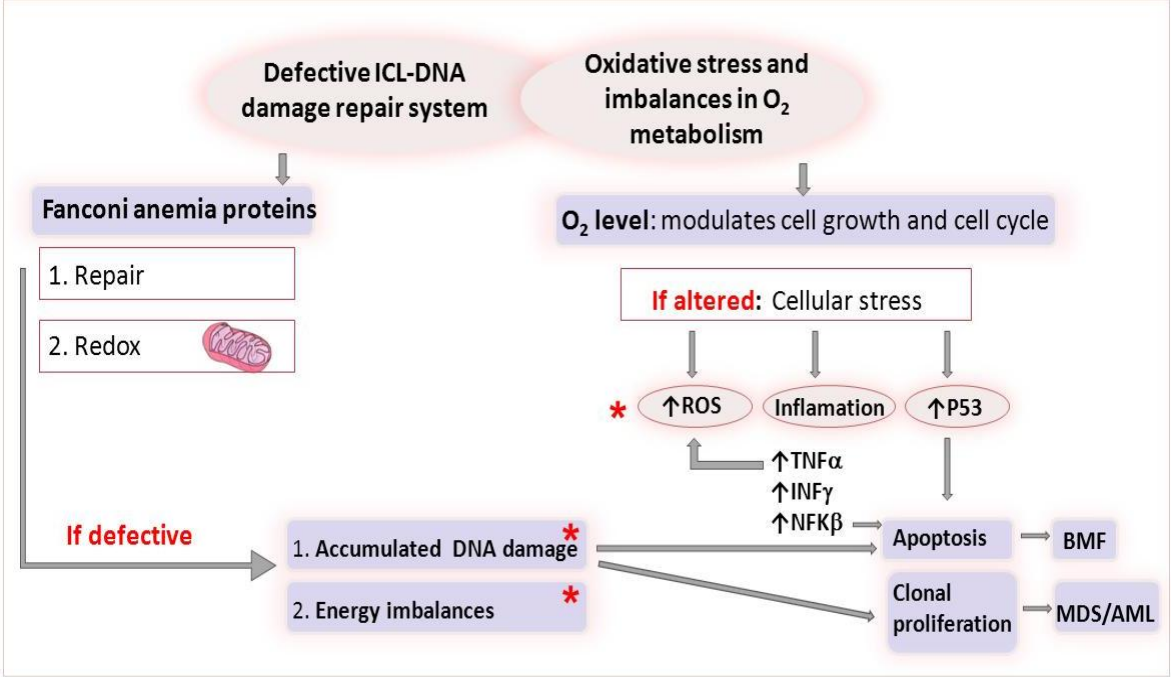


Figure 4 . Scheme of the phenomena that could be responsible for the characteristic FA phenotype.

When exposed to elevated oxygen levels, FA cells alter their cell cycle, leading to an accumulation of cells in S and G2/M phases, reducing viability and increasing the number of chromosomal aberrations (Joenje et al., 1981). They also present an increase in 8-OxodG, one of the most common mutagenic lesions caused by cell rust after exposure to DNA damaging agents (Du et al., 2012).

Recently, a defective mitochondrial function has been proposed to participate in the oxidant sensitive phenotype that characterizes FA cells (Ravera et al., 2013), establishing a relationship of mitochondrial functionality with cellular redox balance and energy metabolism. The mechanisms that involve mitochondrial alterations with oxidative stress and the role of the different FA proteins are not fully elucidated, and only evidences of the role of FANCA protein have been studied in detail. It relates the deleterious effects in mitochondrial physiology of FA cells with the overproduction of TNFα and the increase in ROS levels. The mitochondrial alterations in *FANCA* mutated cells affect complex I of the respiratory chain, resulting in mitochondrial dysfunction at the level of structure, cytoskeleton, electron transfer and as a consequence, in a defective cellular energy metabolism (Kumari et al., 2013; Ravera et al., 2013). Apart from FANCA, FANCC and FANCG there are other FA proteins known to be directly involved in redox homeostasis through interactions with cytochrome p450 in response to cellular damage. More recently, FANCD2 was

found to participate in oxygen damage response, interacting with FOXO3a, ATM and FANCJ to inhibit the oxygen damage sensor hemoxigenase 1 (Pagano et al., 2012).

3.2 SENSITIVITY TO TNFα AND IFNγ

FA is also known to be closely linked to the overproduction of pro-inflamatory and pro-apoptotic cytokines, such as tumour necrosis factor alpha (TNFα), interferon gamma (IFNγ), interleukin 6 (IL-6) or interleukin 1β (IL-1β). As consequence of an alteration in the balance of redox processes these molecules strongly affect the homeostasis of hematopoietic stem cells (HSC) at different levels, including cell proliferation and the process of homing in the bone marrow niche (Du et al.).

The overexpression of TNFα that occurs in the bone marrow of FA patients creates an inflammatory context of oxygen stress and cell death, related with the deregulated expression of molecules, such as p38MAPK, JNK and NFK-β. This process, together with the genomic instability of FA cells, can result in the hyperactivation of clonal proliferation, including leukemic or myelodysplastic episodes and also the increase in the apoptotic rate, that can contribute to the progression of BMF (Du et al., 2014). Nowadays some attempts to ameliorate BMF are being developed with the objective of decreasing the levels of cytokines such as TNFα (Dufour et al., 2003) (Figure 5).

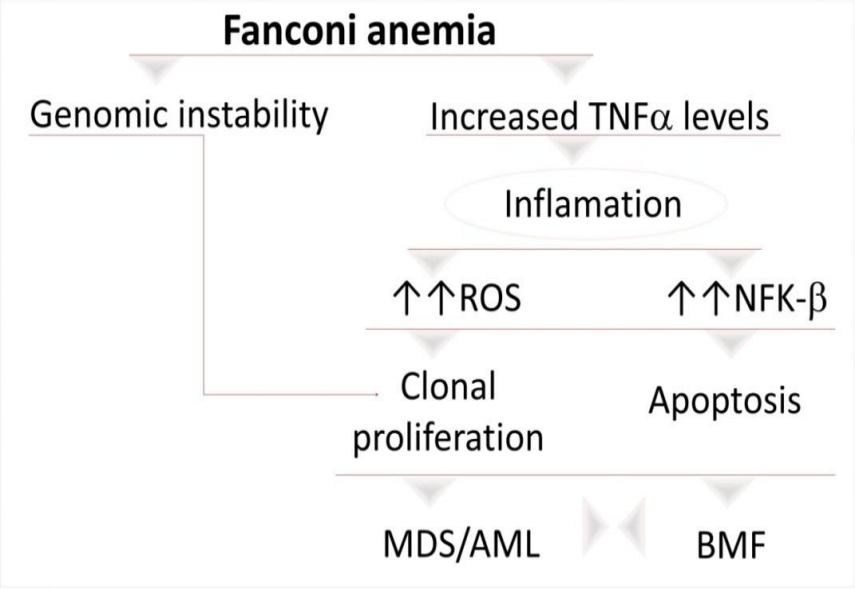


Figure 5. Diagram representing the effects that increased levels of TNFα have in FA cells through the elevated ROS production that leads to clonal proliferation of hematopoietic cells and as consequence, the development of myelodysplastic syndromes (MDS) or acute myeloid leukemia (AML). Another effect of a TNFα excess is the alteration of the NFK-β pathway, increasing the rate of apoptosis that contributes to bone marrow failure (BMF).

3.3 CROSS-LINKING AGENTS AND CELL CYCLE ARREST

FA cells are hypersensitive to DNA crosslinking agents, such as mitomycin C (MMC) or dyepoxibutane (DEB) among others. The exposure of FA cells to these agents generates a high number of chromosomal aberrations such as chromatid breakages, radial chromosomes or chromosomal fusions. It is known that as a consequence of replicative stress and unresolved DNA damage, partially due to the high number of replication forks stalled after ICL generation, the expression of P53 and of its effector P21, is increased in FA cells. FA cells suffer a G2 cell cycle arrest that is usually overcome, but the strong induction of P53 expression gives rise to a new cell cycle arrest in G0/G1, as a protective mechanism to keep the integrity of the genome by prevention of damaged cells to progress in the cell cycle. This mechanism has been proposed to be responsible for the loss of hematopoietic progenitor cells in FA patients that leads to BMF, and it is believed to start early during development (Ceccaldi et al., 2012).

Strategies to prevent cell cycle arrest have been developed in FA cells, mainly to evercome the blockage of G2 checkpoint and downregulation of P53, with the purpose of rescuing the hematopoietic proliferation defects and therefore to recover high numbers of hematopoietic progenitor cells. The main limitation of these strategies arise from the fact that allowing damaged FA cells to continue their cell cycle, bypassing checkpoint controls, could result in the accumulation of genomic damage, thus, with high probability of malignant transformation (Ceccaldi et al., 2012; Dumitriu and Young, 2012).

3.4 DEFECTIVE ADHESION ACTIVITIES THAT AFFECT HOMING

It is known that FA cells have impairment in their cell adhesion and as a consequence in their homing to the bone marrow niche. The mechanisms accounting for the inability of FA cells to attach to the marrow are still unclear, but two molecules have been observed to be altered in FA cells. In *FANCA* deficient cells, the required CDC42 GTPase activity for cell migration, has been reported to be decreased (Zhang et al., 2008b). Furthermore, the characteristic over-expression of TNFα in FA cells could play a role in the down-regulation of CDC42 (Zhang et al., 2008a). Another molecule with a key role in cell adhesion and homing activities, CXCR4 (chemokine receptor 4) was observed to be involved in FA LSK cells (Lin^-, $sca1^+$, $ckit^+$) from different mouse models, limiting their homing capacity. Interestingly, the impaired homing of FA cells can be circumvented by an up-regulation of CXCR4 expression in combination with hypoxic conditions, increasing cell

migration activities and decreasing the generation of ROS caused by oxidative damage (Skinner et al., 2008).

4. CLINICAL FEATURES AND MANIFESTATIONS

As it was previously commented, FA is a very heterogeneous genomic instability syndrome that affects mainly the HSCs and in consequence, all the cells of the hematopoietic system. The disease is characterized by congenital developmental abnormalities in 60-75% of the cases. Although there are several common defects that appear in FA patients, they are variable among patients. The most frequent abnormalities found in FA are: low weight at birth (50%), radius axis defects (absent or very small thumbs or short radius), skeletal abnormalities in hips or ribs, microphthalmia and microcephalia, imperforated anus and mental retardation disorders (Kaplan et al., 1985), (Tischkowitz and Hodgson, 2003).

Skin pigmentation defects are also very frequent (55%), specially the so called “café au lait spots” and fertility problems in females (35%). Less frequent are gastrointestinal problems (7%) (esophageal or duodenal atresia), defects in the kidney and in the urinary tract (21%), endocrine disorders that include defective insulin metabolism or hypothyroidism, ear malformations or alterations in the central nervous and in the cardio pulmonary systems such as ventricular septal defects or pulmonary and aortic stenosis. It is also frequent that FA patients reach adulthood with evidences and features of premature aging (Table 2 and Figure 6).

The main characteristic of the disease is BMF that appears in most of the cases. On the other hand, due to the genomic instability of the cells, FA patients have a high predisposition to develop MDS, AML and SCC in head, neck, oral cavities and genitals (Howlett et al., 2005). In many cases, patients die at an early age due to the severe affection of the hematopoietic system.

There is a phenomenon that takes place in some cases of FA as a consequence of mutations that occur due to the genomic instability of the cells. It is known as “somatic mosaicism” and it was first described in 2002. It consists of naturally occurring mutations, that lead, either to the wild type (wt) form of the mutated FA gene, or to compensatory mutations that generate a mutated, although functional, FA protein. Thus, individuals with somatic mosaicism have a chimeric hematopoietic system composed of defective cells and also of cells in which the mutation has reverted (Gross et al., 2002). Somatic mosaicism is, to a certain extent a natural gene therapy that confers selective advantage to the corrected cell/s, allowing the possibility that only one

genetically corrected HSC could restore the hematologic defect, rescuing the patient from BMF. This long term restoration of the hematologic parameters will occur only if the cell that spontaneously reverts its deficiency is a HSC, because if the genetic correction takes place in a mature cell it would not affect all the hematopoietic progeny.

PHYSICAL ABNORMALITIES IN FANCONI ANEMIA PATIENTS

ABNORMALITY/ DEVELOPMENTAL DEFECT	FRECUENCY
Skin defects: Hyperpigmentation, hypopigmentated areas, large freckles, café-au-lait spots	40%
Short stature	40%
Supernumerary fingers, absent/hypoplastic thumb, radius defects, bifid, clinodactyly	35%
Hypogonadism, undescended/absent testes, bicornuate/malpositioned uterus	25%(male)/2%(fem)
Microcephaly/ hydrocephaly, bird-like face, mid-face hypoplasia, Sprengel´s deformity of neck	20%
Microphtalmia, strabismus, epicanthal folds	20%
Abnormal, ectopic, horseshoe, hypoplastic or absent kidney, hydronephrosis	20%
Deafness, low set ears, abnormal ear shape, narrow canal	10%
Cardio-pulmonary abnormalities: congenital heart disease (situs inversus, atrial/ventricular defects, patent ductus arteriosus)	6%
Gastrointestinal abnormalities: atresia, imperforated anus, tracheoesophageal fistula	5%

Table 2. FA most common pyhysical abnormalities, and the frecuency of occurrence in patients.

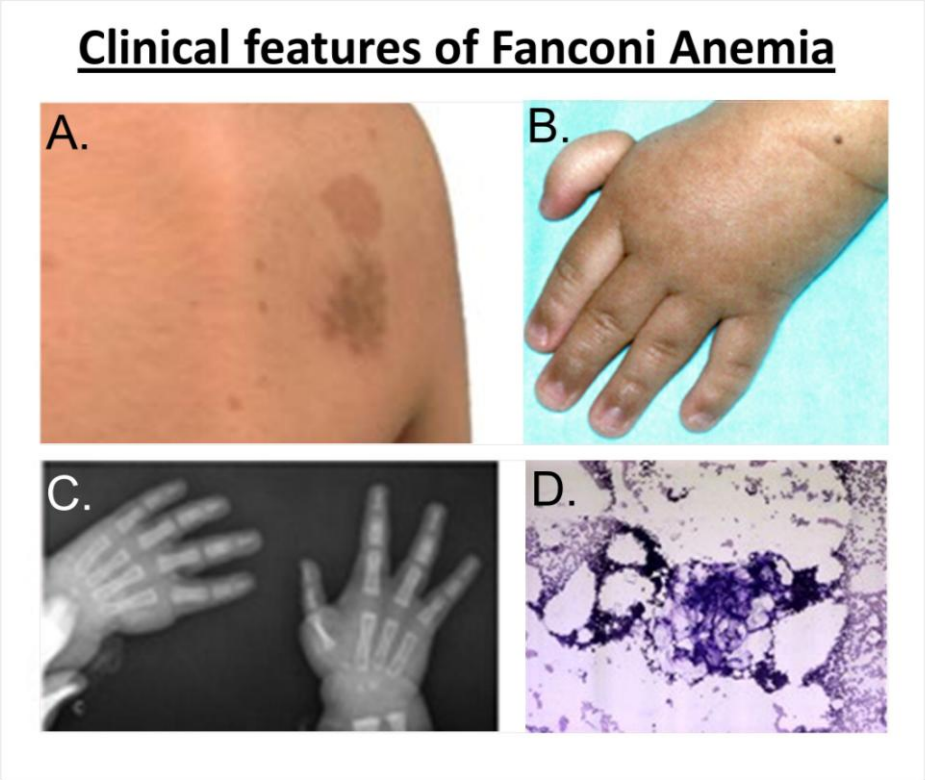


Figure 6. Representation of the most common clinical features observed in FA patients. In the image are represented the characteristic **A)** "café au lait spots", **B, C)** thumb abnormality and **D)** bone marrow hypoplasia.

5. DIAGNOSTIC CRITERIA AND IDENTIFICATION OF FANCONI ANEMIA COMPLEMENTATION GROUPS

The detection of FA patients based on clinical features is difficult because the congenital malformations are not always present or evident, and because sometimes it is not diagnosed until BMF appears. In other cases, the FA patient is diagnosed because of the development of AML. In all these cases, it is recommended to carry out a chromosomal breakage test with DEB or MMC (Giampietro et al., 1997; Kutler et al., 2003).

Symptoms such as haemorrhagic manifestations, fatigue and paleness caused by anemia, petechiae, ecchymosis or epistaxis derived from thrombocytopenia, high temperature or bacterial infections are common, although nonspecific manifestations of FA that difficult the diagnosis of

the disease. A complete hematologic cell count in FA patients frequently reveals decreased numbers of one or all blood cell lineages.

At the time of diagnosis is of critical relevance to discriminate FA from other diseases with similar disease phenotypes, such as Nijmegen Breakage Syndrome (NBS), Bloom syndrome, Seckel syndrome, Diamond-Blackfan anemia, Dyskeratosis congenita or Thrombocytopenia Absent Radius (TAR).

The differential test for FA is the chromosomal breakage test in presence of interstrand-crosslinking agents (ICL agents), being the most commonly used diepoxybutane (DEB) and mitomycin C (MMC). FA cells present increased numbers of chromosomal aberrations in presence of these agents (Carreau et al., 1999). This was first established by Auerbach and Schroeder-Kurth in 1982, who observed that FA patient´s cells were positive for DEB chromosomal breakage induction, while non-FA patient´s cells were negative (Auerbach et al., 1989).

Once the diagnosis of FA is confirmed, bone marrow aspirates or biopsies are studied to confirm the existence of a BMF syndrome, a MDS or specific clonal aberrations indicative of pre-leukemic alterations. Aplastic anemia is associated with hypocellular bone marrow that is occupied by fat and stromal cells. The bone marrow failure can be classified in terms of cellularity as moderate (30% of cellularity, no severe pancytopenia), severe (25% of cellularity; < 50% of cellularity with <30% hematopoietic cells and $<4x10^4$ absolute reticulocyte count/µl; <500 absolute neutrophil count (ANC)/µl or $<2x10^4$ platelet count/µl), or very severe (severe plus absolute neutrophil count (ANC) <200/µl (Young, 2002).

The diagnosis of FA can also be screened by a G2/M phase cell cycle arrest that consists of the accumulation of G2/M cells after treatment with ICL agents (DEB or MMC) due to a cell cycle blockage (Akkari et al., 2001). This test is frequently performed in fibroblasts to confirm the FA diagnosis in cases of mosaicism, because the standard chromosomal breakage test in peripheral blood in these patients can be unclear. In these cases, DEB test must be used as a complementary and confirmatory study (Seyschab et al., 1995). These studies should also be performed in any apparently healthy FA patient sibling, because the disease can be present without clear phenotypic manifestations. It can also be prenatally diagnosed with these tests in amniotic fluid cells (amniocentesis), chorion villus cells of fetal blood (cordocentesis) or mutation analysis in case the mutation of the affected brother is known (Auerbach et al., 1985).

5.1 IDENTIFICATION OF COMPLEMENTATION GROUPS

The screening of the FA complementation groups has been conducted in a four step algorithm represented in Figure 7. First the FA diagnosis is confirmed and it is discerned if the patient has somatic mosaicism, through a DEB and MMC test performed in patient T-cells. The next step is directed to define which of the FA genes is causing the disease.

As previously commented, the identification of the FA affected gene in the patient allows the assignment to a complementation group. One of the methods that has been widely used in our laboratory, consists of the transduction of PB-T-cells, using a battery of vectors, either γ-retroviral (γ-RV) or lentiviral (LV), each one encoding a different FA gene, analyzing which vector corrects the mitomycin C (MMC) hypersensitivity of these cells. The vector that corrects the MMC sensitivity determines the FA complementation group to which the patient belongs (Antonio Casado et al., 2007; Hanenberg et al., 2002).

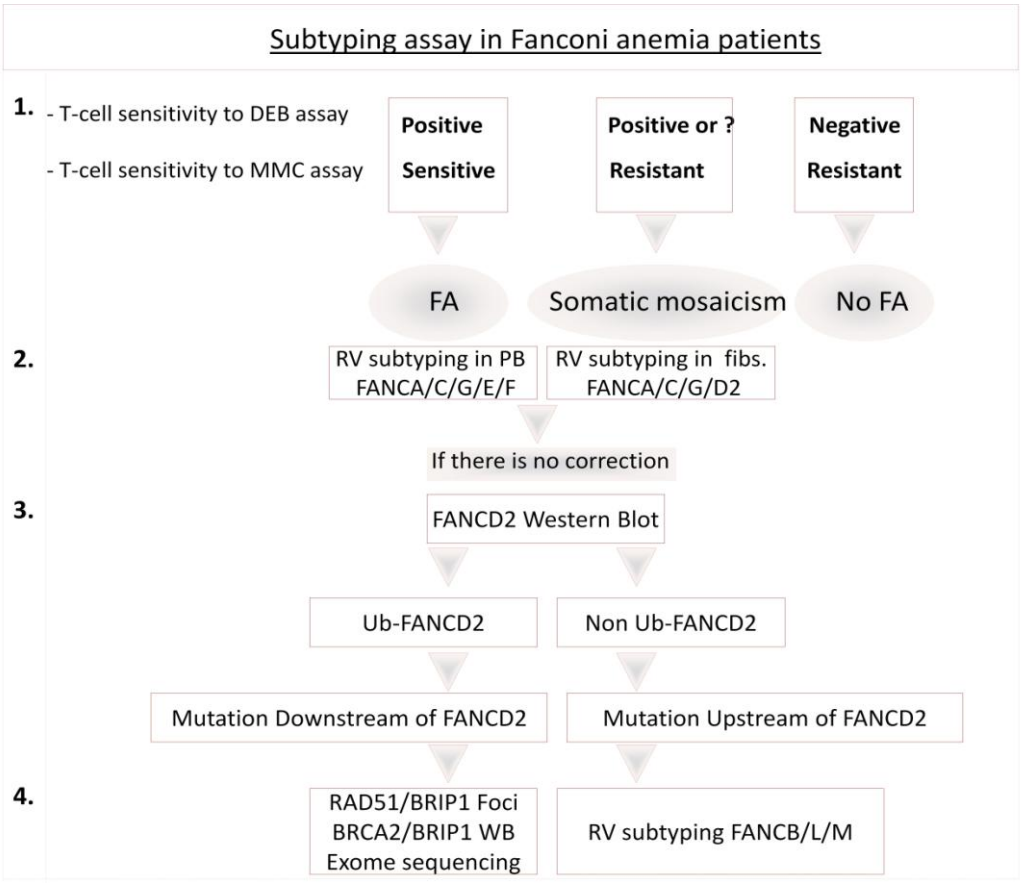


Figure 7. Representation of a four-step algorithm proposed for the subtyping of the FA patients. **Step1:** T-cell sensitivity diepoxybutane (DEB) and mitomycin C (MMC). **Step2:** subtyping of the patient with gamma retroviral vectors (γ-RVs) carrying the most common FA genes. **Step 3**: detection if the defective protein is upstream or downstream of the FA pathway when the most frequent genes do not correct the genetic defect. **Step 4:** detection of the exact mutation of less common FA genes using other existing methods.

In case none of the most common FA genes complements the mutation, Western Blot (WB) of FANCD2 protein allows defining if the FA affected protein is located upstream or downstream of the ID-complex in the FA pathway. The presence of the non-ubiquitinated (FANCD2-S) form of FANCD2 indicates that the affected protein is located upstream. On the other hand, the detection of the ubiquitinated (FANCD2-L) form of FANCD2 indicates that the mutation is located downstream in the pathway. The specific mutated FA protein can be detected by other approaches, such as molecular and functional analysis. For example, the generation of RAD51 *foci* is useful to detect functional alterations in FANCD1/BRCA2. The FANCJ/BRIP1 *foci* formation can be useful to detect certain mutations in this protein. The WB of FA proteins is often used to detect truncated mutations in these proteins (Figure 7) (Antonio Casado et al., 2007).

Due to the advances in exome sequencing and also to the reduced costs of these techniques, the identification of the FA gene responsible for the disease can be efficiently performed through the analysis of any of the 16 FA genes described so far. The analysis of the entire genome by exome sequencing also makes possible the detection of new FA genes. This technique allowed the recent identification of the XPF/FANCQ as a FA gene (Bogliolo et al., 2013).

6. STANDARD THERAPIES OF THE FANCONI ANEMIA HEMATOPOIETIC SYNDROME

Standard therapies of the bone marrow failure are based on the use of androgens, hematopoietic growth factors (Stucki et al., 1998), and if possible hematopoietic stem cell transplantation (HSCT). HSCT is the only treatment that cures bone marrow failure and it is specially recommended when there is a severe or very severe aplasia, because these patients are less likely to respond to other therapies, and have a high risk of bleeding and infection (Ades et al., 2004). Despite its advantages, HSCT does not solve other manifestations of FA, such as SCC. Additionally, it has been discussed wether or not HSCT can increase the risk of head and neck SCC, mainly as a consequence of the development of graft versus host disease (GVHD) (Rosenberg et al., 2005) (Tolar et al., 2012). HSCT in FA is particularly complicated due to the sensitivity of these patients to conditioning regimes used to facilitate the engraftment of transplanted HSCs.

6.1 PALLIATIVE TREATMENTS OF THE HEMATOPOIETIC ASPECT OF THE DISEASE

The palliative treatments to reduce anemia, trobocytopenia or neutropenia in FA patients include the use of androgens, growth factors and transfusions.

- Androgen Therapy

Androgen therapy has been used for more than 50 years and is mainly indicated when the patient does not have a HLA-identical related donor (Tischkowitz and Dokal, 2004).

The clinical symptoms to prescribe androgen therapy are low haemoglobin (HGb) and platelet (PLT) levels. Androgen therapy is known to be more effective when the administration starts before there is a complete BMF. Androgens frequently improve the anemia and, at a less extent thrombocytopenia and neutropenia. Historically, the androgen most widely used was oxymetholone, while in the recent years danazol is more frequently used because it has less virilization side effects. In some cases it is combined with prednisolone to reduce the liver toxicity and premature epiphyseal closure. About 75% of FA patients respond to androgens in a period of 2-12 months. In case there is no hematopoietic improvement with this therapy after three months, the treatment is not recommended any longer (Tischkowitz and Dokal, 2004).

There are several secondary effects derived from the use of androgens, such as accelerated growth followed by an early closure of epiphyses, virilization, changes in the behaviour of the patient (hyperactivity), transaminases raise, cholestatic jaundice or hypertension. Less frequent are hepatic adenoma or hepatic cystic disease.

Several clinical trials are being performed in patients to evaluate the benefits and risks of using different androgens (Scheckenbach et al., 2012).

- Hematopoietic growth factors

The hematopoietic growth factors tested in FA patients are erythropoietin (EPO), granulocyte colony-stimulating factor (rhG-CSF), granulocyte-macrophage colony-stimulating factor (rhGM-CSF) and interleukin-3 (IL-3), being rhG-CSF (granulocyte colony-stimulating factor) the one in which there is more experience (Rackoff et al., 1996).

In general, patients suffering from neutropenia improve the ANC, but the PLT and HGb counts remain unaltered in most of the cases studied. If no increase in ANC is observed after 8 weeks of administration the treatment should be stopped (Rackoff et al., 1996). There is some evidence that the treatment with rhG-CSF could contribute to the early development of MDS or AML. Therefore, treatment with hematopoietic growth factors is not frequently conducted in FA patients.

Blood transfusions are required when BMF is severe. At this stage, HSCT is generally recommended if the patient has a convenient donor.

6.2 CURATIVE TREATMENTS OF THE HEMATOPOIETIC ASPECT OF FANCONI ANEMIA: HEMATOPOIETIC STEM CELL TRANSPLANTATION (HSCT)

As it was previously said, HSCT is the only current treatment to cure BMF (Gupta et al., 2010). HSCT is indicated in FA patients with BMF, MDS or AML, being patients suffering from MDS and AML the ones with a worse prognosis. It is of great importance to find an appropriate donor for HSCT and a good source of HSC cells for HSCT (Figure 8). The ideal donor for FA-HSCT is an HLA-matched sibling donor to limit risks of graft rejection and GVHD.

Apart from adult donors, umbilical cord blood (UCB) is a new and good alternative for allogeneic HSCT. The first successful HSCT using UCB progenitors was performed in the late 80´s by Dr. Gluckman in a patient affected by FA (Gluckman et al., 1989). Since then UCB has emerged as a potential source of HSCs for allogeneic HSCT because it is enriched in immature progenitors ($CD34^+ CD38^-$), although it still remains challenging finding ways of improving rates of engraftment and immune recovery after HSCT (Navarrete and Contreras, 2009).

More recently, peripheral blood (PB) mobilized HSCs gained ground as a good stem cell source for HSCT. It is based on the administration of hematopoietic growth factors that increase the percentage of HSCs circulating in PB and to collect these cells by apheresis (Lemoli et al., 2003). HSC mobilization can be based on the blockage of HSC interactions with the niche, releasing functional progenitor hematopoietic cells to circulation (Pelus and Fukuda, 2008; Ramirez et al., 2009).

Plerixafor (AMD3100, Mozobil) acts reversibly inhibiting the interaction between SDF-1 in bone marrow stromal cells and its receptor CXCR4 (Peled et al., 1999) and has been effective to mobilize HSCs in poor mobilizing patients and also in patients suffering from lymphoma (Farina et al., 2014) or β-thalassemia (Yannaki et al., 2012). There is a sinergy between plerixafor and filgrastim (r-metHuG-CSF) that increases the number of mobilized $CD34^+$ cells. This mobilization regime has not been clinically tested in FA patients. Thus, a phase II clinical trial in FA is currently being developed in Spain to evaluate its efficacy and safety.

- Conditioning regimes of Fanconi anemia patients prior to HSCT

As FA patients are more sensitive to conditioning drugs administered prior to transplant, the conditioning regimes used in FA patients are milder than those used in non FA patients (Gluckman et al., 1995; Guardiola et al., 2004).

For patients receiving HLA-identical related/sibling donor HSCT, Dr. Gluckman showed that a low-dose of cyclophosphamide (CY) (20 mg/kg) with a single dose of thoracoabdominal radiation (TAI) (500 cGy) improved the long-term survival after HSCT, although there is controversy about the convenience of using radiation combined with CY (Zanis-Neto et al., 2005) (Dufour et al., 2001; Farzin et al., 2007; Kohli-Kumar et al., 1994; Medeiros et al., 1999; Socie et al., 1998). Thereafter it was observed that the use of fludarabine had less toxicity and reduced GVHD. For this reason fludarabine has been widely introduced in conditioning regimes for FA patients (Tan et al., 2006).

Since then, several improvements have been made by Drs. Wagner and McMillan for the conditioning of FA patients that tend to limit the dose of radiation, based on the administration of low dose of CY, fludarabine and antithymocyte globulin (ATG*) (Pasquini et al., 2008).

In cases in which there is not an HLA-matched donor available, the conditioning regime is different. It was reported that relatively low doses of CY help to decrease the risk of transplant failure.

A pilot study carried out by MacMillan et al. to scale down the dose of irradiation in matched unrelated donors, partially matched related or unrelated donors consisted of low-dose irradiation, fludarabine, CY and ATG*. In this study it was determined 300 cGy to be the minimum dose of radiation possible in this conditioning regime and it was observed a survival rate of 96% after a three year follow up, with a minimal toxicity and development of GVHD (Yabe et al., 2006). The

latest conditioning regime established for unrelated donors consists of the administration of low dose of CY and fludarabine, combined with ATG*, 300 cGy of radiation and thymic shielding.

In cases of high-risk transplants, that include FA patients with MDS, leukemia or with *BRCA2* gene affected, busulfan was also included in the conditioning regimes (Chaudhury et al., 2008) (Mitchell et al., 2014).

6.2.1 TRANSPLANT FROM SELECTED EMBRYOS

Within the last years, a reduced number of FA patients have been transplanted, after preimplantation genetic diagnosis, with HLA-identical UCB or bone marrow HSCs from a healthy sibling. (Bielorai et al., 2004; Grewal et al., 2004; Rechitsky et al., 2004). These transplants were, thus similar to those performed with hematopoietic grafts from conventional related donors.

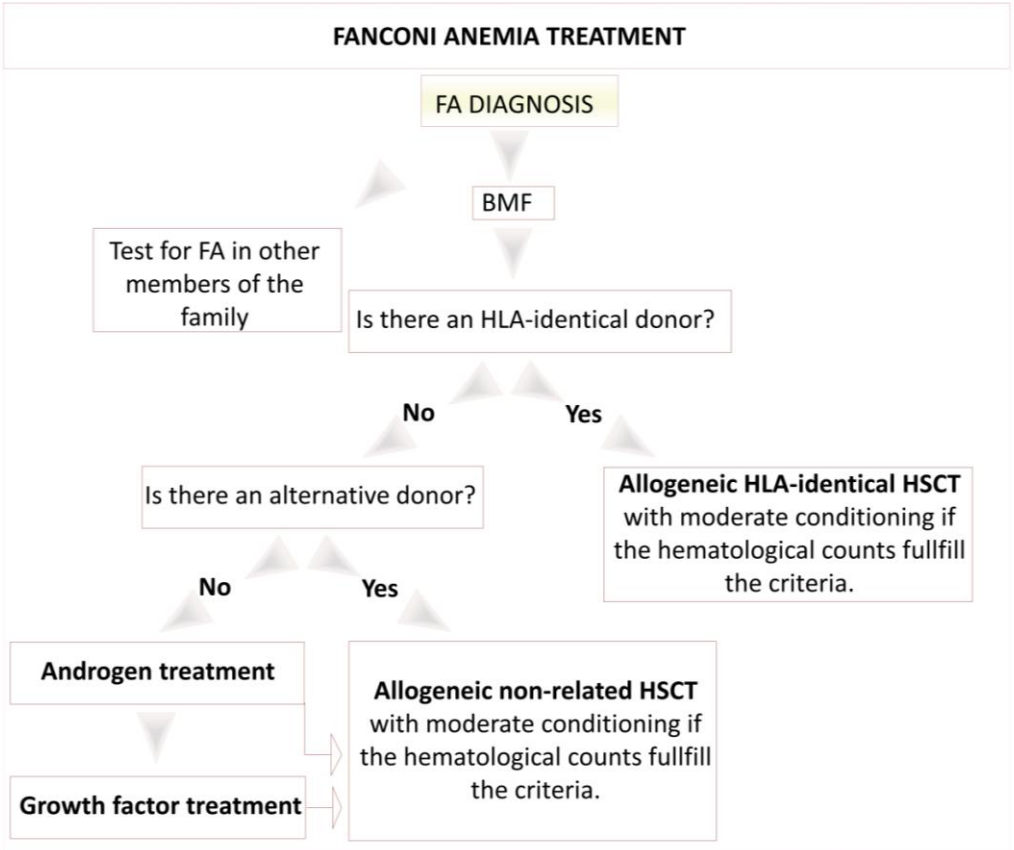


Figure 8. FA treatment algorithm based on the existence or not of bone marrow failure. It represents how to proceed in case there is bone marrow failure depending of the circumstances of the patient, such as the availability or not of an HLA-identical donor.

6.2.2 POST-TRANSPLANT MALIGNANCIES

FA patients that received a HSCT have an increased risk of developing malignancies such as MDS, lymphoproliferative disease, AML or solid tumours, being SCC the most frequent, a side effect that is believed to be enhanced by GVHD. The probability of developing a tumour after the transplant depends on factors like age, the dose of radio and chemotherapy, the severity of the disease, Epstein-Barr virus infection and also on the genetics of the patient. It is difficult to elucidate the contribution of HSCT to the development of malignancies because the administration of immunosuppresive therapy (cyclosporine, ATG etc), also used prior to transplant may contribute to increase the risk of cancer. In a study performed by the European Bone Marrow Transplantation Group it was observed that the incidence of tumour development 20 years post-transplant was 42% in contrast to the 14% observed in patients with severe aplastic anemia with a different aetiology (Dufour et al., 2001).

7. NOVEL THERAPIES FOR FANCONI ANEMIA

7.1 GENE THERAPY

Gene therapy is one of the most promising new therapeutic approaches to overcome BMF in FA, because it has already shown its potential to cure other monogenic diseases. It consists of modifying the cellular genome by the introduction or substitution of genes mediated by viral (integrating or non-integrating) or non-viral vectors. Gene therapy is of special interest in monogenic recessive diseases caused by the loss of gene function, because it offers the possibility of restoring the defective gene by the introduction and expression of the correct copy of the gene (therapeutic gene) in the target cells. The outcome of gene therapy can be improved using methods to confer a proliferative or survival advantage to the target cells over non-transduced cells to increase the proportion of corrected cells in the patient (Qasim et al., 2007) (Corrigan-Curay et al., 2012).

In this decade several clinical trials have been initiated with promising results in adenosine deaminase-severe combined immunodeficiency (ADA-SCID) (Aiuti et al., 2002b; Thrasher et al., 2006), Wiskott-Aldrich syndrome (WAS), chronic granulomatous disease (CGH), adenoleukodystrophy and hemoglobinopathies, among others. In Table 3 representative HSC gene therapy protocols are shown.

DISEASE	INJECTED CELLS	VECTOR	REFERENCE
> **X-SCID** (X-linked Severe Combined Immunodeficiency)	**HSC**	**γ-RV**	(Cavazzana-Calvo et al., 2000)
> **ADA-SCID** (Adenosine Deaminase Deficiency-Severe Combined Immunodeficiency)	**HSC**	**γ-RV**	(Aiuti et al., 2002a)
> **X-CGD** (X-linked Chronic Granulomatosus Disease)	**HSC**	**γ-RV**	(Ott et al., 2006)
> **ALD** (Adrenoleukodystrophy)	**HSC**	**SIN LV**	(Cartier et al., 2009)
> **WAS** (Wiskott-Aldrich Syndrome)	**HSC**	**γ-RV**	(Boztug et al., 2010),
> **β-Thalassaemia**	**HSC**	**SIN LV**	(Cavazzana-Calvo et al., 2010)
> **X-SCID** (X-linked Severe Combined Immunodeficiency)	**HSC**	**γ-RV**	(Cavazzana-Calvo et al., 2000)
> **ALD** (Adrenoleukodystrophy)	**HSC**	**SIN LV**	(Cartier et al., 2009)
> **LDM** (Metachromatic leukodystrophy)	**HSC**	**SIN LV**	(Biffi et al., 2013)
> **WAS**	**HSC**	**SIN LV**	(Aiuti et al., 2013)

Table 3. Modified from Kaufmann (2013) (Kaufmann et al., 2013). Table representing the clinical trials carried out until now in hematologic and immune diseases, in which hematopoietic stem cells were modified and transplanted into patients.

Gene therapy can be classified in three types depending on the strategy followed:

1- Gene addition: introduces the correct and functional gene to be expressed in the target cells at a degree that allows the restoration of the genetic defect. In this type of gene therapy the expression of the transgene is usually conferred by an exogenous promoter.

2- Gene substitution: is based on the replacement of a defective gene by a correct form of it, either by substitution of a functional copy of the gene by HR or modifying only the non-functional part of the gene by exchanging defective nucleotides by the correct ones, to finally obtain a functional gene.

3- Gene suppression: consists of the elimination or reduction of the expression of a gene by knocking-out or knocking-down strategies, respectively, using techniques such as gene targeting or mRNA interference (Davidson and McCray, 2011).

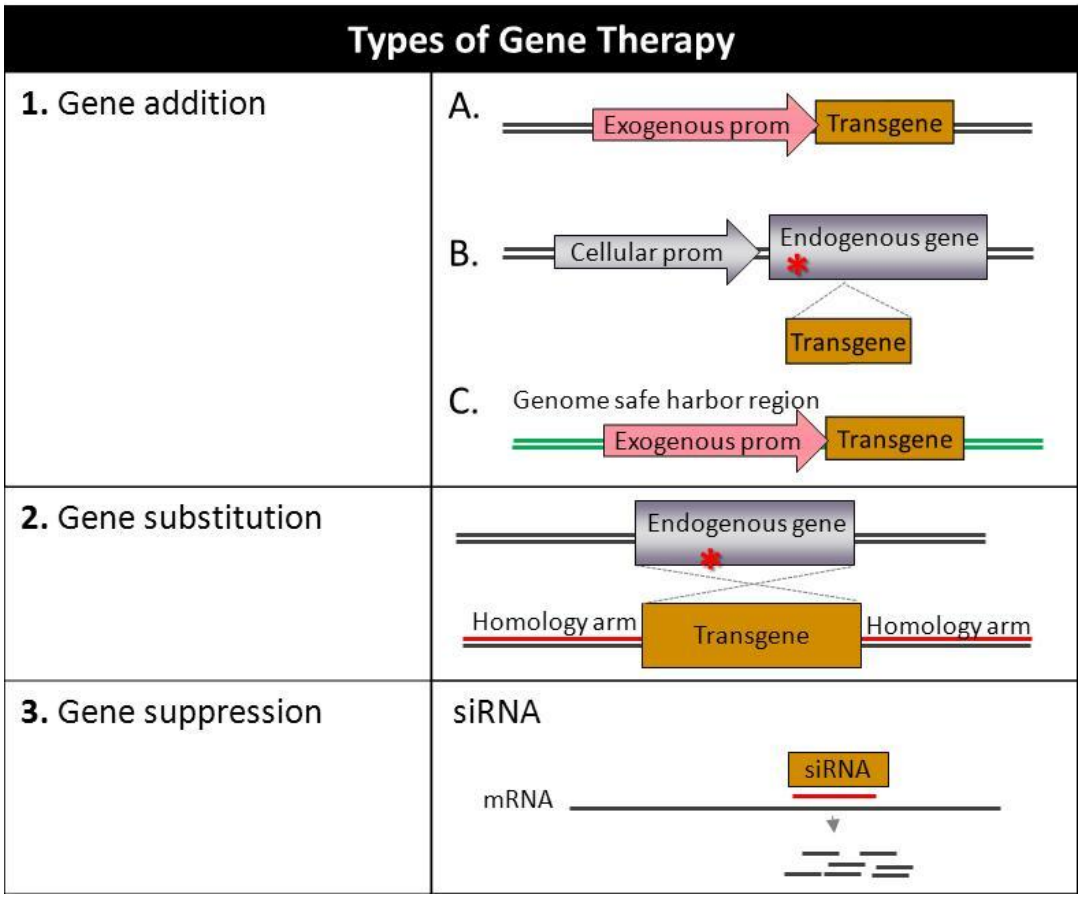


Table 4. Types of gene therapy strategies. **1. Gene** a**ddition** of the therapeutic gene **A)** Semi-random integration and expression driven by the promoter of the vector introduced. **B)** Knock-in strategy in which the transgene is introduced by homologous recombination (HR) in a determined genomic region and the expression is regulated by the cellular promoter. **C)** Consists of the safe harbour strategy by which the vector is introduced in a secure genomic site by HR and the expression is regulated by the exogenous promoter. **2. Gene substitution** of the mutation causing the disease by the correct nucleotides by the design of arms flanking the therapeutic gene, that have homology with the genomic region flanking the mutation and the substitution of the defective nucleotides by HR. **3. Gene suppression** is based on the design of vectors that can express siRNAs or specific nucleases to knock-out the gene.

According to the modality of treatment, gene therapy can be classified as ***in vivo* gene therapy** and ***ex vivo* gene therapy.**

In vivo gene therapy consists of the introduction of the therapeutic gene directly into the patient with the therapeutic vector.The *ex vivo* gene therapy is based on the *in vitro* transduction of the

cells. Once corrected, transduced cells are infused back into the patient, being this strategy more efficient and the most widely used in HSC gene therapy (Figure 9).

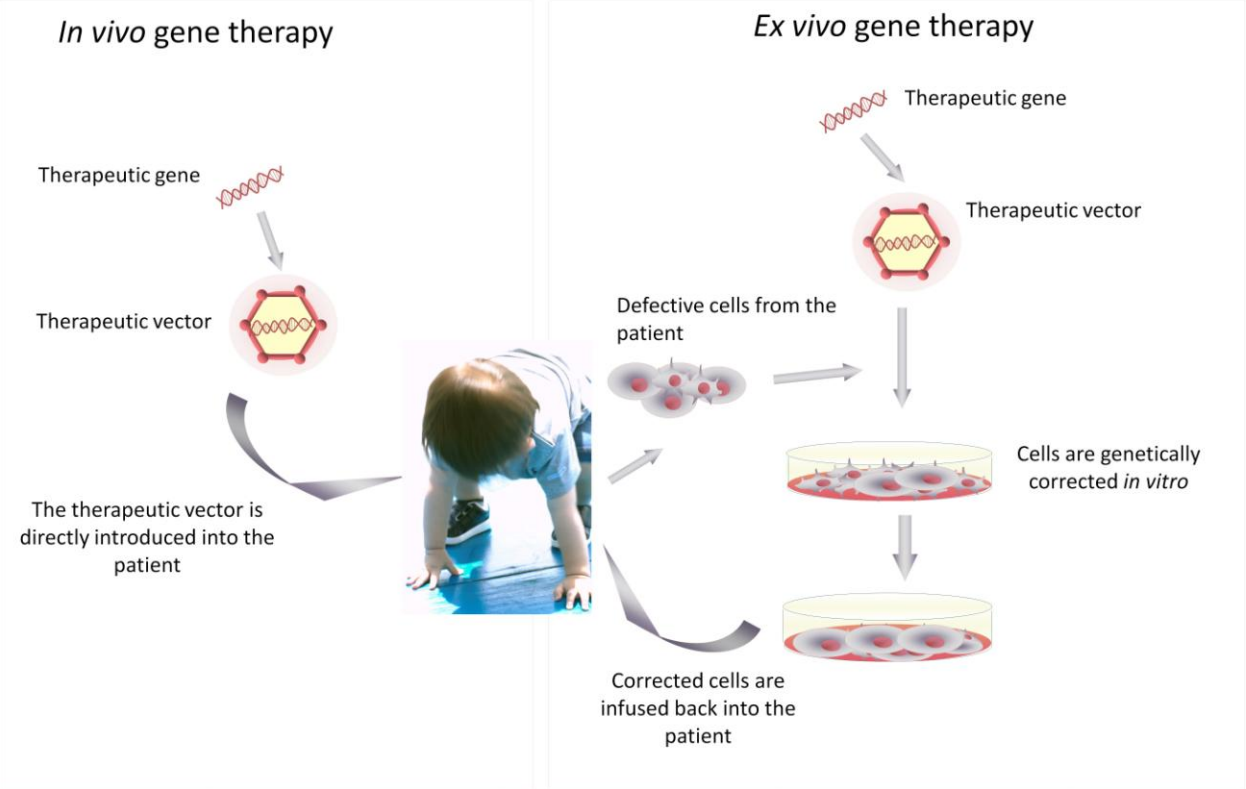


Figure 9. Basic diagram representing *in vivo* and ex *vivo* gene therapy protocols.

7.1.1 CONSIDERATIONS FOR GENE THERAPY

Several aspects must be considered for the development of efficient and safe gene therapy.

- **Type of gene**

The main limitation of the gene to be expressed is the size of the gene to be introduced in the gene therapy vector. The promoter chosen to activate the gene expression is also critical for the adequate level and stability of transgene expression, something that can be affected by the differentiation state of the cells and epigenetic changes that may occur *in vivo* (Manilla et al., 2005).

- **Type of vector**

Finding a good system to introduce the DNA of interest in the target cells is another critical aspect in the design of a safe and efficient gene therapy strategy.The viral vectors more widely used for hematopoietic gene therapy are the γ-retroviral (γ-RV) and lentiviral vectors (LV). They will be

described in detail later but in general they present certain limitations of semi-random integration and the maximum size of DNA that can be introduced. Relevant recent non-viral systems have been developed to deliver DNA into the cell genome using plasmids or transposons (Ivics et al., 1997) (Izsvak et al., 1997).

- *Ex vivo* manipulation

Ex vivo manipulation in gene therapy protocols allows the isolation of cells from the patient, the correction of the genetic defect in the cells and the introduction of the corrected cells back into the patient. This manipulation is generally more efficient and safer than the *in vivo* modification of the cells, because it prevents the patient to be directly exposed to the vector, and also allows, in some instances, the selection of transduced cells (Kaufmann, Buning et al. 2013).

- Efficiency of transduction and selective advantage

One relevant aspect that needs to be considered when performing gene therapy is the number of cells available to be transduced. In the case of HSCs, this number is frequently very limited. This limitation is particularly relevant if the percentage of transduced cells is low and transduced cells do not have a significant survival and/or proliferation advantage over non-transduced cells (Hacein-Bey-Abina et al., 2010; Hacein-Bey-Abina et al., 2002).

In some cases, cells corrected by gene therapy can develop a proliferative advantage that allows the efficient reconstitution of the affected system, with only a small number of corrected cells avoiding, in some cases, the use of conditioning regimes prior to the infusion of corrected cells. This was the case of gene therapy trials performed for X1-SCID patients (Kaufmann et al., 2013). In FA this selective advantage of cells corrected by gene therapy would be even stronger because in this case the affected cells include the HSC.

- Immunogenicity

It is also important to consider the possible reaction of the immune system to the vector and/or the transgene. To prevent this undesired reaction, different strategies, such as modifications of the viral capsid, have been developed to avoid the recognition of the viral capsids by the immune system of the recipient (Raper et al., 2003).

- **Genotoxicity or insertional oncogenesis**

Insertional oncogenesis is an important factor to consider mainly due to two aspects. The so called "clonal dominance" by which a determined cell population can grow at a higher rate leading to MDS, or leukemia (Kaufmann et al., 2013). Related to the potency of the promoter/enhancer element of the vector and the nature of the vector itself, the use of strong LTRs from γ-RVs and the preferential insertion close to the TSS of genes have generated some problems.

Due to these undesired events, integrating viral vectors have been modified to prevent them, by using self-inactivating LTRs (Sokolic et al., 2008). Weak promoters or promoters with specific activity in defined cell lineages are nowadays widely used in HSC gene therapy trials (van Til et al., 2012) (Vassilopoulos et al., 2001) (Throm et al., 2009).

7.1.2 GENE THERAPY VECTORS USED IN THIS WORK:

-Gamma-retroviral vectors (γ-RV)

γ-RVs are developed from the genome of γ-retroviruses that have a diameter of 80-100 nm and an encapsidated RNA based genome. Their envelope is formed by the membrane of the host cell and glycoproteins from the envelope encoded by the virus. The viral cycle goes from ssRNA through a double stranded DNA (dsDNA) intermediate that is able to integrate in the host genome, allowing this proviral DNA to replicate with the host DNA. The γ-RV genome usually has a size of 8-12 kb and is composed by two molecules of single stranded RNA (ssRNA) linked by hydrogen bridges, with positive polarity and a "capping" region at 5´and a "polyadenylation" region at 3´. There is also a transfer RNA that the virus needs for its replication. The complexity of the retroviruses is different among retroviral *genera*, having the simplest ones only the basic information and others also have regulatory elements. There are three genes that all γ-RVs share that are always located in the same order: 5´-gag-pol-env-3´ (Figure 10).

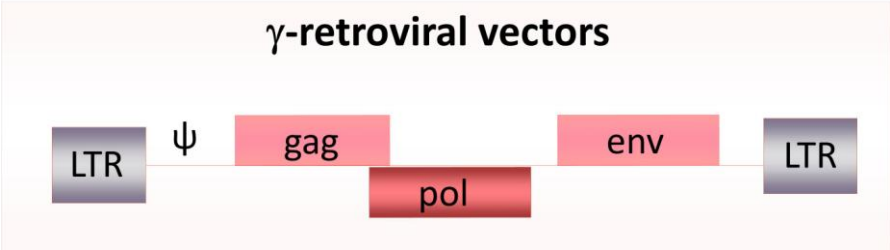


Figure 10. General representation of γ-RVs used in gene therapy assays, with the principal elements. LTR are the long terminal repeats located at both sides of γ-RVs that control essential activities such as transcriptional and post-transcriptional processes. Ψ is the encapsidation signal. gag, pol and env are the structural genes needed for vector production.

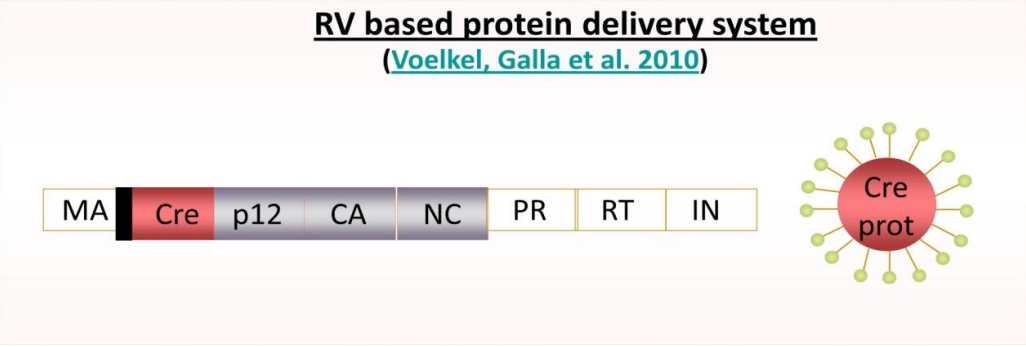
The principal components of integrating γ-RVs are:

- gag: this gene encodes the information for core and structural proteins: the proteins that form the capsid (CA), the matrix (MA) and the nucleo-capsid (NC).

- pol: encodes the reverse transcriptase (RT), the integrase (IN) and the protease that are essential proteins for viral integration.

- env: is the gene that encodes for the viral envelope. It is processed by the cellular proteases to generate the glycoprotein of its surface and the transmembrane protein. These two proteins form the complex that interacts with the cellular receptors and is the specificity of this interaction the one that determines the type of cells that the virus can infect (tropism).

As γ-RVs tend to integrate in areas both upstream and downstream of the TSS (Wu et al., 2003), in this basic structure of γ-RVs several modifications were introduced to prevent from possible adverse effects of integration into the host genome. Some of these modifications are the generation of **self-inactivating** (SIN) **γ-RVs** or the **γ-retroviral based protein delivery system** that was used in this work. This is based on the use of γ-retroviral particles specifically designed to transduce proteins in a targeted, controlled, dose dependent and transient manner into cultured cells. Precursors of the protein of interest are inserted into gag-protein precursors from γ-RVs and they assembly into infectious retroviral particles to escape the action of proteases in the endosome. They carry nuclear localization signals to facilitate that proteins are released by the retroviral protease in the nucleus of transduced cells (Voelkel et al., 2010) (Figure 11). This method results useful for vector excision in induced pluripotent stem cells (iPSCs) by the specific release of the Cre recombinase enzyme.



(Schwarze et al., 1999), (Dalba et al., 2007), (Anton and Graham, 1995), (Miura et al., 2009)

Figure 11. Retroviral based protein delivery system in which the Cre recombinase was cloned to be used as a vector removing system for vectors with LoxP sites.

-Lentiviral vectors

These vectors derive from a genus of the *Retroviridae* family and have the property of being able to transduce both dividing and non-dividing cells. They have the basic structure of γ-RVs (5´-gag-pol-env-3´) but they are more complex since they have accessory proteins that are involved in the regulation of the viral cycle (in human immunodeficiency virus (HIV)-derived vectors: vif, vpr, vpu, tat, rev, nef) (Manilla et al., 2005) (Figure 12).

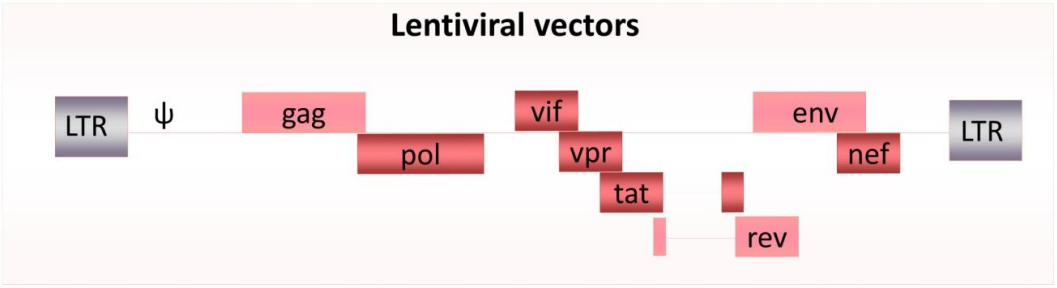


Figure 12. General representation of the lentiviral vectors backbone used in gene therapy with the principal elements. LTR are the long terminal repeats located at both sides of LVs that control essential activities such as transcriptional and post-transcriptional processes. Ψ is the encapsidation signal. Gag, pol and env are the structural genes needed for vector production. vif, vpr, vpu, tat, rev, nef correspond to accessory proteins that participate in the regulation of the viral cell cycle.

LVs, particularly the ones that derive from HIV, integrate preferentially in transcriptionally active genes. In some instances this can lead to the production of aberrantly spliced transcripts and deregulated gene expression (De Palma et al., 2005) (Yang et al., 2008) (Yang et al., 2008) (Cesana et al., 2012; Moiani et al., 2012). In contrast to γ-RVs, LVs do not have a preferential integration in regions close to the TSS of genes, something that markedly limits the trans-activation potential of LVs. In the so called "Self-Inactivating (SIN) LV vectors" the U3 region of the LTR is inactivated, allowing the use of different enhancer-promoters with improved safety properties. The design of more sophisticated vectors also includes the option of inserting strong RNA termination/polyadelination signals that act as insulators to prevent undesired gene trans-activation (Richard et al., 2001).

The genetic engineering of gene therapy vectors facilitates the modification of the natural tropism of the vector, and in consequence the cell type to be transduced. This can be done by replacing the natural viral envelope by other viral envelopes or by chimeric proteins, in a process known as vector pseudotyping. The most widely used envelope for lentiviral pseudotyping is the Vesicular Stomatitis Virus' G-protein (VSV-G) that is able to transduce many cell types (Cronin et al., 2005).

As many LVs derive from HIV, improved packaging systems have been progressively developed in which the vector production involves three plasmids (2nd generation LV vectors) or four plasmids (3rd generation LV vectors) (Figure 13).

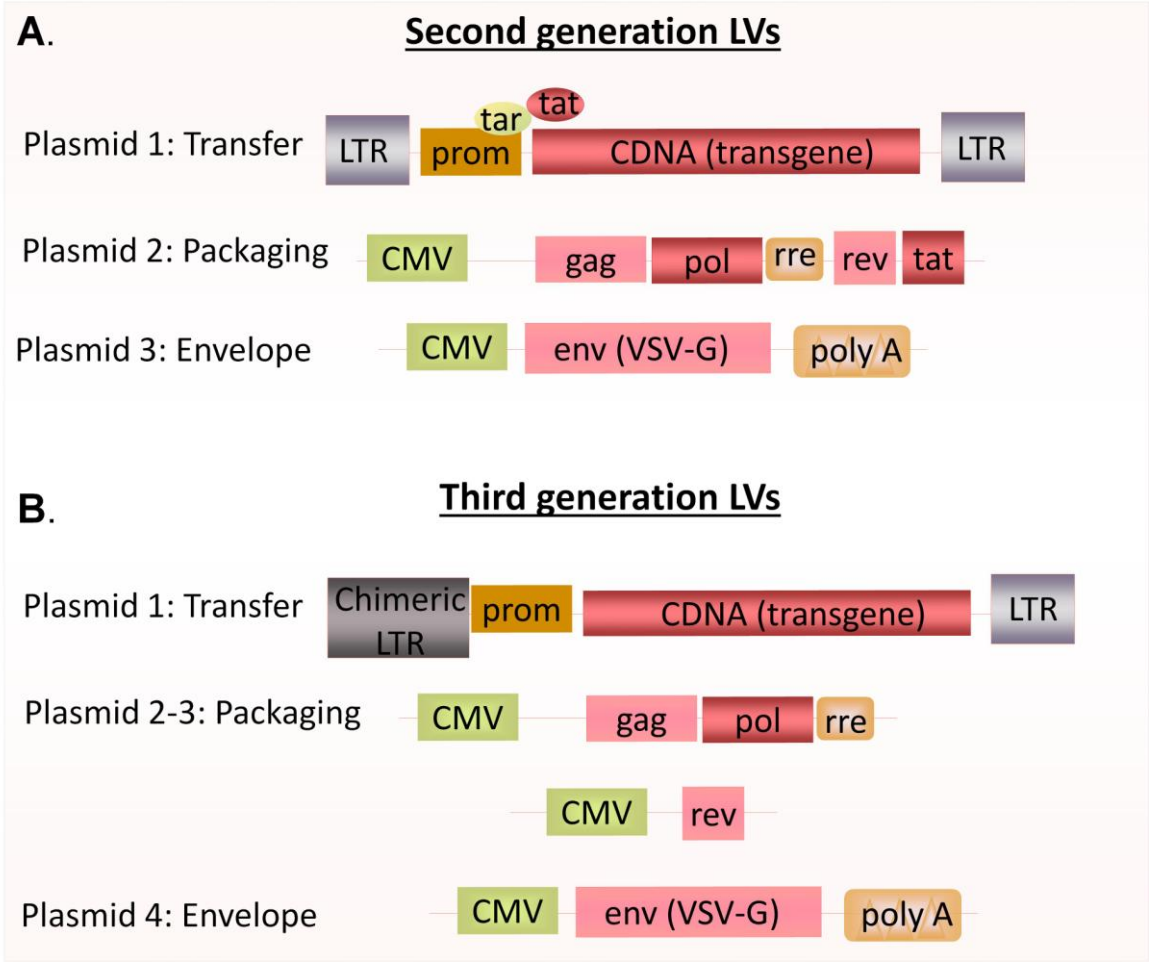


Figure 13. A) Plasmids used in 2nd generation LV production that involves the use of three plasmids. **Plasmid 1** corresponds to the construct that contains the gene of interest under the control of the adequate promoter. **Plasmid 2** contains the structural genes gag and pol and some regulatory elements and **plasmid 3** contains the viral envelope gene. **B)** Plasmids used in 3rd generation LV production that involves the use of four plasmids to increase the biosecurity of the process. **Plasmid 1** corresponds to the construct that contains the gene of interest under the control of the adequate promoter and 5' LTR is modified for the expression to be driven only by the internal promoter. **Plasmid 2** contains the structural genes gag and pol and some regulatory elements, **plasmid 3** contains the regulatory element rev. **Plasmid 4** harbours the envelope gene.

Several modifications have been made in lentiviral vectors, for example through the development of integration defective lentiviral vectors (IDLV) (Figure 14) in which the integrase is mutated to block the integration process (Brussel and Sonigo, 2004) and to avoid undesired effects of integrating LVs (Kulkosky et al., 1992; Shibagaki and Chow, 1997; Yanez-Munoz et al., 2006) even though a residual integration of these vectors has been already reported. IDLV vectors are competent at transcription and they efficiently express the transgene of interest for a short

period of time given that non-integrated episomes are lost within cell divisions (Philippe et al., 2006; Yanez-Munoz et al., 2006) (Figure 14).

Another kind of modified LVs are the ones designed to be induced or repressed in response to the administration of certain drugs “drug inducible LVs”.

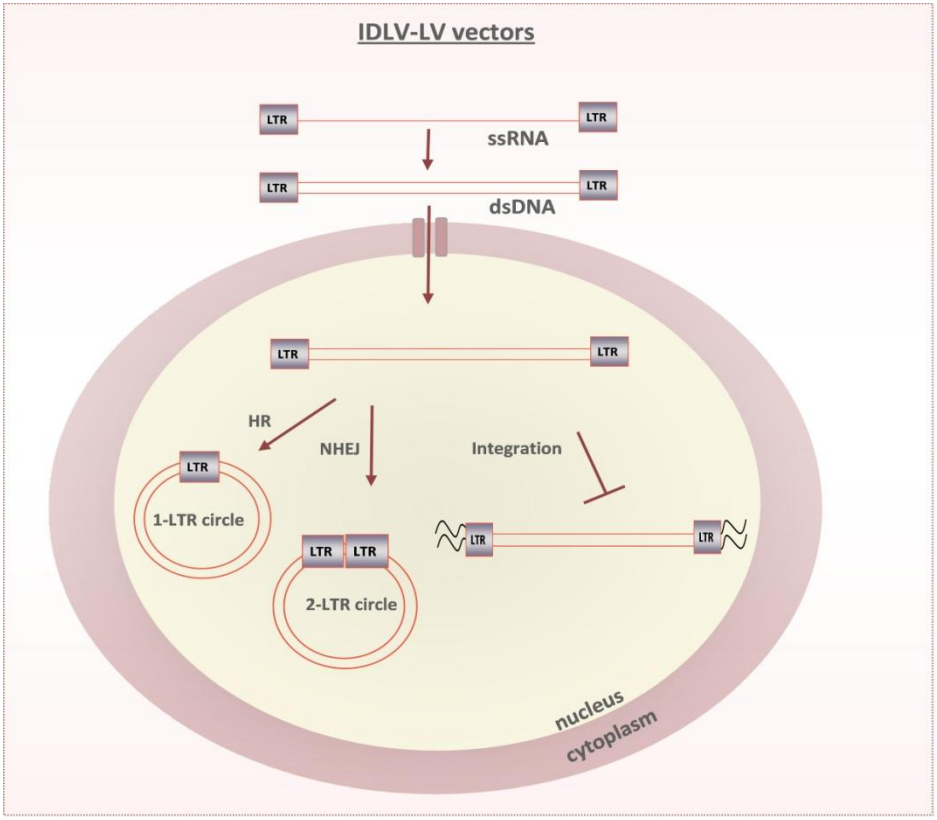


Figure 14. Non-integrating lentiviral vectors and their mechanism of action in the cell. The different LTR conformations that take place in the cell are represented. 1-LTR circle is generated after intramolecular homologous recombination between the LTRs with one single final LTR. 2-LTRs are generated when there is non-homologous- end-joining of linear episomal DNA.

7.2 NEW TOOLS FOR TARGETED GENE THERAPY BY HOMOLOGOUS RECOMBINATION

Recently, more refined strategies of gene modification have been developed that consist of the genome modification at specific sites, trying to avoid dangerous genomic localizations with a high risk of proto-oncogene activation and other undesired effects.

Tools based on HR have been designed to specifically modify the gene of interest by gene editing. As HR occurs at a low frequency in mammalian cells, efforts are directed to increase the HR rate, using a donor DNA construct, flanked by regions with homology to the target site. Although many studies have been performed to find optimal systems that increase the rate of HR in the cells, it

seems that the mechanisms that have a stronger effect on the rate of HR are the ones based on the generation of DSB in the target site (Vasquez et al., 2001), (Porteus and Baltimore, 2003), (Deng et al., 2012). These strategies use specific endonucleases that can recognize unique sites of the genome where they are able to generate a DSB and promote its repair by HR, thus improving the efficiency to introduce the gene of interest. Several endonucleases are used in gene targeting, being meganucleases, zinc finger nucleases, TALE nucleases and more recently the CRISPR/CAS nucleases, the most widely used (Grizot et al., 2010), (Mussolino et al., 2014; Pabo et al., 2001) (Wijshake et al., 2014)

7.3 GENE THERAPY IN FANCONI ANEMIA

FA constitutes a good candidate disease for being treated by HSC gene therapy strategies.
The gene therapy approaches proposed for FA aim the introduction of the correct sequence of the gene that is defective in the FA patient into his HSC. These corrected HSCs, would have a selective advantage over uncorrected cells, and therefore, the transduction of a small number of HSCs with the therapeutic vector would give rise to disease-free HSCs and finally to definitive differentiated cells. Gene therapy approaches for FA are particularly relevant for patients who do not have a HLA-identical donor for bone marrow transplantation (Rio et al., 2008).

So far, two gene therapy trials have been developed for FA patients with γ-RVs. The first one was carried out by Liu and colleagues in 1997 to treat FA patients of the FA-C complementation group. In this study the authors observed an increased number of bone marrow cells in treated patients but no clinical improvement was observed (Liu et al., 1999). The second study was developed for FA-A patients and was performed in 2007 by Dr. Williams. This study neither generated clinical improvements in the patients (Kelly et al., 2007).

There are certain obstacles that slowed down the gene therapy for FA to be a reality, like the difficulty of obtaining high numbers of HSCs from patients, once BMF was developed. Improved vectors were also required to achieve high transduction rates with the therapeutic vector.

The transduction efficiency of vectors carrying the required therapeutic gene was improved by reducing the culture time during transduction (Jacome et al., 2006), preventing oxidative stress and by the development of more sophisticated vectors, such as SIN LVs (Kee and D'Andrea, 2012) (Gonzalez-Murillo et al., 2010).

The lack of success observed in previous FA gene therapy trials led researchers to think about improvements and considerations that should be taken into account to develop safer and more efficient gene therapy trials to treat this disease. Thus, at the International FA Gene Therapy Working Group that was held in 2011, worldwide experts in the field, set up the conditions for future FA gene therapy trials to reduce genotoxicity and to assure stable expression of the transgene (Tolar et al. 2011). In Europe these studies are coordinated by our laboratory, and another gene therapy protocol is being conducted at the Fred Hutchinson Center in Seattle, USA. In our laboratory a SIN LV vector developed by Gonzalez-Murillo et al. (Gonzalez-Murillo et al., 2010) was designated as an Orphan drug in 2010 by the European Comission (EU 3/10/822) and was approved for a Phase I/II Gene Therapy Trial for FA-A patients, the most common complementation group in Spain (Antonio Casado et al., 2007). The proposed protocol will be carried out by multiple centres all over Europe and it is believed to improve the clinical efficacy observed in the two protocols previously performed.

8. FANCONI ANEMIA MOUSE MODELS

Mouse models represent a powerful tool in basic and translational research because they allow to deeply investigate the mechanisms underlying the disease and also to evaluate the efficacy and toxicity of new experimental therapies.

In FA, different mouse models have been generated to improve the understanding of the molecular basis of the disease and to mimic the phenotype observed in the affected patients. In the mouse models developed until now, several FA genes have been deleted by knock-out strategies, in which one FA gene or part of it was deleted in ESCs by HR, allowing the inheritance of the genetic modification by the offspring. With the generation of these models, important information was revealed about how FA pathway protects against genomic instability and how to develop safer and more efficient therapies for FA. In this respect, it was observed that the disruption of FA pathway affects viability in mice, especially in certain backgrounds, being the C57/BL6J mouse strain the one that produces more lethality (van de Vrugt et al., 2009).

Most of the mouse models developed so far present some of the typical features observed in FA patients, such as cell hypersensitivity to DNA ICL agents (MMC and DEB) and to some cytokines like IFNγ or TNFα, and also impaired fertility. From the single FA gene mutants, none of these models was able to reproduce the BMF observed in patients. Among these models, the

Slx4/Fancp$^{-/-}$ *(Btbd12*$^{-/-}$*)* mouse model that presents low white blood cell (WBC) and platelet (PLT) counts (Crossan et al., 2011), the *Brca2/Fancd1*$^{\Delta 27/\Delta 27}$ hypomorphic model, with proliferation defects in hematopoietic progenitor cells (McAllister et al., 2002a) (Houghtaling et al., 2003) (Navarro et al., 2006) are the ones that more closely resemble the disease observed in FA patients. Cells lacking *FANCD1/BRCA2* gene are the only ones from all FA gene deficiencies that do not form RAD51 *foci* after damaging their DNA with ICL agents, although cells deficient in BRCA2 partner *FANCN/PALB2*, also have an impaired capacity to form RAD51 *foci (Xia et al., 2007)*, thus proving the importance of this protein in repairing the DNA by HDR (Godthelp et al., 2006).

The most frequent developmental abnormality observed in FA mouse models is microphtalmia (Carreau et al., 1998; Crossan et al., 2011; Houghtaling et al., 2003; Wong et al., 2003), although it has not been described in all the models. A high percentage of *Slx4/Fancp*$^{-/-}$ *(Btbd12*$^{-/-}$*)* mice show an abnormally shaped and enlarged skull in C57/BL6J strain (Crossan et al., 2011).

The embryonic and perinatal lethality is common to different FA mouse models observed in *Fancd1*$^{\Delta 27/\Delta 27}$*, Fancn*$^{-/-}$*, Fanco*$^{-/-}$*, Fancl*$^{/-}$ *Fancd2*$^{-/-}$*, Fancm*$^{-/-}$*and Fancp*$^{-/-}$ (Agoulnik et al., 2002; Bakker et al., 2009; Crossan et al., 2011; Houghtaling et al., 2003; Marmorstein et al., 1998; Rantakari et al., 2010). *FANCD1* and *FANCN* are lethal in humans as well, and this is the reason why some of these mouse models are hypomorphic rather than knock-out (Alter, 2007; Tischkowitz and Xia, 2010). In some models the embryonic lethality was bypassed by generating conditional models. In other cases, genes such as *p53* were also deleted to increase viability (Jonkers et al., 2001).

Apart from the fact that very few models show evidences of BMF, mouse models do not show many of the hematologic defects observed in FA patients. Some FA models have a predisposition to develop cancer. This is the case of *Fancd1*$^{\Delta 27/\Delta 27}$ (high incidence of epithelial cancer) (McAllister et al., 2006) or *Fancm*$^{-/-}$. Nevertheles, it is very rare that FA mouse models spontaneously develop AML. Recently, the hypothesis that aldehydes could contribute to the pathology of FA has been supported by studies performed in which double mutants for *Fancd2* and *Aldh2,* one of the enzymes in charge of metabolizing aldehydes, were not viable and the very low proportion of surviving mice had a high γH2AX induction, indicative of DSBs in the DNA (Langevin et al., 2011).

The FA mouse models existing nowadays are described in Table 5.

FANCONI ANEMIA MOUSE MODELS			
GENE	**DISRUPTION**	**REFERENCE**	**PHENOTYPE**
Fanca	Exons 4-7 replaced by *neo*	Cheng et al. *(Hum. Mol. Genet., 2000; 9:1805)* (Cheng et al., 2000)	Infertility, hypogonadism, genetically unstable and hypersensitive to ICL agents (MMC) fibroblasts. No signs of anemia, although there is a small PLT decrease and rise of erythrocytes.
Fanca	Exons 1-6 replaced by *LacZ*	Wong et al. *(Hum Mol Genet, 2003; 12(16):2063-76) (Wong et al., 2003)*	Microphtalmia, infertility and defects in meiosis. Retarded growth, chraneal malformations, germ line apoptosis and hypersensitivity to ICL agents. No hematologic abnormalities.
Fancc	Exon 8 replaced by *neo*	Chen et al. *(Nature Genet., 1996; 12:448)* (Chen et al., 1996)	Infertility, hypersensitivity of spleen cells to ICL agents (DEB and MMC). No hematologic or developmental anomalies until 9 months of life.
Fancc	Exon 9 replaced by *neo*	Whitney et al. *(Blood, 1996; 88:49) (Whitney et al., 1996)*	Infertility, hypersensitivity to ICL agents. Reduced capacity of forming progenitor colonies in vitro from BM cells although no anemia was observed in PB. HPC sensitive to IFNγ.
Fancg	Exons 2-9 replaced by *neo*	Yang et al. *(Blood, 2001; 98: 3435)* (Yang et al., 2001)	Reduced fertility. Low repopulating ability. Defects in mobilization and homing. Normal telomere length. Spontaneous chromosomal breakage and sensitivity to MMC.
Fancd1 *(Brca2$^{\Delta 27/\Delta 27}$)*	Exon 27	McAllister et al. (Cancer Res. 2003; 62: 990) (McAllister et al., 2002a)	Infertility, hypersensitivity to ICL agents (MMC). Light reduction of perinatal lethality. Increased probability of cancer and reduced survival. Low short and long-term repopulating ability and low HPC *in vitro* proliferation.
Fancd2	Replacement of exons 26-27 with *neo*	Houghtaling et al. (Genes & Dev. 2003; 17: 2021) (Houghtaling et al., 2003)	Microphtalmia, infertility, hypersensitivity to ICL agents. Perinatal lethality, especially in C57/BL6J strain, deficiencies in meiosis, low repopulating ability. Increased cell cycle entry and loss of quiescence. High incidence of epithelial tumours and telomere dysfunction.
Fancd2	Intron 1 replaced by *pur*	Parmar et al. (2010) (Parmar et al., 2010)	

Usp1		**Kim et al. *(Dev. Cell, 2009, 16(2):314-20)(Kim et al., 2009)***	**Male infertility, hypersensitivity to ICL agents (MMC). Increased perinatal lethality.Hypoplasia in BM.**
Fancd2/p53		**Houghtaling et al. (*Cancer Res, 2005;65:85–91*) (Houghtaling et al., 2005)**	**Infertility, hypersensitivity to ICL agents. Increased tumours in comparison with the single *Fancd2* mutant. Cells do not stop at S-phase due to genotoxic stress derived from *p53* suppression.**
Fancc/ Sod1		**Hadjur et al. *(Blood, 2001; 98:1003)* (Hadjur et al., 2001)**	**Infertility, hypersensitivity to ICL agents. Increased production of superoxide by hepatocytes *in vitro*. Hypocellular bone marrow and decreased WBC and red cells in PB. Low number of lineage commited progenitors *in vitro.***
Fancc/p53		**Freie et al. *(Blood, 2003; 102: 4146)* (Freie et al., 2003)**	**Infertility, hypersensitivity to ICL agents .Tumour development early in life.**
Fancd2/Mlh1		**van de Vrugt HJ et al. *(Cancer Research, 2009, 69(24):9431-8) (van de Vrugt et al., 2009)***	**Infertility, hypersensitivity to ICL agents in fibroblasts (MMC). Delayed growth.**
Fancm	**Exon 2 replaced by *LoxP neo***	**Bakker et al. *(Hum Mol Genet, 2009; 18(18):3484-95)* (Bakker et al., 2009)**	**Perinatal lethality; in C57/BL6J background there are less females in the offspring. Infertility, hypersensitivity to ICL agents Increased incidence of cancer and reduced lifespan.**
Fancc/Fancg		**Pulliam-Leath et al. *(Blood, 2010, 116(16):2915-20)* (Pulliam-Leath et al., 2010)**	**Infertility, hypersensitivity to ICL agents. High incidence of bone marrow failure and tendency to develop AML and MDS.**
Fancp (Slx4)	**Insertion of *b-geo* in intron 3 and *LoxP* mediated deletion in exon 3**	**Crossan et al. *(Nat Genet., 2011, 43(2):147-52)* (Crossan et al., 2011)**	**Infertility, hypersensitivity to ICL agents. Microphtalmia and in some cases abnormally shaped and enlarged skull. Prerinatal lethality. Reduced WBC and PLT counts. Premature senescence and tendency to accumulate spontaneous DNA damage.**
Fancl	**150kb on chr. 1 (exons 4-14) replaced by goat *b-globin* gene**	**Agoulnik et al., 2002, *Hum. Mol. Genet.* (2002) 11(24): 3047-3053 (Agoulnik et al., 2002)**	

Fancn	**Intron 1 replaced with *b-geo***	**Rantakari et al. 2010, *Hum. Mol. Genet.* (2010) 19(15): 3021-3029 (Rantakari et al., 2010) Bouwman et al.,2011, J. Pathol., 224: 10–21 (Bouwman et al., 2011)**	
Fandf	**Exon 1 replaced by *neo***	**Bakker et all. J Pathol. 2012 Jan;226(1):28-39 (Bakker et al., 2012)**	**Infertility, hypersensitivity to ICL agents. Reduced capacity to monoubiquitinate *Fancd2*. Compromised survival and high incidence of tumours.**
Fanco	**Introduction of G>A splice-site mutation in intron 5**	**Smeenk et al., 2010, Mutat Res. 2010 Jul 7;689(1-2)(Smeenk et al., 2010)**	
Fanco	**Exons 2-3 replaced with *LoxP neo***	**Kuznetsov et al.,2007, J Cell Biol. 2007February26;176(5) (Kuznetsov et al., 2007)**	
Fancd2/Aldh2		**Garaycoechea et al. *(Nature, 2012, 489(7417):571-5) (Garaycoechea et al., 2012)***	**Infertility, hypersensitivity to ICL agents. Developmental defects and susceptibility to develop AML and to the toxic effects of ethanol. Adults that do not develop spontaneous leukemia develop aplastic anemia with accumulated damage in CPHs/CMHs.**

Table 5. Fanconi anemia existing mouse models.

The *Brca2* hypomorphic mouse model (*Fancd1/Brca2*$^{\Delta 27/\Delta 27}$) was generated in 2002 by McCallister et al. (McAllister et al., 2002a) by a deletion of exon 27, last exon of *Brca2* gene. This model was characterized in 2006 by Navarro et al. (Navarro et al., 2006) from our laboratory and by that time it was the model that more closely resembled the phenotype of human FA patients. Importantly, *Brca2*$^{\Delta 27/\Delta 27}$ mice showed spontaneous chromosomal instability, even in the absence of DNA damage, and also reduced proliferative potential of CFCs and HSCs compared to control mice or to other FA mouse models. Finally, these mice showed a defective competitive repopulating potential *in vivo*, compared to wild type (wt) cells. Because of the relevance of this model in the field of FA, this was the model that we chose in this PhD work.

B. CELL REPROGRAMMING

1. STEM CELLS

Stem cells have the ability to self-renew, differentiate and to undergo two different types of cell division, symmetric division, in which the two daughter cells are either differentiated cells or stem cells and asymmetric division, in which the progeny is composed by a stem cell and a partially differentiated cell (Morrison and Kimble, 2006; Tajbakhsh et al., 2009). Stem cells can be divided in four categories based on their potency to generate different cell types (Figure 15 and Table 6).

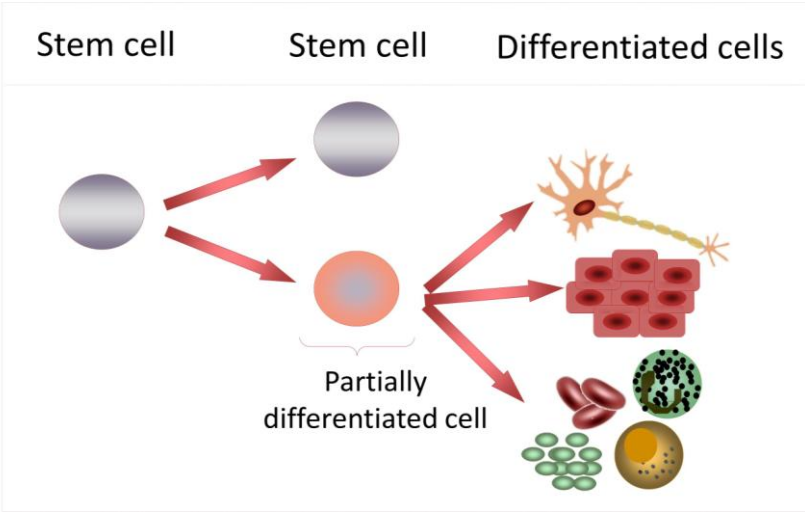


Figure 15. Stem cells and the differentiation pattern that they can follow to maintain the stem population and also to generate differentiated cells. Stem cells have the particularity that they can divide simetrically, giving rise to new stem cells (self-renew) or to differentiated cells and assimetrically, differentiating into a stem cells and a maturing cell type (differentiation).

STEM CELL CLASSIFICATION IN TERMS OF DIFFERENTIATION POTENTIAL		
Totipotent	They can generate **all cell types** (embryonary and extra-embryonary tissues)	Zigote and 1st blastomere division
Pluripotent	They generate **all lineages** of the individual (embryonary tissues)	Oocyte, ESC, MAPCs..... iPSCs
Multipotent	Capacity to generate **all the cells from a lineage** (Somatic cells)	Hematopoietic Stem Cells (HSCs)
Unipotent	Generation of **only one type of cells**	Spermatogonia

Table 6. Stem cell classification in terms of their differentiation potential.

1.1 EMBRYONIC STEM CELLS (ESC)

Embryonic stem cells are pluripotent cells able to generate cells from all the lineages of the individual, including germ cells, with the exception of the extra-embryonary tissues.

ESC isolation dates from the 80´s, when the researchers Evans and Kaufman in the UK and Martin (Martin, 1981) in the US, derived ESC from the inner cell mass (ICM) of 2.5 days blastocysts.

It is important to mention that ESCs have a high telomerase activity, a ribonucleoprotein enzyme in charge of compensating for telomere shortening during cell division by synthesizing telomeric DNA, thereby maintaining the telomere length. This activity allows ESCs to self-renew without undergoing senescence (Hiyama and Hiyama, 2007).

ESCs have the capacity to form teratomas, tumours of embryonic origin that contain cells of the three embryonic layers, endoderm, mesoderm and ectoderm. Altough human ESC (hESC) and mouse ESC (mESC) are very similar, they differ in their properties. Some molecular and functional characteristics of mouse ESCs (mESCs) are represented in Table 7.

MOUSE EMBRYONIC STEM CELL (mESC) PROPERTIES	
SSEA-1 ----------Positive Alkaline phosphatase (AP) -----Positive Oct3/4 ----------Positive Nanog ----------Positive Sox2 ----------Positive Telomerase activity----------Positive Teratoma formation ----------Positive Chimera formation----------Positive	**Growth characteristics *in vitro*:** Round and tight colonies, 3D growth, syncytia formation. **Feeder cell dependant**: Inactivated embryonary fibroblasts (MEFs) either by irradiation or by treatment with MMC. **LIF** (leukemia inhibitory factor) required for their maintenace *in vitro*.

Table 7. Main properties of mouse embryonic stem cells.

Despite the promising future of hESC to be used in cell therapy, there are several biologic limitations, mainly derived from aspects related to allogeneic transplantation that should be circumvented for their use. There are also important ethical problems regarding the obtainment and use of these cells.

1.2 HEMATOPOIETIC STEM CELLS (HSC)

HSCs are adult stem cells with multipotent capacity. These cells are very rare in bone marrow, representing only $1/10^4$-$1/10^5$ in total bone marrow cells. They cannot be recognizable by morphology and have a low rate of renewal. The hematopoietic system is a hierarchical system in which HSCs generate committed progenitors that can only be recognizable by cellular markers or by *in vitro* clonogenic cultures (Lemieux et al., 1995).

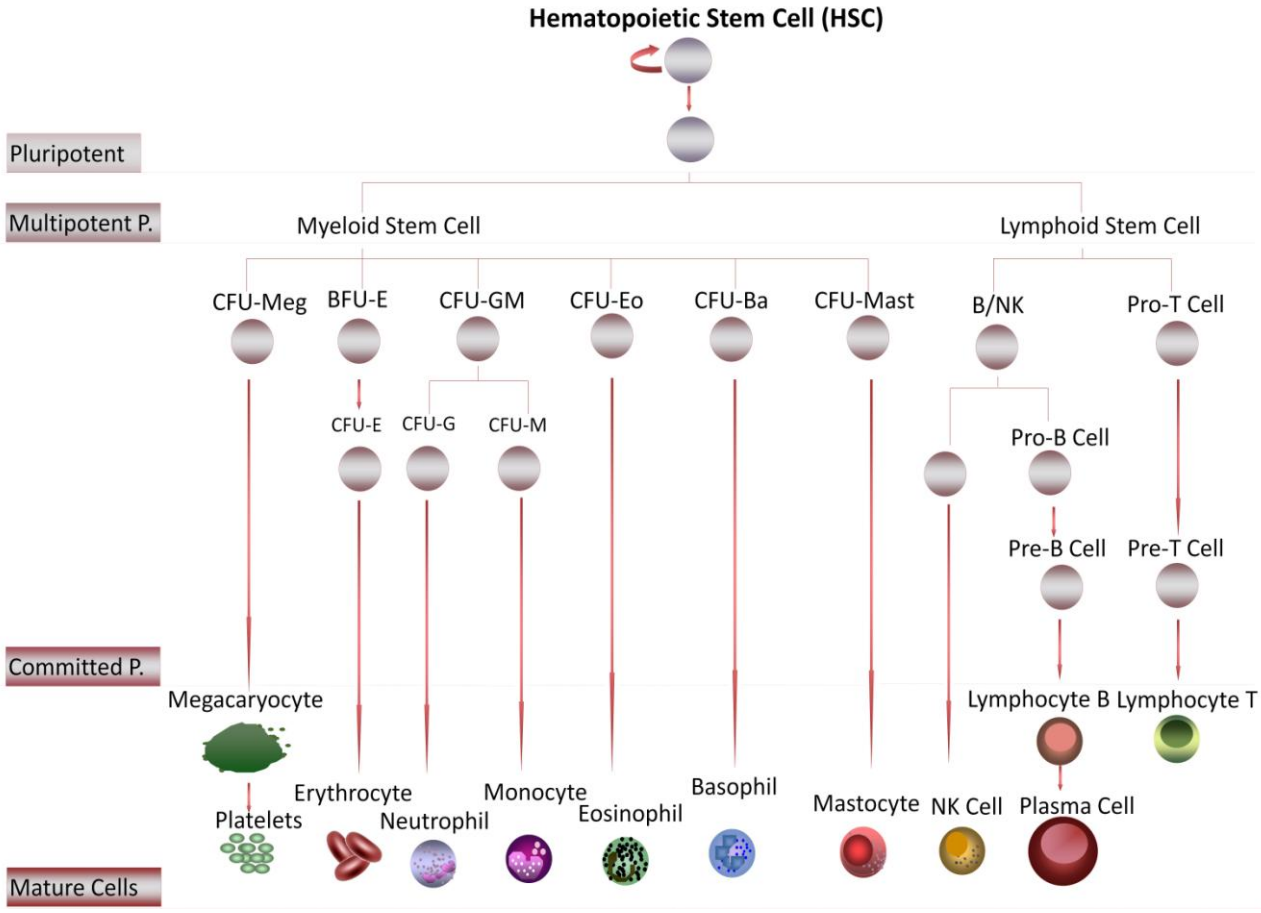


Figure 16. Diagram of the hematopoietic system with the different cell populations along differentiation of the hematopoietic stem cell to give rise to all cell types present in blood.

HSCs were identified by the expression of a variety of surface markers. Their functional properties could be determined by transplants into lethally irradiated mice (Morrison and Weissman, 1994). The assays that best define the HSCs are the *in vivo* competitive repopulating assays in which the functionality of these cells is proven by their ability to restore and reconstitute the hematopoietic system of irradiated mice (Harrison, 1980).

The phenotypic characterization of the HSCs has shown that these cells are characterized by the lack of expression of lineage markers, either erythroid, monocytic, granulocytic or lymphoid, together with an elevated expression of $Sca1^+$ and $ckit^+$ specific markers of undifferentiation. This population is known as LSK (Lin^-, $Sca1^+$, $ckit^+$) (Figure17).

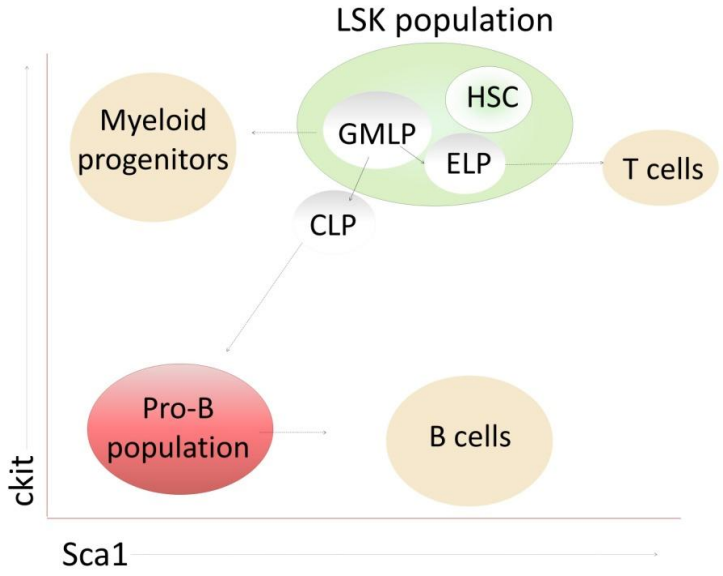


Figure 17. LSK population (Lin^-, $Sca1^+$, $ckit^+$) represented in green colour that is double positive for *ckit* and Sca1. The population to which these cells give rise when differentiated are also represented in beige and red colours.

Among LSK population, other markers have been found in the long-term HSCs (LT-HSCs -Lin^-, $Sca1^+$, $ckit^+$, $Flk2^-$, $Thy1^{low}$, $CD34^-$-), in the short term HSCs (ST-HSC - Lin^-, $Sca1^+$, $ckit^+$, $Flk2^-$, Thy1, $CD34^+$ -) and in the primitive progenitors (MPP - Lin^-, $Sca1^+$, $ckit^+$, $Flk2^+$, $CD34^+$) (Ratajczak, 2008).

More recently, the SLAM code was proposed as a system for HSC characterization based on the surface markers CD150, CD244 and CD48. Based on this, the HSC are characterized by the $CD150^+$, $CD244^-$ and $CD48^-$ phenotype, while primitive progenitors express $CD150^-$, $CD244^+$ and $CD48^-$, and most restricted progenitors express $CD150^-$, $CD244^+$and $CD48^+$ (Kiel et al., 2005) (Figure 18).

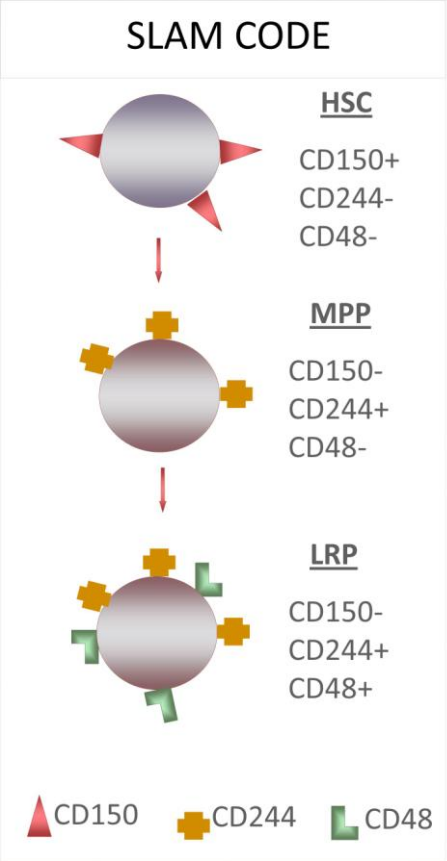


Figure 18. SLAM code for the characterization of HSC populations based on the expression of CD150, CD244 and CD48.

During embryogenesis, mouse HSCs, follow a differentiation that passes through an intermediate stage called hemangioblast defined by the expression of the mesodermal markers Flk1^{+} and Brachyury^{+}, and then they differentiate to hemogenic endothelium (Tie2^{high}, ckit^{+}, *VE cadherin*$^{+}$ and CD41^{-}) from which cells would differentiate both to endothelium (CD31^{+}) and to primitive (CD41^{+}, CD45^{-}) and definitive hematopoiesis (CD41^{+}, CD45^{+}). In this transition from endothelial to hematopoietic fate, the cells undergo several changes in their expression of adhesion molecules and their pattern of expression of endothelial related genes, losing the expression of hemogenic endothelial surface markers and acquiring hematopoietic like markers and features (ie; round shape and mobility) (Figure 19) (McKinney-Freeman et al., 2009), (Lancrin et al., 2012).

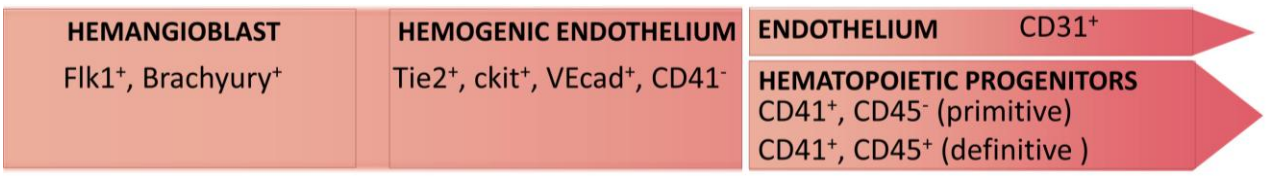


Figure 19. Model of hematopoietic differentiation through an intermediate hemogenic endothelium that gives rise both to endothelial cells and to hematopoietic progenitor cells.

1.3 INDUCED PLURIPOTENT STEM CELLS (iPSCs)

In the year 2006, a new horizon of future opportunities in basic and translational research opened when Shinya Yamanaka and Kazutoshi Takahashi demonstrated for the first time that mouse fibroblasts could be reprogrammed to an embryonic state through the delivery of only four pluripotency genes into their genome. These transferred genes were *Oct3/4* (encoded by the *Pou5f1* gene), *Sox2* (cooperates with *Oct3/4* to regulate the expression of target genes involved in pluripotency), *Klf4* (Kruppel-like factor 4 highly expressed in undifferentiated cells) and *c-Myc* (a proto-oncogene that regulates genes involved in cell proliferation, differentiation and apoptosis) (Figure 20).

The resulting cells had an ESC-like morphology and other properties such as 3D compacted colonies, high proliferation rate, teratoma formation capacity etc. In contrast, these cells did not reproduce the ESC genetic expression pattern and methylation status (Yamanaka and Takahashi, 2006). One year later Yamanaka´s group refined the technique solving that problem and being able to generate chimeras (Okita et al., 2007).

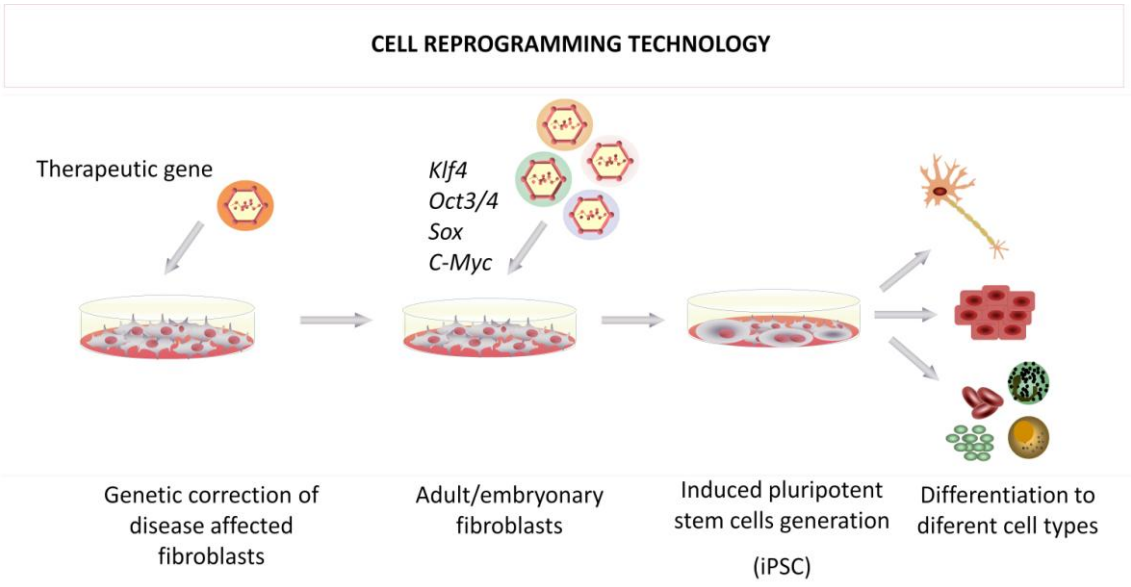


Figure 20. Cell reprogramming protocol after correction of the genetic defect by gene therapy.

Jaenish et al. contributed to the very new field of cell reprogramming obtaining similar results to the ones published by Okita, without introducing selection markers into the fibroblasts (Takahashi et al., 2007). Simultaneously, this approach was improved and extended to human cells, both healthy and affected by a variety of genetic defects (Eminli et al., 2009; Hanley et

al., 2010), using several cell sources like neonatal and adult fibroblasts (Park et al., 2008a; Park et al., 2008b) as well as blood progenitors (Loh et al., 2009). Later on, many different strategies were developed for the reprogramming of mouse and human cells. The most commonly used methods for the introduction of the reprogramming factors are represented in Figure 21.

Cell reprogramming technology provides an optimal source of cells to investigate embryonic development. Furthermore, iPSCs derived from patients or animal models of disease constitute important tools for modelling and understanding the pathogenesis of the molecular defects underlying the disease. The use of iPSCs is particularly important in BMF diseases, such as FA where HSCs are severely affected. They can also serve as a platform to test different methods or compounds that can restore the normal phenotype in disease-affected cells.

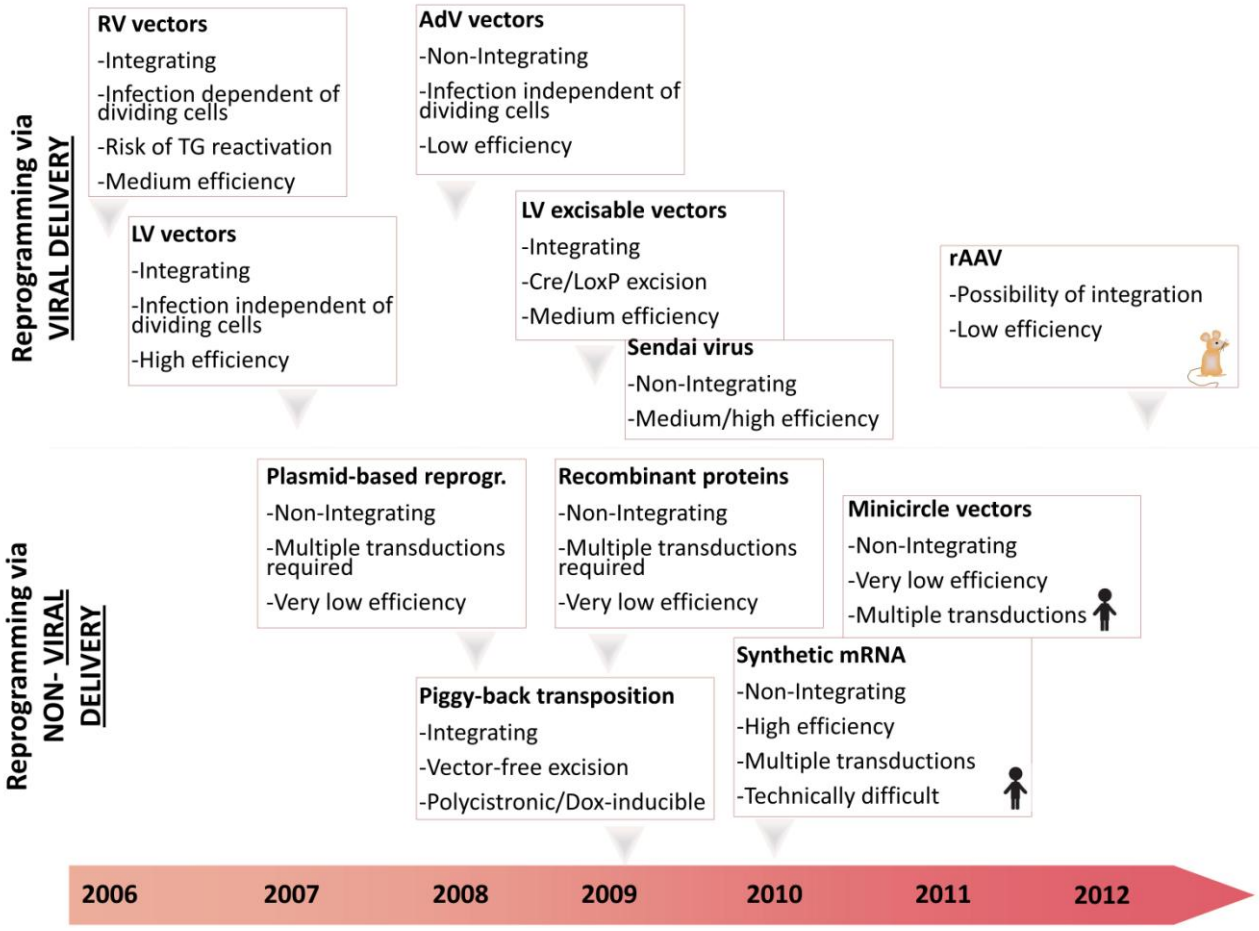


Figure 21. Different methods for cell reprogramming based on viral and non-viral systems and the moment in which each technology was developed.

2. SPECIFIC LIMITATIONS IN CELLULAR REPROGRAMMING

Studies performed since the cell reprogramming technology was developed showed different patterns of gene methylation among different cell sources, suggesting that there might be a certain epigenetic memory intrinsic to the cell type of origin and that this may condition both the reprogramming and the differentiation capacity of these cells (Chin et al., 2009), (Deng and Xu, 2009). Another hypothesis supported that these differences could be due to variability among different iPS clones (Bock et al., 2011) (Lengner et al., 2010) (Newman and Cooper, 2010).

So, it seems that there are some critical factors to consider in cell reprogramming that can influence the expression of the reprogramming factors. For example, the age of the cells used for reprogramming is important and their DNA should be as less damaged as possible.

At a molecular level, it has recently been shown that cells with telomere shortening, with deficiencies in DNA repair pathways, or with DNA damage caused by exogenous factors, are not able to efficiently reprogram their genome. Similarly, *p53* was shown to be critically involved in the abrogation of the reprogramming of cells with harmed DNA (Raya et al., 2009) (Marion et al., 2009) (Marion and Blasco, 2010) (Muller et al., 2012a). The studies of Raya et al. and Müller et al. showed that cells deficient in FA genes cannot be efficiently reprogrammed due to their characteristic phenotype. These last authors reported that this impairment in cell reprogramming efficiency could be overcome by using low oxygen concentration during iPSC generation (Muller et al., 2012b).

It is also known that cells in which the telomere homeostasis is altered and that have mutations in genes of signalling pathways involved in DNA damage repair systems, such as signal transducer genes (*ATM* or *53BP1*), nucleotide excision repair genes (*XPC* and *RAD23B*) are not able to efficiently wind back their genetic program to a pluripotent state (Marion and Blasco, 2010).

Prior to our studies, the implication that proteins located downstream in the FA pathway, such as FANCD1/BRCA2, had in cell reprogramming was unkown.

3. HEMATOPOIETIC DIFFERENTIATION OF iPSC

The arose of the iPSC technology allowed the envision of a new field in regenerative medicine through the generation of iPSCs-derived cells capable of generating a large repertoire of cell types.

In the field of hematopoietic diseases, the generation of iPSC-derived hematopoietic progenitors has been challenging, due to the difficulties to generate hematopietic repopulating cells.

Although many different hematopoietic differentiation protocols have been proposed, the most efficient ones were based on the generation of embryoid bodies (EBs) and the sequential addition of determined cytokines. This allowed the differentiation of iPSCs towards mesoderm, and then to the hematopoietic populations of interest. In some cases the co-culture of iPSC-derived cells with hematopoietic stromas secreting hematopoietic cytokines and adhesion molecules improved the hematopoietic differentiation of iPSCs. It has also been described that genes from the LIM homeobox family play an important role in the differentiation and expansion of HSCs both *in vitro* and *in vivo*. Thus, the ectopic expression of *HOXB4* (Sauvageau et al., 1995) or *LHX2* (Kitajima et al., 2011) has been used to improve self-renewal of HSC cells (Kitajima et al., 2013). Strikingly, Hanna´s group engrafted the hematopoietic system of mice affected by Sickle cell anemia with gene corrected iPSCs-derived HSCs from a mouse model of this disease (Hanna et al., 2007).

One of the main limitations of HSC gene therapy of FA is the limited number of HSCs that are present in most FA patients. Therefore, the possibility of generating HSCs from genetically corrected FA iPSCs results of great interest as a new therapeutic approach to treat the BMF characteristic of this disease. Our group, in collaboration with Izpisúa-Belmonte's group and J. Surrallés, generated iPSC-derived hematopoietic progenitor cells from two FA complementation groups, one located in the FA core complex (FA-A) and the other one, FA-D2 that belonged to the ID-complex. Significantly, only after correction of the genetic defect, iPSCs from these patients could be generated (Raya et al., 2010). Based on this study, in the present work we have investigated the generation of iPSC from a FA mouse model with hypomorphic mutations in the *Brca2* gene. The relevance of correcting the genetic defect of this FA-D1 model to reprogram and re-differentiate these cells towards the hematopoietic system is shown in this work.

FA is a genetic disease associated with developmental abnormalities, bone marrow failure (BMF) and cancer predisposition. Nowadays, the only curative treatment of the BMF of FA patients is the HSCT from a healthy donor. Novel therapies, such as gene therapy, are being investigated to find new therapeutic alternatives for patients without a compatible donor.

One of the current limitations of FA gene therapy is the limited number of HSCs present in many of these patients. As there are no protocols capable of expanding these cells *in vitro*, we have proposed that gene therapy combined with cell reprogramming would facilitate the generation of corrected HSCs from non hematopoietic tissues.

The objective of this thesis was to investigate for the first time the process of cell reprogramming in a mouse model of FA-D1 and to explore the feasibility of using this approach to generate disease-free HSCs that could rescue the hematological defects that characterize these animals.

To achieve this main objective, three intermediate objectives were investigated:

1. Study of the relevance of *Brca2* in cell reprogramming.

2. Generation and characterization of disease-free iPSCs from *Brca2*$^{\Delta 27/\Delta 27}$ mouse fibroblasts.

3. Differentiation of gene corrected *Brca2*$^{\Delta 27/\Delta 27}$ iPSCs towards disease-free HSCs.

1. ANIMAL MODELS

Several FA mouse models and their correspondent wild type (wt) mouse strains used as controls were used in the different experiments performed in this work.

***Fanca*$^{-/-}$:** This mouse model was generated by Dr. Arwert (Department of Clinical Genetics and Human Genetics, Free University Medical Center, Amsterdam, The Netherlands) (Cheng et al., 2000) by the elimination of exons 4 to 7 of the gene and they were substituted by the *LacZ-Neo* by homologous recombination in a Fvb/129 background. Wt Fvb/129 litter mates were used as controls.

***Brca2*$^{\Delta 27/\Delta 27}$:** This mouse model was first generated by Dr. McAllister at Dr. Wiseman laboratory (Laboratory of Women's Health, National Institute of Environmental Health Sciences, Research Triangle Park, NC) on a Balb/c background (McAllister et al., 2002a). As *Brca2* knock-out mice are not viable, the transgenic mice were generated by the deletion of exon 27 of the *Brca2* gene (*Fancd1*) resulting in a hypomorphic mutation, that does not generate embryonic lethality (Sharan et al., 1997). Wild type Balb/c litter mates were used as controls.

NOD/SCID γc^{null}: This is an immunodeficient mouse model, which is double homozygous for the severe combined immunodeficiency (SCID) mutation and interleukin-2R_ (IL-2R_) mutation (γc^{null}). It was used in the teratoma formation experiments for the characterization of iPSCs.

All experimental procedures and animal maintenance were carried out according to Spanish and European regulations (Spanish RD 223/88 and OM 13-10-89 of the Ministry of Agriculture, Food and Fisheries; and European convention ETS-123, for the use and protection of vertebrate mammals used in experimentation and other scientific purposes).

2. PRIMARY CELLS

-Adult fibroblasts (MAFs)

The first set of reprogramming experiments was performed using mouse adult fibroblasts (MAFs) from the ears of two different FA mouse models, *Fanca*$^{-/-}$ and *Brca2*$^{\Delta 27\Delta 27}$ (*Fancd1*) mice and their control wt littermates.

The generation of mouse adult fibroblasts (MAFs) was done by extraction of small biopsies from the ears of one mouse from each strain. They were cut in small fragments and then digested for 6 hours at 37°C with shaking and 0.25 % collagenase A (0.240 U/mg) lyophilized *Clostridium histolyticum* – Roche, diluted in DMEM + glutaMAX TM-I Dulbeccos's Modified Eagle's Medium (Gibco 61965-026) with 0.1 µg-U/µl penicillin/streptomycin (Invitrogen). Once the tissues were digested the fibroblasts were cultured in F25 flasks (Nunc) in DMEM + glutaMAX TM-I Dulbeccos's Modified Eagle's Medium (Gibco 61965-026) medium supplemented with 10 % FBS (Biowhitaker) and 0.1 µg-U/µl antibiotics (penicillin-streptomycin, Gibco 15140-148) filtered by 0.1 µm. MAFs were maintained in culture for two to three passages.

-Mouse embryonic fibroblasts (MEFs)

Mouse embryonic fibroblasts (MEFs) were extracted from the chorion of 13.5E pregnant females. The procedure was performed by removing the uterus with the embryos. Once the embryos were obtained, the head and viscera were removed and the remaining embryonic parts were dissociated with 0.05 % trypsin (Gibco) with the help of scissors until complete cell dissociation. The big fragments remaining after dissociation were discarded by centrifugation and fibroblasts were seeded at a concentration equivalent to two embryos per 145 cm^2 plate (Nunc), in DMEM + glutaMAX TM-I Dulbeccos's Modified Eagle's Medium (Gibco 61965-026) medium supplemented with 10 % FBS (Biowhittaker) and 0.1 µg-U/µl antibiotics (penicillin-streptomycin, Gibco 15140-148), filtered by 0.1 µm, and cultured under hypoxic conditions (37°C, 5 % CO_2, 5 % O_2) in 145 cm^2 plates (Nunc). When confluent, MEFs were split 1/5 and expanded as required for the experiments.

MEFs for cell reprogramming were expanded for one passage and were then either reprogrammed of frozen for future reprogramming experiments. MEFs to be used as feeder layers were expanded until passage 4 to obtain a high number of cells trying to minimize the

number of passages. Mouse embryonic fibroblasts from ATTC (SCRC-1008) from C57BL/6 males were also used as feeder layers.

For MEFs inactivation, cells were trypsinized and added into tubes at a concentration equivalent to 10 confluent 145 cm^2 plates (Nunc) per 50 ml tube in a final volume of 20 ml. For the inactivation, cells were irradiated with X-rays at a dose of 60 Gy and when inactivated with MMC, cells were exposed to 12 mg/ml of MMC for 2.5 hours. After inactivation, cells were either maintained in culture to use as feeders for ESC or iPSC culture or frozen at -80°C at a concentration equivalent to two 145 cm^2 plates (Nunc) per cryotube with half a mililitre of 80 % DMSO freezing solution and half a mililitre of culture medium to obtain a final freezing solution concentration of 10 %.

3. CELL LINES

-NXE cell line (ATCC: SD 3444)

It is a packaging cell line used for γ-RV production in a transient or stable manner. This cell line was generated by modification of the previously described 293T cells in which the retroviral proteins (gag-pol, env) required for the packaging of the γ-RVs had been stably transduced.

This cell line was cultured in DMEM + glutaMAX TM-I Dulbeccos's Modified Eagle's Medium (Gibco 61965-026) medium supplemented with 10 % FBS (Biowhittaker) and 0.05 µg-U/µl (penicillin-streptomycin, Gibco 15140-148). Suplemented medium was filtered by 0.1 µm.

-293T cell line (ATCC: CRL-11268)

It is a cell line used to produce non replicative LVs. It is a human embryonic kidney epithelial cell line that was modified by the insertion of a temperature sensitive gene codified by the simian SV40 T antigen that is constitutively expressed to achieve higher transfection efficiency. The inserted sequence allows the episomal replication of plasmids that contain the replication origin (ORI) in the early promoter region of the SV40.

-NIH/3T3 (ATCC-CRL-1568 TM)

It is a cell line of embryonic origin from mouse sarcoma derived from the MIH/Swiss strain. The NIH/3T3 cell line is highly sensitive to sarcoma virus focus formation and leukemia virus propagation and has proven to be very useful for DNA transfection.

It was cultured in DMEM + glutaMAX TM-I Dulbeccos's Modified Eagle's Medium (Gibco 61965-026) medium supplemented with 10 % FBS (Biowhittaker) and 0.05 µg-U/µl antibiotics (penicillin-streptomycin, Gibco 15140-148). Supplemented medium was filtered by 0.1 µm.

This cell line was cultured in DMEM + glutaMAX TM-I Dulbeccos's Modified Eagle's Medium (Gibco 61965-026) medium, supplemented with 10% FBS (Biowhittaker) and 0.05 µg-U/µl antibiotics (penicillin-streptomycin, Gibco 15140-148). Supplemented medium was filtered by 0.1 µm.

-HT1080 cell line (ATCC-CCL-121)

It is a human male fibrosarcoma cell line with adherent morphology that contains an activated *N-RAS* oncogene. This cell line was used for vector titration.

It was cultured in DMEM + glutaMAX TM-I Dulbeccos's Modified Eagle's Medium (Gibco 61965-026) medium supplemented with 10 % FBS (Biowhittaker) and 0.05 µg-U/µl antibiotics (penicillin-streptomycin, Gibco 15140-148). Supplemented medium was filtered by 0.1 µm.

-mESJ1 mouse embryonic stem cell line

This cell line (ATCC (SCRC-1010)) of male mouse of the 129S4/SvJae strain was used as a control cell line for the characterization of the iPSCs generated in this study and also in the hematopoietic differentiation protocols of iPSCs. mESC and iPSCs were cultured in KO-DMEM^{TM} medium (Gibco 10829-018) supplemented with 15 % Hyclone (Thermo Scientific, Sv 30160.03), 1 % glutamax, 0.1 µg-U/µl antibiotics (penicillin-streptomycin, Gibco 15140-148), 1x NEAA (non-essential aminoacids 100x, Biowhittaker, PE13-114C), 50 µM 2β–mercaptoethanol (Gibco 31 350-010), 100 U/ml LIF (Mouse Leukemia Inhibitory Factor) (10^7 Chemicon, CA92590). Supplemented medium was filtered by 0.1 µm.

-Hematopoietic stromal cell line OP9 (CRL-2749 TM)

This cell line derives from a mouse hematopoietic stroma with fibroblastic morphology. It was obtained from C57BL/6 x C3H F2$^{-op/-op}$ mice. These cells are unable to produce functional macrophage colony stimulating factor (M-CSF) due to an osteopetrotic mutation in the gene that codifies for the GM-CSF, factor that inhibits the hematopoietic differentiation. This line

was grown and amplified one week prior to initiate the differentiation of iPSCs. OP9 cells were grown in α-MEM medium (MEM-Alpha + glutamax), Minimum Essential Medium 1x, supplemented with 10 % FBS, 0.1 µg-U/µl penicillin/streptomicin (Gibco), 1x NEAA (non-essential aminoacids 100x, Biowithttaker, PE13-114C), 50 µM 2β–mercaptoethanol (Gibco 31 350-010) and filtered by 0.1 µm.

The cells were split 1/5 - 1/8 when confluent to prevent contact self-inactivation and the generation of cytoplasmic vacuoles, decreasing the quality of the cells. OP9 were frozen in 10 % DMSO and stored in liquid nitrogen until needed.

-AM20.1B4 stromal cell line

It is a stromal cell line from the aorta-gonada-mesonephro region obtained from mouse embryos at day E11 of development. This line was immortalized by transduction of the T antigen of the SV40 virus and was used in hematopoietic differentiation protocols.

AM20.1B4 cell line was kindly provided by Dr. Majlinda Lako's laboratory. It was amplified in culture medium composed of 50 % Myelocult long term medium M5300 (Stem Cell Technologies), 30 % α-MEM (MEM-Alpha +glutamax), Minimum Essential Medium 1x, 12 % Hyclone (Thermo Scientific, Sv 30160.03), 4 mM L-Glutamine and 10 µM β-mercaptoethanol (Gibco 21350-010 50MM). Cells were amplified and split 1/10 and were frozen when reached 80 % confluence in 10 % DMSO and were stored in liquid nitrogen.

-SC1#6: Cre reporter

Cell line used for the functional titration of the retroviral based protein delivery system, to excise the reprogramming cassette (Ma.Cre.prot). This cell line was genetically modified to express a vector cassette in which the green fluorescent protein (eGFP) and the red fluorescent protein (RFP) were inserted. The vector was designed for eGFP open reading frame to be interrupted by LoxP sequences that, in between harboured the RFP. When the Cre recombinase was expressed in these cells, the sequence in between the two LoxP sites of SC1#6 cells, was removed allowing the eGFP protein to be expressed.

This cell line was kindly provided by Dr. Melanie Galla from the Experimental Hematology Institute, MHH, Hannover.

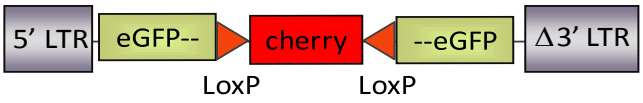


Figure 22. Schematic representation of the construction that SC1#6 cell line harboured to perform the functional titration of Ma.Cre.prot supernatant for reprogramming vector excision.

4. VIRAL VECTORS

4.1 VECTORS FOR PHENOTYPE CORRECTION OF THE FA CELLS

-PCL1-BRCA2

Lentiviral vector in which the expression of the human *BRCA2* gene was driven by the SFFV promoter (Rio et al., 2008). The *BRCA2* fragment had a size of 10,254 base pairs. It was used for the genetic correction of *Brca2*$^{\Delta 27/\Delta 27}$ fibroblasts for further generation of disease-free *Fancd1*$^{\Delta 27/\Delta 27}$ iPSC (Figure 23).

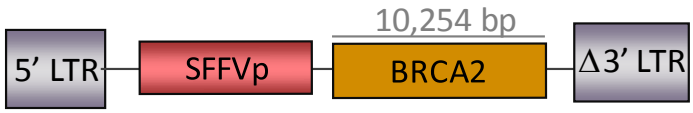


Figure 23. Schematic representation of the therapeutic PCL1-BRCA2 lentiviral vector used for the genetic correction of the *Brca2*$^{\Delta 27\Delta 27}$ mouse cells.

-PGK-hFANCA-wpre (pCCLsin18.ppt.hPGKhFANCAWpre)

This monocistronic lentiviral vector was also developed in the laboratory and designed for its clinical use in FA patients. It carries the human *FANCA* gene under the control of the phosphoglycerate kinase promoter (PGK). In this vector, *FANCA* expression was stabilized by a woodchuck hepatitis virus posttranscriptional regulatory element (wpre) in which the ATG that could give rise to a protein and part of the coding sequence were eliminated (Egelhofer y col., 2004; Schambach y col., 2006).

Figure 24. Schematic representation of the therapeutic lentiviral vector pCCLsin18.ppt.hPGKhFANCA.Wpre* used for genetic correction of the *Fanca*$^{-/-}$ deficiency in mouse cells.

4.2 VECTORS FOR CELL REPROGRAMMING

-**Monocistronic γ-RVs:** **pMXs.Sox2** (Addgene plasmid 13367)**, pMXs.Klf4** (Addgene plasmid 13370)**, pMXs.Oct3/4** (Addgene plasmid 13366)**, pMXs.c-Myc.T58A** (Addgene plasmid 13372)**.**

pMXs based monocistronic 4 γ–RV vectors containing the mouse factors for reprogramming: *Sox2*, *Klf4* (Kruppel-like factor 4), *Oct3/4* (POU domain, class 5, transcription factor 1) and mouse *c-Myc* (myelocytomatosis oncogene T58A mutant) were used in the first experiments of cell reprogramming. They were generated by Dr. Toshio Kitamura from Tokyo University and the Institute of Medical Science from Shinya Yamanaka's laboratory and were obtained from Addgene.

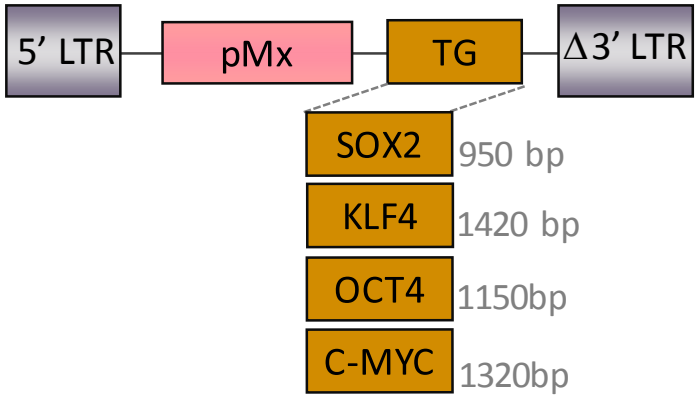


Figure 25. Schematic representation of the monocistronic 4 γ-RV vectors carrying the *Sox2* gene (pMXs.Sox2), *Klf4* gene (pMXs.Klf4), *Oct3/4* gene (pMXs.Oct4) and *c-Myc* gene (pMXs.c-Myc) used for cell reprogramming.

-Polycistronic lentiviral vectors for cell reprogramming: **1)** EF1αSTEMCCA LoxP (pHAGE - EF1αFull-Oct4 .F2a.Klf4.IRES. Sox2. E2A. cMYC. Wpre.LoxP). **2)** EF1α STEMCCA REDLIGHT LoxP (pHAGE - EF1αFull-Oct4 .F2a.Klf4.IRES. Sox2. E2A.Cherry. Wpre.LoxP)

Polycistronic vectors carrying the four reprogramming factors (*Oct4*, *Klf4*, *Sox2* and *c-Myc*) under the control of the EF1α promoter with a F2A and an IRES sequence to allow the expression of the four factors at a similar level were kindly provided by Gustavo Mostoslavsky (Gianotti-Sommer et al., 2008).

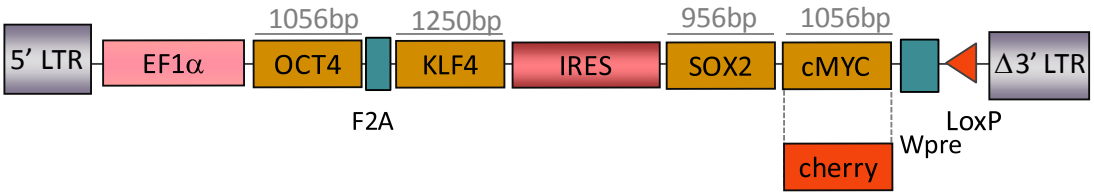


Figure 26. Schematic representation of the polycistronic lentiviral vectors carrying either the four reprogramming factors (*Oct4*, *Klf4*, *Sox2* and *c-Myc*) or three of the reprogramming factors (*Oct4*, *Klf4* and *Sox2*) followed by the fluorescent cherry protein, used for cell reprogramming. The vector expression was driven by the EF1α promoter, followed by the *Oct4* gene, a F2A sequence that cleaved the first polyprotein by a ribosomal skipping mechanism and the *Klf4* reprogramming factor. Then there was an IRES to continue the expression of the *Sox2* and c-*Myc* or the Cherry protein reprogramming genes. The vector contained a stabilizing wpre sequence and a LoxP site to allow the removal of the cassette by the Cre recombinase activity.

4.3 VECTORS FOR THE EXCISION OF THE REPROGRAMMING CASSETTE

-Integrase deficient lentiviral vector carrying the Cre recombinase (pLBid-nlsCre-SF LV: IDLV)

This vector was used for the excision of the polycistronic lentiviral vector from the iPSC. It is a bidirectional, integration deficient lentiviral vector carrying the Cre recombinase under the control of the CMV promoter and the cherry fluorescent protein controlled by the SSFV promoter. It was built and kindly provided by Drs. Tobias Mätzig and Mellanie Galla from the Experimental Hematology Institute, MHH, Hannover.

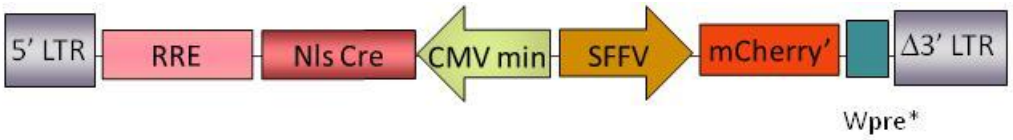


Figure 27. Schematic representation of the integration deficient lentiviral vector carrying Cre recombinase and cherry protein as a reporter that was used to remove the reprogramming cassette from the iPSCs.

-Mlv.Ma.prot.nlsCreco

This vector was generated by Dr. Schambach and Dr. Schedlmeier at the Experimental Hematology Institute, MHH, Hannover. It consisted of a retroviral vector specifically designed to transfer non-retroviral proteins in a directed, controlled and transient manner. This vector was designed to express the Cre recombinase from a MLV structural protein precursor harbouring a nuclear localization signal that allowed the protein of interest to be released in the nucleus of the target cells. It was produced in a 3rd generation packaging system (described in materials and methods). A functional titration was done by FACS using the previously described "SC1#6: Cre reporter" cell line. The excision of the reprogramming cassette in the iPSCs by this method occurred through a site specific delivery of the DNA Cre recombinase in the gag position of the retroviral matrix, after the proteolysis that allowed its release (Voelkel et al., 2010).

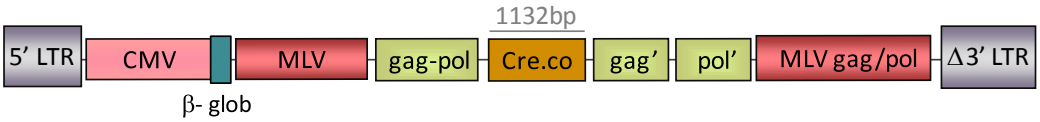


Figure 28. Schematic representation of the retroviral based protein transfer system used for the reprogramming vector excision.

4.4 VECTORS FOR HEMATOPOIETIC DIFFERENTIATION PROTOCOLS

-MSCV-HOXB4-IRES-GFP

A γ-RV carrying the *HOXB4* LIM-homeobox gene was used to promote the expansion of HSCs by the obtainment of a selective advantage of transduced cells by the expression of this gene during the hematopoietic differentiation process of mESC and iPSCs.

This vector was kindly provided by Christopher Baum's laboratory at Experimental Hematology Institute, MHH, Hannover, and drove the expression of *HOXB4* gene by the MSCV promoter followed by an IRES sequence that also allowed the expression of the reporter eGFP protein (Zhou et al., 1998) (Mizuguchi et al., 2000).

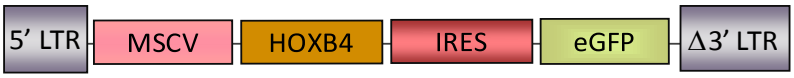


Figure 29. Schematic representation of the MSCV-HOXB4-IRES-GFP vector that expressed the *HOXB4* homeobox gene driven by the MSCV promoter. This vector contained an IRES sequence to express the eGFP protein.

4.5 GENERATION OF RETROVIRAL AND LENTIVIRAL SUPERNATANTS

The production of γ-RV and LV was performed both by transient co-transfection of the transfer plasmid in Nxe and 293T cells, respectively, and also the plasmids encoding for the VSV-G and for the gag/pol structural proteins, and the rev protein in case the production was in 3rd generation.

The viral vector production in 2nd generation was performed as previously commented, using three plasmids, the transfer plasmid of interest, the envelope that in all cases was the VSV-G and the helper plasmid that was commercial but different in γ-RVs (Pasmid Factory–pCDNA3) or LVs (Pasmid Factory -PCMV.dR.8.74) production. The vector production in 3rd generation involved the use of the additional plasmid that encoded for the rev protein.

For that purpose Nxe/293T cells were seeded the day before transfection on gelatin coated (0.1 %) 10 cm^2 plates, at a concentration of 5 x10^6 cells per plate. The day after 293T cells were at 80 % confluence and the medium was changed 2-4 hours before the transfection (except for the $CaCl_2$ based transfection method).

Different transfection methods were used for viral supernatant production that will be described, together with the vectors that were produced by each of the methods:

-FuGENE transfection method

The vectors that were produced by this method were:

- pMXs.Sox2 (reprogramming experiments)
- pMXs.Klf4 (reprogramming experiments)
- pMXs.Oct3/4 (reprogramming experiments)
- pMXs.c-Myc.T58A (reprogramming experiments)

- Four factor polycistronic excisable lentiviral vector for cell reprogramming. EF1αSTEMCCA LoxP (pHAGE-EF1αFull-Oct4.F2a.Klf4.IRES.Sox2.E2A.cMYC. Wpre.LoxP)
- Three factor polycistronic excisable lentiviral vector for cell reprogramming EF1α STEMCCA REDLIGHT LoxP (pHAGE-EF1αFull-Oct4.F2a.Klf4.IRES.Sox2.E2A. Wpre.LoxP)
- MSCV-HOXB4-IRES-GFP (hematopoietic differentiation experiments)

FuGENE (FuGENE HD Transfection Reagent - Roche) is a non-liposomal compound to transfect cells with a high efficiency and low toxicity even in non-serum free conditions. It is an expensive transfection method that we used in cases in which other methods were not successful, due to the size of the transfer vector or to the need of transfecting a high number of plasmids. For transfection with FuGENE, 51 µl of FuGENE were diluted in 600 µl of serum free DMEM glutaMAX TM-I Dulbeccos's Modified Eagle's Medium (Gibco 61965-026) DMEM added in 10 cm^2 plates and incubated for 5 minutes at room temperature. In another tube the DNA mix was prepared at a total concentration of 11-12 µg of total DNA per 10 cm^2 plates (8 µg transfer DNA, 2 µg helper 1 DNA, 2 µg helper 2 DNA (3^{rd} generation) and 2 µg envelope-VSV-G.

The FuGENE-DMEM mix was added to the DNA mixture drop by drop, avoiding FuGENE to touch the tube walls to allow the proper formation of DNA precipitates. This mixture was incubated for 15 minutes and then added to the cells (650 µl/plate). Cells were incubated for 24 hours and then the medium was replaced by 9 ml of IMDM 10 % FBS y 0.05 µg-U/µl antibiotics (penicillin-streptomycin, Gibco 15140-148) per 10 cm^2 plate and after 24 hours the supernatant was collected, filtered by 0,45 µm and concentrated by ultracentrifugation in polypropylen tubes at 25,000 rpm for 2.5 hours in an Optima™ L-100 XP Beckman Coulter ultracentrifuge using a Beckman Culture SW32 Ti S/N 10V 2610 rotor. After centrifugation the viral particles were resuspended in PBS or medium and stored at -80°C. The day after, the same procedure was repeated by collecting the supernatant 48 hours post-transfection.

-PEI transfection method (Polyethylenimine)

By this method the following vectors already described in materials and methods were produced:

- PCL1-BRCA2 (correction of FA *Brca2*$^{\Delta 27/\Delta 27}$ cells)
- PGK-hFANCA-wpre (correction of FA *Fanca*$^{-/-}$ cells)

Previous to transfection, the culture medium was replaced with 4 ml/10 cm^2 plate of DMEM glutaMAX TM-I Dulbeccos's Modified Eagle's Medium (Gibco 61965-026) supplemented with 10 % Hyclone and without antibiotics. The PEI stock was prepared to obtain a final concentration of 40 µM = 1 mg/ml. The transfection was carried out in 10 cm plates and the preparation of the transfection mixture was performed in the following proportions per 10 cm^2 plate.

In one 15 ml tube (Tube1) 20 µL PEI 1 mg/ml were added over 980 µL of non supplemented DMEM glutaMAX TM-I Dulbeccos's Modified Eagle's Medium (Gibco 61965-026) and mixed. In a different 15 ml tube (Tube2) 14 µg of total DNA (2nd genereation) or 16.8 µg (3rd generation) 8.4 µg Transfer DNA and 2.8 µg of each plasmid (helper 1, helper 2 (3rd generation) and envelope)- were mixed with 993 µl of non supplemented DMEM glutaMAX TM-I Dulbeccos's Modified Eagle's Medium (Gibco 61965-026). The PEI-DMEM mixture (Tube 1) was pipetted over the DNA-DMEM mix (Tube2), mixed and incubated for 20 minutes at room temperature. After the incubation the 2 ml mixture was added per plate, moved and stored in the incubator for 24 hours. The day after, the medium was replaced by 9 ml of IMDM 10 % FBS y 0.05 µg-U/µl antibiotics (penicillin-streptomycin, Gibco 15140-148) per 10 cm^2 plate and after other 24 hours the supernatant was collected from the medium, filtered by 0.45 µm and concentrated by ultracentrifugation in polipropilen tubes at 25,000 rpm for 2.5 hours in a Beckman Coulter Optima^{TM} L-100 XP ultracentrifuge in a Beckman Culture SW32 Ti S/N 10V 2610 rotor. After centrifugation, the viral particles were resuspended in PBS or medium and stored at -80ºC. The day after, the same procedure was repeated collecting the 48 hours supernatant.

-$CaCl_2$ transfection method

The vectors that were produced by this method were the ones used for the excision of the reprogramming cassette.

- Integrase deficient lentiviral vector carrying the Cre recombinase (pLBid-nlsCre-SF LV) (excision of the reprogramming cassette)

- Mlv.Ma.prot.nlsCreco. For the production of this vector only the envelope plasmid (VSV-G) was necessary because gag/pol was already encoded in the vector construction (excision of the reprogramming cassette)

For transfection by the HBS and $CaCl_2$ method, plasmids were mixed in a 15 ml tube with 2.5 M $CaCl_2$ in the following proportions per plate: 5 µg transfer DNA, 10 µg helper 1.5 µg helper 2 (3^{rd} generation), 1.5 µg envelope, 50 µl $CaCl_2$ and distilled water in a final volume of 500 µl. In another 15 ml tube 500 µl/plate of HBS (HEPES buffered saline solution (274 mM NaCl, 50 mM HEPES, 1.5 mM Na_2HPO_4 y H_2O, optimal pH between 6.95 and 7.05) were added. While gently bubblig up, the DNA-$CaCl_2$ mixture was added drop by drop with a p20 micro-pipette to allow the formation of calcium precipitates. This mixture was incubated at room temperature without moving for 20 minutes. While incubating the DNA mixture, the culture medium was replaced by the "transfection medium" composed of: DMEM + glutaMAX TM-I Dulbeccos's Modified Eagle's Medium (Gibco 61965-026) medium supplemented with 10 % FBS (Biowhitaker), 1 % Natryum pyruvat, 0.1 µg-U/µl antibiotics (penicillin-streptomycin, Gibco 15140-148) and 20 mM HEPES. Additionally, just before use, cloroquine was added to the medium at a concentration of 25 mM (Chloroquine Diphosphate, Sigma-Aldrich). After incubation, the transfection mixture was added to the cells (1 ml/plate) and 6-7 hours post-transfection, the medium was changed again for "tansfection medium" without cloroquine, to avoid the toxicity of this drug. Thirty six hours post-transfection the supernatant was collected from the cell plates and filtered by 0.45 µm to eliminate cellular debris, and was stored at 4°C. At 48 hours post-transfection, the supernatant was collected and filtered again and together with 36 hour supernatant was concentrated by ultracentrifugation in polypropylene tubes at 25,000 rpm for 2.5 hours in a Beckman Coulter Optima™ L-100 XP ultracentrifuge using a Beckman Culture SW32 Ti S/N 10V 2610 rotor. After centrifugation, the viral particles were resuspended in PBS or culture medium and stored at -80°C.

4.6 TITRATION OF VIRAL SUPERNATANTS BY FACS AND RT-qPCR

The titration of vector supernatants was performed by transducing HT1080 cells (human fibrosarcoma cells) with serial dilutions of the supernatant. Fifty thousand HT1080 cells per well were seeded in p24 well plates, one day before the supernatant collection, in DMEM + glutaMAX TM-I Dulbeccos's Modified Eagle's Medium (Gibco 61965-026) medium supplemented with 10 % FBS (Biowhitaker) and 0.05 µg-U/µl antibiotics (penicillin-streptomycin, Gibco 15140-148) filtered by 0.1 µm. Once the supernatant was prepared HT1080 cells were transduced with serial dilutions of the supernatant, ranging from 10^{-2} to 10^{-7}. Just before transduction, the number of cells in one well was counted to use this data for the titre calculation. Twenty four hours post transduction, the medium was replaced by fresh medium. When vectors carried fluorescent reporter genes, cells were collected 5 days after transduction and the percentage of cells expressing the reporter gene was analyzed by flow cytometry. When the vector did not contain a reporter gene, cells were collected 10-11 days after transduction. Genomic DNA was extracted (DNeasy Tissue Kit, Qiagen GmbH) and the number of viral particles per microlitre was determined by RT-qPCR-Rotor-Gene RG-3000 (Corbett Research Products), analyzing the number of integrated copies of the provirus, using either probes or primers that specifically recognized sequences of the provirus. The quantification of the number of viral particles in the samples was performed normalizing the data obtained with a standard curve that was performed by 10 fold serial dilutions, starting from 10^6 molecules of a plasmid containing the housekeeping human albumin gene gene (h*ALB*)- pRRL.SIN.cPPT.PGK.EGFP.hALB (provided by Fr. Charrier;(Gennethon, Evry, France). This plasmid also harboured the ψ (psi) lentiviral encapsidation signal, only present in LV-transduced cells. Samples were loaded in triplicates with the appropriate negative control corresponding to non-transduced cells and DNA of a sample known to contain one provirus/cell. The titre of the supernatant was given by the number of infective particles per mililiter, accordingly to the formula represented in Table 8.

Titration of infective supernatant with RVs or LVs harbouring a fluorescent marker

$$T\ (UT/mL) = [Nº\ cells \times \%\ fluorescent\ cells/100] \times DF \times CF$$

Titration of infective supernatant with RVs or LVs not harbouring a fluorescent marker

$$VCN/cell = VCN_{psi} \times 2 / VCN_{hAlb} \times 2$$

$$T\ (UT/mL) = [Nº\ cells \times normalized\ VCN/cell/100] \times DF \times CF$$

Table 8. T is the titre of the viral supernatant. **Nº of cells** is the number of cells counted at the moment of transduction. **Percentage of fluorescent cells** corresponds to the percentage of cells expressing the transgene, obtained by FACS. **DF** is the dilution factor that corresponds to the dilution used for the titre calculation. **CF** is the factor used to obtain the titre in UT/mL, when the total volume at infection is not higher than 1 ml. (200 µl/well x 5= titre/ml). **VCN** is the vector copy number value.

5. GENETIC CORRECTION AND MODIFICATION ASSAYS

5.1 GENETIC CORRECTION OF FANCONI ANEMIA CELLS WITH GAMMA-RETROVIRAL VECTORS

The FA cells used were adult or embryonic fibroblasts, either deficient in FA A gene (*Fanca*$^{-/-}$) gene or in FA-D1 gene (*Brca2* $^{\Delta 27/\Delta 27}$).

In the first set of experiments in which the defective genes were corrected, *Fanca*$^{-/-}$ and *Brca2*$^{\Delta 27/\Delta 27}$ mouse ear adult fibroblasts were transduced with lentiviral vectors carrying the gene of interest and their correspondent control vectors PGK.iGFP, PGK FANCA Wpre (Gonzalez-Murillo et al., 2010), PCL1.EGFP, PCL1.BRCA2 (Rio et al., 2002). Viral supernatants were produced and titrated following the previously described method.

5.2 CELL REPROGRAMMING ASSAYS

5.2.1GENERATION OF INDUCED PLURIPOTENT STEM CELLS

The generation of mouse iPSCs from two FA mouse models, *Fanca*$^{-/-}$ and *Brca2*$^{\Delta 27/\Delta 27}$, was performed using γ-RVs in a first attempt and LVs only in *Brca2*$^{\Delta 27/\Delta 27}$ deficient cells.

-Generation of induced pluripotent stem cells (iPSC) using γ–RVs reprogramming vectors

Fifty thousand mouse adult fibroblasts (MAFs) per well were infected with a 1:1:1:1 proportion mixture of *Oct3/4, Sox2, Klf4* and *c-Myc (T58A)* retroviral supernatants with 8 μg/mL of polybrene. The plate was centrifuged at 700 g and 32°C for 45 min without brake and then the cells were incubated for 24 hours. A second cycle of transduction was repeated following the same procedure. The day after, the medium was replaced by fresh DMEM + glutaMAX TM-I Dulbeccos's Modified Eagle's Medium (Gibco 61965-026) medium supplemented with 10 % FBS (Biowhittaker) and 0.1 μg-U/μl antibiotics (penicillin-streptomycin, Gibco 15140-148).

At day 4 post-transduction, transduced cells were trypsinized and plated at a concentration of $5x10^4$ cells on irradiated MEFS seeded the day before over 0.1 % gelatine pre-coated 10 cm^2 plates. The day after, the culture medium was replaced for a medium for ESC/iPSC culture (mES medium). mES medium was composed of 15% FBS Hyclone, 1x NEEA (Biowhittaker), 1 % Glutamax (Invitrogen), 0.1 μg-U/μl P/S (penicillin-streptomycin, Gibco 15140-148), 50 μM β-Mercaptoethanol (Gibco), 100 U/mL leukemia inhibitory factor (LIF) (Chemicon), KO-DMEM (Invitrogen). From this moment the culture medium was replaced daily and between 9-13 days post-transduction iPSC colonies arose in the plates showing a compact and round morphology.

The iPSC colonies were selected and marked for individual colony collection. To pick the colonies, 5 ml of 0,25 % trypsin per plate were added and rapidly removed to prevent the complete detachment of the cells from the plate, but allowing them not to be too tightly attached. Single colonies were picked under the microscope with a p200 micropipette and were seeded in new 10 cm^2 plates over irradiated MEFs trying to dissociate the cells as much as possible.

-Generation of induced pluripotent stem cells (iPSC) using an excisable polycistronic lentiviral vectors

To increase the efficiency of reprogramming of *Brca2*$^{\Delta 27/\Delta 27}$ mouse embryonic fibroblasts (MEFs) and polycistronic reprogramming LVs were used. Several conditions were included in these experiments: uncorrected fibroblasts, fibroblasts corrected prior to reprogramming and fibroblasts that were corrected and reprogrammed at the same time. In each condition, $2.5x10^4$ mouse embryonic fibroblasts (MEFs) were transduced with supernatants from a polycistronic excisable vector (STEMCCA-LV) produced as previously described by Sommer (Sommer, Stadtfeld et al. 2009), every 24 h at 37 °C, 5 % CO_2 in two cycles of infection.

Four days after transduction the culture medium was replaced by mES Medium, composed by: KO-DMEM (Invitrogen), 15 % FBS Hyclone, 1x NEEA (Biowhittaker), 1 % Glutamax (Invitrogen), 0.1 µg-U/µl P/S (penicillin-streptomycin, Gibco 15140-148), 50 µM β-mercaptoethanol (Gibco), 100 U/mL leukemia inhibitory factor (LIF) (Chemicon). The medium was replaced daily and between 9-11 days post-transduction iPSC arose in the plates showing a compact and round morphology. iPSC colonies were expanded as already described.

5.2.2 INDUCED PLURIPOTENT STEM CELL CHARACTERIZATION

-Alkaline Phosphatase

Two kits were used for alkaline phosphatase (AP) detection: Alkaline Phosphatase Detection Kit (Millipore, SCR004) and Vector Blue Alkaline Phosphatase Substrate Kit III Cat. No. SK-5300 (Vector laboratories). Alkaline phosphatase is a hydrolase enzyme that acts dephosphorylating nucleotides and other molecules under alkaline conditions. This activity, together with the expression of other surface markers is often used to evaluate the pluripotent state of ESC and iPSC, one of the first criteria in the characterization of these cells. Alkaline phosphatase (AP) activity was evaluated in the cell membrane of colonies fixed with 2 % paraformaldehyde according to the protocol described by the manufacturer (Millipore). Undifferentiated, thus pluripotent cells appeared red or purple, whereas differentiated cells appeared colorless.

-OCT3/4, NANOG and SSEA1 Immunostaining

The expression of the pluripotency markers OCT3/4, NANOG and SSEA1 was analyzed by immunostaining in the iPSC colonies. Fifty thousand cells per chamber were cultured in gelatin-coated polystyrene two slide chambers BD Falcon, fixed with 4 % paraformaldehyde and then permeabilized for 1 hour by a treatment with 1% BSA, 10 % FBS, 0.3 M glycine, 0.1 % Tween 20 diluted in PBS.

Fixed colonies were stained with the corresponding antibody hOCT3/4-FITC (1:20) (R&D, Minneapolis, MN). SSEA1-PE (1:200) (R&D Systems) and Nanog-PE (1:20) (R&D), overnight incubated at 4ºC together with DAPI (1:500) (Roche). Preparations were mounted with Moviol (Fluka) for analysis.

-Teratoma formation

iPSCs were cultured under normal conditions and when confluent, one million iPSCs were subcutaneously injected into the dorsal flanks of NOD.Cg-Prkdcscid IL2rgtm1Wjl mice (NOD/SCID γc^{null}). 4-8 weeks after injection tumours were extracted and paraffin sections of formalin-fixed teratoma specimens were prepared. Teratoma evaluation was performed by histological analysis of hematoxilin and eosin (H&E) stained sections. Aditionally, teratomas from iPSCs generated by transduction with the four reprogramming RVs were stained for specific tissues: AFP (Abcam) for teratoblastoma and mesendoderm cells, FOXA2 (Abcam) for endoderm, BRACHYURY (Abcam) and α-ASMA (Abcam) for mesoderm and TUJ1-Neuronal Class III β-Tubulin (Abcam) and GFAP (Abcam) for ectoderm.

-Quantitative RT–PCR and transgene expression

The h*BRCA2* relative transgene expression was determined by the 2Δ Ct method (Livak and Schmittgen, 2001), in which Ct corresponds to the cycle threshold. The primers used for h*BRCA2* gene expression are represented in Table 9. The murine glyceraldehyde-3-phosphate dehydrogenase (m*Gapdh*) housekeeping gene was used as an expression control (Table 9). The expression of endogenous mouse pluripotency genes was also determined by this method, in which $Brca2^{\Delta 27/\Delta 27}$ MEFS were used as a negative control. Primers used for pluripotency gene expression analyses are detailed in Table 9.

-Promoter methylation analyses

The methylation profile of the internal *Oct3/4* and *Nanog* promoters in the iPSC and their original fibroblasts was analyzed by the sodium bisulfite method.

This method was based on the conversion of non-methylated cytosine (C) into uracil (U) after bisulfite treatment. As this technique produced a high rate of DNA degradation, gDNA from at least $1x10^6$ iPSCs, mES cells or fibroblasts was used. DNA was then amplified by RT-qPCR in which non-methylated C that became U were read as T, while the methylated ones were read as C. For *Oct3/4* promoter analyses, a nested PCR was carried out obtaining a 460 bp long product with 15 CpG islands and for *Nanog* promoter analyses a single PCR was carried out obtaining a 366 bp long product with 6 CpG islands. Primers used for the amplification of *Oct3/4* and *Nanog* promoters are described in Table 9. "Hot start" PCR was performed for 35 cycles consisting of denaturation at 95°C for 1 minute, annealing at 56°C for 1 minute and extension at 72°C for 1 minute for all primer sets.

The sequence obtained after amplification was run on a 2 % agarose gel and cloned in a TOPO vector (TOPO-TA Cloning® Kit for Sequencing; Invitrogen) for DNA sequencing. The result was obtained by differences in the amount of C that did not become T, at locations where there was a CpG island.

Primer/probe	Primer/probe sequence
mOct4-F (RV)	5 -GGATGCTGTGAGCCAAGG-3
mOct4-R (RV)	5-GAACAAAATGATGAGTGACAGACAG-3
mNanog–F (RV)	5 -CACCCACCCATGCTAGTCTT-3
mNanog-R (RV)	5 -ACCCTCAAACTCCTGGTCCT-3
BRCA2-F	5 -CTTATGTTGCACAATGAGAAAAGAAATTAG-3
BRCA2-R	5-CGGAATCGGGCAAAACG-3
mNanog–F	5 -TTGCTTACAAGGGTCTGCTACT-3
mNanog-R	5 -ACTGGTAGAAGAATCAGGGCT-3
mOct4-F	5 -TAGGTGAGCCGTCTTTCCAC-3
mOct4-R	5 -GCTTAGCCAGGTTCGAGGAT-3
mSox2-5 F	5 -GAAACGACAGCTGCGGAAA-3
mSox2-R	5 -TCTAGTCGGCATCACGGTTTT-3
mMyc-F	5 -TAACTCGAGGAGGAGCTGGA-3
mMyc-R	5 -GCCAAGGTTGTGAGGTTAGG-3
mUtf-F	5 -GTGCACGCACCTCTACCTG3
mUTF-R	5 -AGAAAAAGCGGGCAACCT-3
Oct3/4 Promoter F1	5'- GTAAGTAAGAATTGAGGAGTGG-3'
Oct3/4 Promoter R1	5'- TCCAAACCCACCTAAAAACC-3'
Oct3/4 Promoter F2	5'- GATGGTTGAGTGGGTTGTAAGG-3'
Oct3/4 Promoter R2	5'- CCAACCCTACTAACCCATCACC-3'
Nanog Promoter F	5'- GATTTTGTAGGTGGGATTAATTGTGAATTT-3'
Nanog Promoter R	5'- ACCAAAAAAACCCACACTCATATCAATATA-3'
mGapdh-F	5 -TCCAAGGAGTAAGAAACCCTGGA-3
mGapdh-R	5 -GAAATTGTGAGGGAGATGCTCAG-3
mGapdh-Probe	5 -TexasRed-GAGCAAGAGAGAGGCCCTATTCCAACTC-BQH23
B-actin-F	5 -ACGGCCAGGTCATCACTATTG-3
B-actin-R	5 - ACTATGGCCTCAGGAGTTTTGTCA-3
B-actin-Probe	5 -Texas Red-AACGAGCGGTTCCGATGCCCT-BQH2-3
WPRE-F	5'-GGCACTGACAATTCCGTGGT-3'
WPRE-R	5'-AGGGACGTAGCAGAAGGACG-3'

Table 9. Schematic representation of primers and probes used for verification of genetic correction of gene complemented *Brca2*$^{\Delta 27/\Delta 27}$ cells and for analysis of pluripotency gene expression in iPSCs.

-Excision of the reprogramming cassette and analysis of proviral copy number by RT-qPCR

The excision of the polycistronic reprogramming cassette was carried out with two different types of excision vectors. First $10x10^5$ cells from diploid iPSC selected clones were transduced with an integration-deficient pLBid-nlsCre-SF LV, either in suspension in a tube or over gelatin-coated plates where they were maintained for 24 hours on irradiated feeder cells (irr MEFs).

When cells were confluent, gDNA was extracted from each individual colony derived plates and the proviral copy number was quantified by RT-qPCR in a Rotor Gene RG-3000 (Corbett Research Products) using primers against *wpre* sequence present in the reprogramming vector (Table 9). The housekeeping gene used to normalize the expression data obtained was *mβ-actin* (Table 9). Amplification was performed using SYBR Green Amplification Kit (Applied Biosystems) and the determination of the copy number was done by absolute quantification using a standard curve of a mouse cell line. Those colonies in which viral copies were still detected, were re-infected with the retroviral protein delivery system described in materials and methods. After re-excision, the iPSC colonies in which no amplification of w*pre* signal was detected were considered for further characterization and differentiation assays.

-Southern Blot for detection of the presence of reprogramming cassette

Genomic DNA (5-10 µg) from the samples was digested with PstI and EcoRV restriction enzymes (New England Biolabs) and a 0.8 % agarose gel electrophoresis was then preformed. The result of the electrophoresis was transferred to nylon membranes (Hybond-N + Amersham Biosciences) in a 0.4 M NaOH solution that was overnight hybridized with α^{32}P-δXTP labelled IRES and Wpre probes, with the labelling kit Rediprime II (Amersham Pharmacia Biotech) and fragments obtained from the digestion of the STEMCCA 4F plasmid with PstI and EcoRV enzymes.

Hybridized filters were washed twice in SSC 2X (0.03 M sodium citrate, 0.3 M NaCl) at room temperature. Filters were then washed again twice with SSC 2X + SDS 0.2 % (v/v) at 65°C for 20 minutes, followed by a room temperature wash with SSC 2X. For the detection of the labelled probe bound to the DNA, the Phosphorimaging system was used (Bio-Rad –molecular Imager FX).

- **GENOMIC STABILITY ASSAYS**

-Cell cycle

For cell cycle analysis, cells were trypsinized and then transferred to flow cytometry tubes to be fixed. For fixation, cells were washed with 300 µl of cold PBS while vortexing and then 800 µl of pure ethanol, while carefully vortexing, and were stored at -20°C for at least two hours.

Before analysis, cells were washed with PBS and centrifuged at 4ºC and 600 g for 10 minutes. Propidium iodide (5 µg/ml Molecular Probes) and RNase A (100 µg/ml Sigma) were added to the samples, incubated for 15 minutes at room temperature and then analyzed by flow cytometry (Coulter XL) with linear fluorescence of propidium iodide or DAPI, indicating the DNA content and eliminating doublet cells.

-Chromosomal stability studies

These studies were performed in actively dividing iPSCs at 70 % confluence in cultures, either treated or not with 1-3 butadiene diepoxide DEB (Sigma) to damage the cells, three hours before starting the cell cycle arrest treatment. To determine structural chromosomal abnormalities, cells were incubated with 10 µg/ml colcemid (Invitrogen) for 4 h at 37 °C after the incubation for 2 hours with or without 0,1 µg/mL of diepoxybutane (Sigma). Cells were then treated with 0.56 % KCl for 15 min at 37°C and fixed in methanol:acetic acid (3 : 1). Cell suspensions were dropped onto clean slides and air-dried for 24 h before hybridization. FISH was carried out as described by Samper, E. et al. 2002, using a Cy3-labeled LL (CCCTAA) 3 PNA telomeric probe and a FITC-labeled LL (ATTCGTTGGAAACGGGA) PNA centromeric probe (Eurogentec). The post-hybridization washes (3 x 10 min; each one) were performed in PBST 0.1 % Tween 20 at 60 ºC and dehydrated in ethanol (70 %, 90 % and 100 %; 5 min each). Slides were then counterstained and mounted in Vectashield H-1200 mounting medium with DAPI (Vector Laboratories, CA, USA). Fluorescence images were acquired with a Nikon 90i microscope (Nikon Instruments, Melville, NY, USA) fitted with a 100 × planfluor 1.3 N/A objective, appropriate filters, and an Hg Intensilight fluorescence unit. Digital images were acquired with Cytovision Genus software (Genetix, Boston, MA, USA). At least 20 metaphases of each cell line and condition were analyzed.

Chromosomal aberrations were identified as follows: chromatid or chromosomal breakages: gaps in one or two chromatids whose corresponding centromere was identified or loss of telomere signal in case of terminal breakage; chromosomal fusions: two chromosomes joined without telomere signals at the fusion point with one centromeric signal; dicentrics: chromosomal fusions between two chromosomes joined without telomere signals at the

fusion point with two centromeric signals, radials: a structure thought to result from the fusion of the broken arms of non-homologous chromosomes that can form trirradials, tetrarradials. The percentage of each type of aberration in each cell line and condition was used for statistical analysis.

-Comparative Genomic Hybridization arrays (aCGH)

A whole genome analysis was conducted by the company NIMGENETICS, in selected iPSC clones to determine the existence of more detailed chromosomal abnormalities. This technique combined genome-wide screening for copy number variations (CGH) with microarrays in which slides were arrayed with small DNA fragments as targets. A control DNA sample (wt-Balb/c mouse MEFs) and the DNA samples to be tested (wt iPSC and A16Ex7 iPSC) were differentially labelled and competitively hybridized with a commercial 180 kb oligonucleotide mouse array-CGH (AMADID 027411, Agilent Technologies, Santa Clara, CA), following manufacturer's protocol 1 (Barrett et al., 2004).

Relative fluorescence intensity data from the microarray were extracted and visualized using Feature Extraction software v10.7 and Agilent Genomic Workbench v5.0 (Agilent Technologies). Copy number altered regions were detected using ADM-2 (set as 6) statistic, provided by DNA Analytics, with a minimum number of 5 consecutive probes. Genomic build UCSC mm8 (NCBI36) was used for the experiment (Barrett et al., 2004).

5.2.3 STUDY OF THE FANCONI ANEMIA PHENOTYPE REVERSION

-BRCA2 expression

In corrected *Brca2*$^{\Delta 27/\Delta 27}$ iPSC clones, the expression of human *BRCA2* was analyzed by RT-qPCR to verify if upon gene complementation, the expression of this gene had been restored. When cells were confluent, RNA was extracted from each of the iPSC pellet samples (Qiagen kit) and it was retro-transcribed to cDNA to analyze the expression of *BRCA2* by RT-qPCR in a Rotor Gene RG-3000 (Corbett Research Products) with the primers and probes designed to detect the expression of this gene (Table 9). The housekeeping gene used to normalize *BRCA2* expression was the glyceraldehyde-3-phosphate dehydrogenase *mGapdh* (Table 9). Amplification was performed using PCR Taqman (Universal PCR Master Mix. Roche) and the data obtained were analysed by the 2Δ Ct method (Livak and Schmittgen, 2001).

-RAD51 *foci* formation studies

RAD51 *foci* formation assays were performed in fibroblasts and in iPSCs as a functional test to verify that the genetic defect of *Brca2*$^{\Delta 27/\Delta 27}$ cells that had been gene complemented with the therapeutic PCL1-BRCA2 vector, restored their ability to recruit RAD51 to the DNA repair *foci*, a sign that BRCA2 protein functioned properly and therefore, the HDR pathway.

To detect RAD51 *foci*, cells were cultured in gelatin-coated polystyrene two-slide chambers BD Falcon ($5x10^4$ cells/chamber). Two conditions from each sample were performed: one with mitomycin C (MMC) 40 nM (Sigma), to activate the DNA repair pathway that involves the generation of RAD51 *foci*, and another one without MMC, as a control. After 16 hours cells were fixed for 15 minutes with 3.7 % paraphormaldehyde and cell membranes were permeabilized for 5 minutes with 0.5 % Triton X-100. After washing up the permeabilizing solution with Tris-buffered saline (TBS, 50 mmol/l Tris-HCl, pH8, 150 mmol/l NaCl) samples were blocked for 30 minutes with blocking solution (10 % FBS,0,1 % NP-40 diluted in PBS) to prevent non-specific binding of the antibody. The primary antibody used for cell staining was anti RAD51 Rabbit polyclonal antibody (Calbiochem) diluted 1:250 in blocking solution and overnight incubated in a humid and dark chamber. The primary antibody was washed with TBS and the preparation was then incubated for 30 minutes at room temperature in the dark with a fluorescent secondary antibody (anti-mouse Alexa 488- FITC (Molecular Probes, Leiden, Netherlands), diluted 1:1,000) and with DAPI (4,6-diamidino-2phenylindole, diluted 1:1,000) to stain the nuclei. Finally, preparations were mounted with Moviol and air dried in the dark. The analysis of RAD51 *foci* formation was performed by fluorescence microscopy.

5.2.4 STUDY OF APOPTOSIS DURING THE REPROGRAMMING PROCESS

-Annexin V based apoptosis study

To analyze apoptosis during the reprogramming process, cells were transduced with the polycistronic excisable lentiviral vector harbouring the reprogramming factors *Oct3/4*, *Klf4* and *Sox2* and the reporter cherry protein. Apoptosis studies were performed only in the cherry positive population by Annexin V and DAPI staining. Cells were analyzed by flow cytometry at day 9 after transduction with the reprogramming vector. Freshly obtained cells were washed with cold PBS and were resuspended in binding buffer 1x. Cells were then incubated with FITC-

annexin V (BD Pharmigen) antibody together with DAPI (1/300) for 15 minutes at room temperature in the dark. After incubation cells were analyzed by flow cytometry in a maximum period of one hour. Control cells were wild type (wt) fibroblasts.

Analyses performed by annexin V, together with DAPI staining allowed the detection of four different cell populations that are represented in Figure 30, based on the principle that annexin V binds to residues of phosphatidilserine that start appearing in the cell membrane in the first steps of apoptosis. Within the advance of the apoptotic process, apoptotic cells became positive for annexin V and DAPI. In dead necrotic cells the binding of annexin V to PS residues is lost and only DAPI staining is detected.

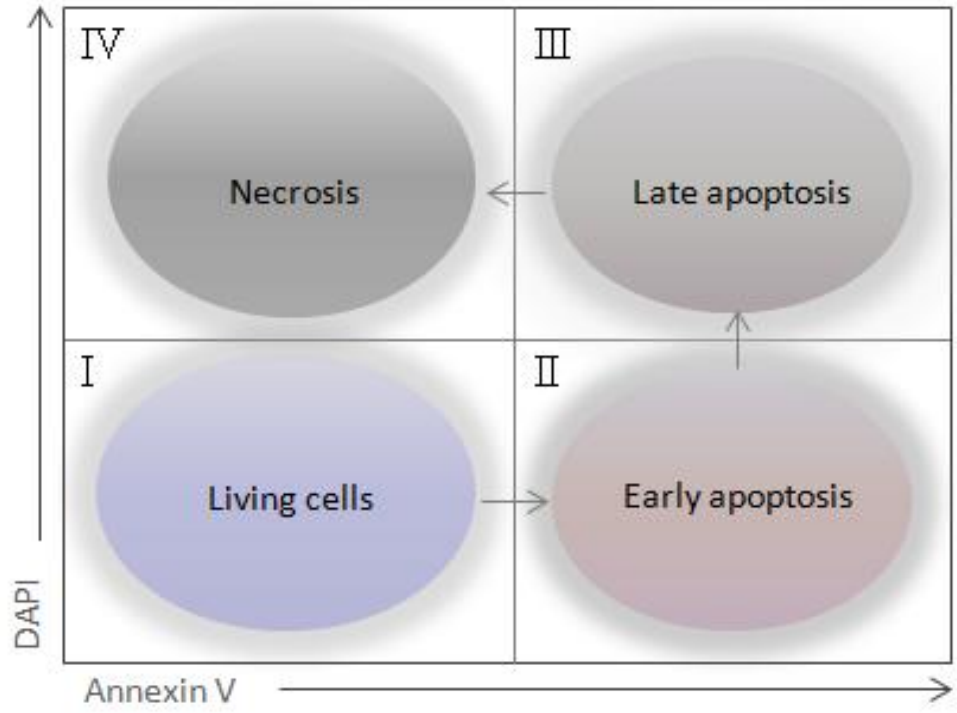


Figure 30. Representation of the flow cytometry analysis strategy used to study apoptosis during the cell reprogramming process by annexin V and DAPI staining. This strategy allowed the identification of four populations. **I:** Living cells that do not have phosphatidilserine (PS) residues exposed in the membrane to bind annexin V and do not allow DAPI entrance across the cellular membrane. **II:** Early apoptotic cells that have PS residues exposed in their membrane being possible to bind and detect annexin V fluorescence but not DAPI. **III:** Late apoptotic cells in which annexin V could be detected and DAPI was able to enter the cellular membrane. **IV:** corresponds to necrotic cells that lost their ability to bind annexin V to PS residues but DAPI entered the nucleous.

6. HEMATOPOIETIC DIFFERENTIATION ASSAYS OF MOUSE iPSC AND mESC

To achieve the hematopoietic differentiation of mouse embryonic stem cells (mESC) and genetically corrected iPSCs from *Brca2*$^{\Delta 27/\Delta 27}$ fibroblasts, several protocols were carried out with the objective of promoting hematopoietic differentiation (Table 10).

6.1 *IN VITRO* HEMATOPOIETIC DIFFERENTIATION PROTOCOLS

- *In vitro* hematopoietic differentiation of HOXB4 transduced cells on OP-9 stroma.

This hematopoietic differentiation protocol was a serum free method that consisted of transducing mESC and iPSC with a retroviral vector carrying the *HOXB4* LIM homeobox gene (MSCV.HOXB4.IRES.GFP). Forty eight hours before embryoid body generation, the medium in which cells were normally cultured (DMEM) was exchanged by IMDM medium (Biowhittaker, Walkersville, MD USA) for the cells to adapt to the medium used for embryo body generation.

Embryoid bodies were generated by natural aggregation (48 hours) of transduced cells after trypsinization with Trypsin/EDTA 0.25 % 1X (Gibco). Cells were seeded onto 10 cm^2 bacterial petri dishes at a final volume of 9 mL per plate ($1.8x10^5$ cells/plate) or ultralow-attachment 6 well plates (Corning, Costar) at a final concentration 10^6 cells/mL to prevent the cells to be attached to the surface. Cells were plated carefully, avoiding bubble generation that could alter the good condition of the cells, and were incubated at 37°C in a low percentage of O_2 (3 %) and high percentage of CO_2 (7 %) atmosphere, protected by a plastic box and a plate of sterile water to provide humidity to the environment. The medium used for embryo body formation was StemPro34-medium (500ml) (Gibco) + Nutrient Mix (~13 ml), 1 % Glutamax supplemented with 0.1 µg-U/µl P/S, 200 µg/ml Holotransferrin, monothioglycerol (MTG) (4,5 mM), 50 µg/ml ascorbic acid (added just before use due to its instability).

At this time, recombinant human bone morphogenetic protein (rhBMP4), at a final concentration of 4 ng/mL, was the only cytokine added to induce the differentiation of the cells towards mesoderm. Forty eight hours after aggregation, half of the medium was replaced by StemPro34 enriched with nutrient supplement and cytokines, to induce the differentiation towards hemangioblast and hematopoietic progenitors, added at a final concentration of 5

ng/mL mouse fibroblast growth factor (mFGF) (Prepotech), 5 ng/mL mActivin (R&D Systems), 5 ng/mL human vascular endothelial growth factor (hVEGF) 20 ng/mL (Prepotech), 20 ng/mL human thrombopoietin (hTPO) (Prepotech), 100 ng/mL mouse stem cell factor (mSCF) (R&D Systems). Embryoid bodies were maintained for 4 more days in this medium and at day 6, embryo bodies were collected and dissociated by Tryple Select treatment (Invitrogen). The resulting cells were counted and seeded at a concentration of $2x10^5$ cells/mL in a final volume of 2 mL over an irradiated OP9 stroma, in 6-well plates (Nunc, New York, USA).

Co-cultures were maintained for 14 days in OP9 co-culture medium: Iscove medium (Biowhittaker, Walkersville, MD USA) 20 % FBS (Gibco), 2 mM Glutamax (Invitrogen) , 2 mM P/S (penicillin-streptomycin, Gibco 15140-148), 100 ng/mL Flt-3, 100 ng/mL mSCF (R&D), 40 ng/mL TPO (Prepotech) and 40 ng/mL VEGF (Prepotech). Every other day, half of the medium was replaced until the final analysis by FACS (Figure 31).

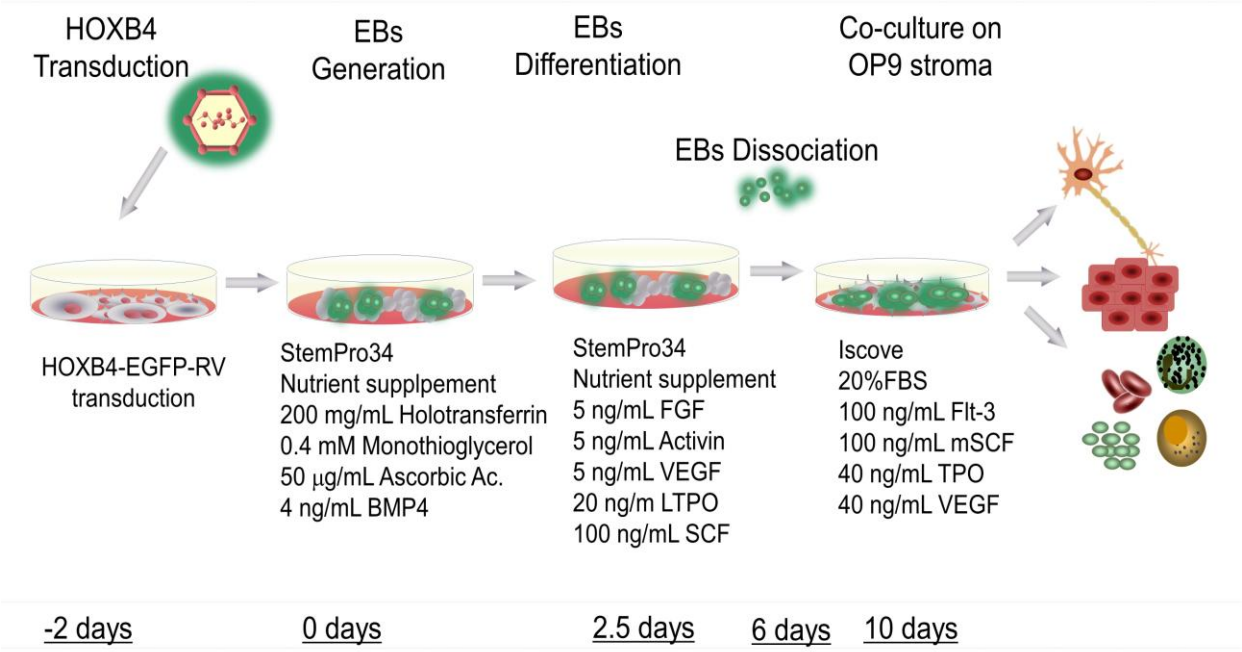


Figure 31. Schematic representation of the hematopoietic differentiation protocol, based on transduction of mESC and iPSCs with *HOXB4* and embryo body generation combined with co-culture on an OP-9 stroma.

- *In vitro* hematopoietic differentiation on AM.20 stromas

For hematopoietic differentiation in stromas derived from aorta-gonada-mesonephros region (AGM) AM20.1B4, mESJ1 and iPSCs clones were differentiated to embryo bodies (EBs) by natural aggregation at a concentration of 10^6 cells/mL in Iscove medium (Biowhittaker, Walkersville, MD USA) supplemented with 15% FBS (Gibco), 4.5 mM monothioglycerol (Sigma),

50 μg/mL ascorbic acid (Sigma) and 200 μg/mL iron-saturated holotransferrin (Sigma) at 37°C, 5 % CO_2, 21 % O_2. During the period of EB formation that lasted 6 days, hematopoietic cytokines were sequentially added as follows (Figure 32): at day 0, 4 ng/mL Bmp4, at day 2.5, 5 ng/mL hFGF and 5ng/mL Activin A and at day 4.5 ng/mL VEGF. At day 6, EBs were dissociated with Tryple Select (Invitrogen), counted and seeded at a concentration of $1.5x10^4$ cells/cm^2 in 6-well plates over a mitomycin C (MMC)-inactivated and confluent AM20.1B4 stroma. Cultures were maintained up to 21 days removing cellular debris and changing the culture medium every 3 to 4 days. By day 21, the expression of hematopoietic markers was evaluated by flow cytometry.

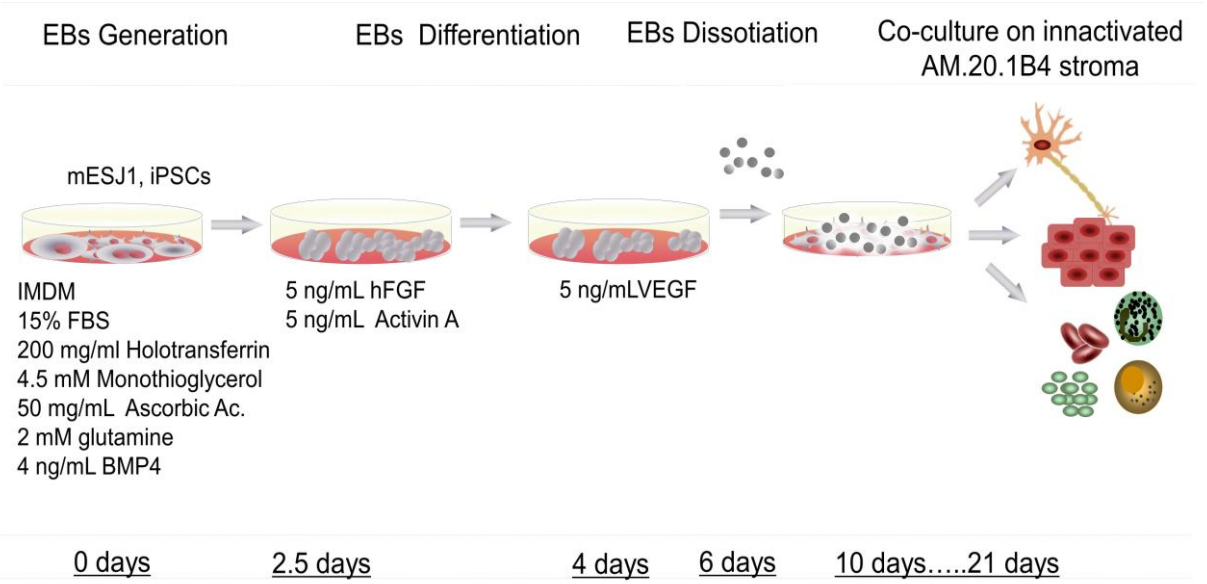


Figure 32. Schematic representation of the hematopoietic differentiation protocol, based embryo body generation combined with co-culture on an aorta-gonada-mesonephros region (AGM) stroma.

- Transplantation of iPSC derived hematopoietic progenitors into sub-lethally irradiated *Brca2*$^{\Delta 27/\Delta 27}$ mice

Cells from wt iPSCs and also from the A16Ex7 *Brca2*$^{\Delta 27/\Delta 27}$ iPSC clone were obtained after *in vitro* differentiation, following the protocol based on *HOXB4* transduction. After cell collection for FACS analysis at day 10 of differentiation, cells were collected and dissociated passing them through a syringe and were counted with Trypan blue.

One million cells per mouse were intravenously injected, into sub-lethally irradiated (3Gy) *Brca2*$^{\Delta 27/\Delta 27}$ mice, in a total number of two mice per condition, due to the limited number of cells.

The percentage of chimerism and expression of eGFP was analyzed by FACS since day 15 after transplant.

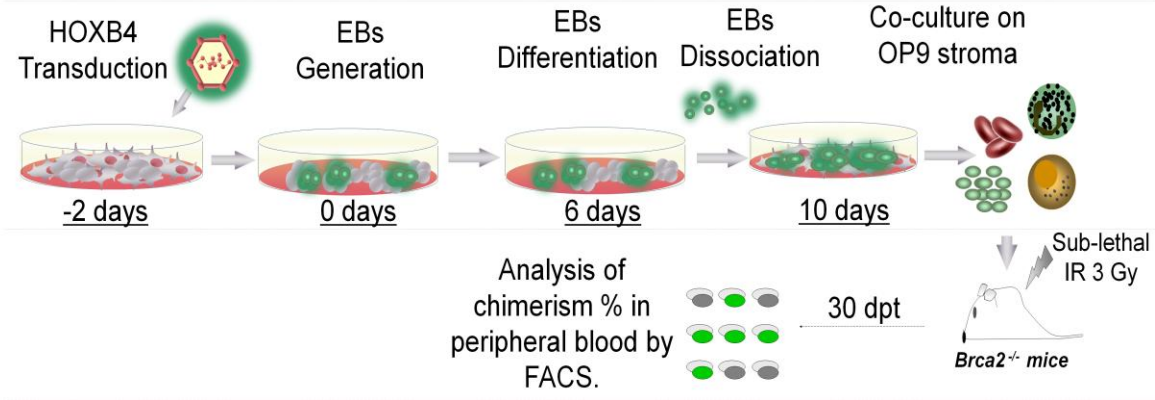


Figure 33. Schematic representation of the *in vivo* differentiation protocol, based on intravenous injection of EB-derived differentiated cells into sub-lethally irradiated *Brca2*$^{\Delta 27/\Delta 27}$ mice.

6.2 FLOW CYTOMETRY ANALYSES OF PLURIPOTENCY AND HEMATOPOIETIC MARKERS

The phenotype of iPSCs was analyzed by flow cytometry (FACS), using surface and intracellular antibodies linked to different fluorophores. The analysis of pluripotency by FACS was performed using SSEA-1 antibody (RyD Systems) linked to phycoerythrin (PE) fluorochrome. To analyze the phenotype of hematopoietic progenitors the antibodies used were: anti-SCA1-PE (BD Pharmigen), that binds to pluripotent cells, anti-FLK1-PE (BD Pharmigen), marker of hemangioblast, anti-CD31-PE (BD Pharmigen) that marks hemogenic endothelial cells, anti-cKit (BD Pharmigen), anti-CD41-PE (BD Pharmigen), anti-CD45-PE (BD Pharmigen), anti-CD34-biotin (eBioscience), anti-AA4.1-FITC (BD Pharmigen), all markers of hematopoietic stem cells at different stages. Gating was done with matched isotype IgG1-PE, FITC and -PE-Cy5 monoclonal antibodies used as controls, all from Pharmigen. Cells were washed with PBA (PBS with 0.1% BSA and 0.01% sodium azide), resuspended in PBA plus 2 µg/ml propidium iodide and analyzed using an EPICS ELITE-ESP cytometer (Beckman Coulter Inc). Off-line analysis was done with CXP Analysis 2.1 software.

A.

In vitro differentiation protocols

	Pluripotent stem cell	Mesoderm		Hemangioblast	Hematopoietic stem cell Progenitors		
	Transduction pre-differentiation	EBs Generation and Differentiation		Co-culture with hematopoietic stroma	Transduction post-differentiation	Co-culture with hematopoietic stroma	
	HOXB4	CK	MC	OP9	LHX2	OP9	AM20
1	√	√	√			√	
2		√	√				√

B.

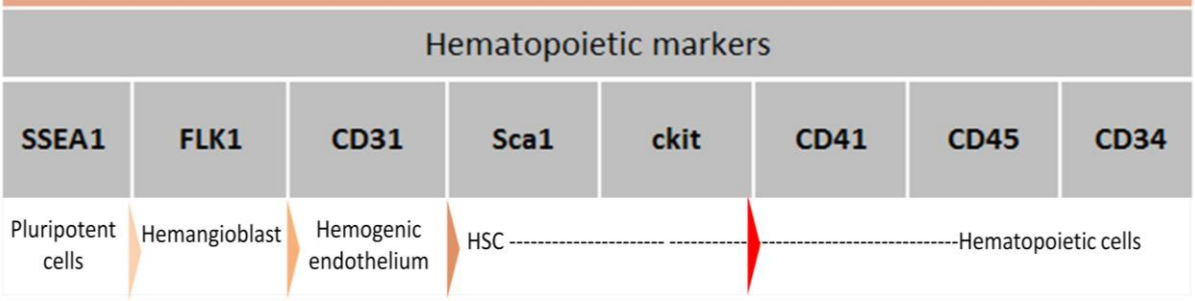


Table 10. A) Representation of the different ***in vitro*** hematopoietic differentiation protocols performed. **Protocol 1** and **protocol 2** involved the embryo body generation and culture with hematopoietic differentiation cytokines in a semisolid methylcellulose culture medium and co-culture over hematopoietic stromal cells. The differences between protocol 1 and 2 were that protocol 1 involved the transduction of the mESC and iPSCs with HOXB4 prior to the EB generation and co-culture over OP-9 stroma while protocol 2 employed the aorta-gonada-mesonephros (AGM) stroma for cell co-culture. **B)** Specific surface markers that sequencially appear in the cells during the embryonic hematopoietic development.

7. STATISTICAL ANALYSIS

Data obtained from the different experiments represented the mean and standard error of the mean (SEM). The significance of differences was determined by two non-parametrical tests. For comparisons between two groups we applied Mann-Whitney test. When comparing more than two groups we applied Kruskal-Wallis test. Differences were considered significant at $p<0.05$. All statistical analyses were performed using GraphPad Prism Software, Version 5.0 a.

1. INDUCED PLURIPOTENT STEM CELLS GENERATED BY TRANSDUCTION WITH RETROVIRAL VECTORS HARBOURING SINGLE REPROGRAMMING FACTORS

1.1 STUDY OF THE RELEVANCE OF FANCA AND BRCA2 IN CELL REPROGRAMMING

To investigate the relevance of two important members of FA/BRCA pathway (FANCA and BRCA2 proteins) in cell reprogramming and to search for new sources of HSCs in FA gene and cell therapy, we standardized the reprogramming method in wt mouse adult fibroblasts (MAFs) to then generate induced pluripotent stem cells (iPSC) from two FA mouse models (*Fanca*$^{-/-}$ and *Brca2*$^{\Delta 27/\Delta 27}$).

1.1.1 CELL REPROGRAMMING OF WILD TYPE CELLS

Three reprogramming attempts were performed using wt adult ear fibroblasts from C57BL/6 mice with γ-RVs harbouring the reprogramming factors *Oct3/4*, *Sox2*, *Klf4* and *c-Myc* described by Yamanaka. Cells were transduced with these γ-RVs, in two rounds of infection with either three (*Oct3/4*, *Sox2* and *Klf4*) or four of these factors (*Oct3/4*, *Sox2*, *Klf4* and *c-Myc*). Four days after transduction, cells were split and 5 x 10^4 cells were cultured over an irradiated mouse embryonic fibroblast (MEF) feeder layer, in the presence of mES medium (materials and methods). At day 11-13 the colonies arising in the culture were identified by their mESC-like morphology and were individually picked.

Despite it is described in bibliography that the transduction with the three reprogramming factors *Oct3/4*, *Sox2* and *Klf4* is sufficient to generate completely reprogrammed cells (Nakagawa et al., 2008), in our hands, colonies with a good ESC-like morphology could only be obtained when cells were transduced with the four reprogramming factors *Oct3/4*, *Sox2*, *Klf4* and *c-Myc*. In the condition that lacked *c-Myc*, colonies that arose did not perfectly resemble a mESC-like morphology as a consequence of being only partially reprogrammed.

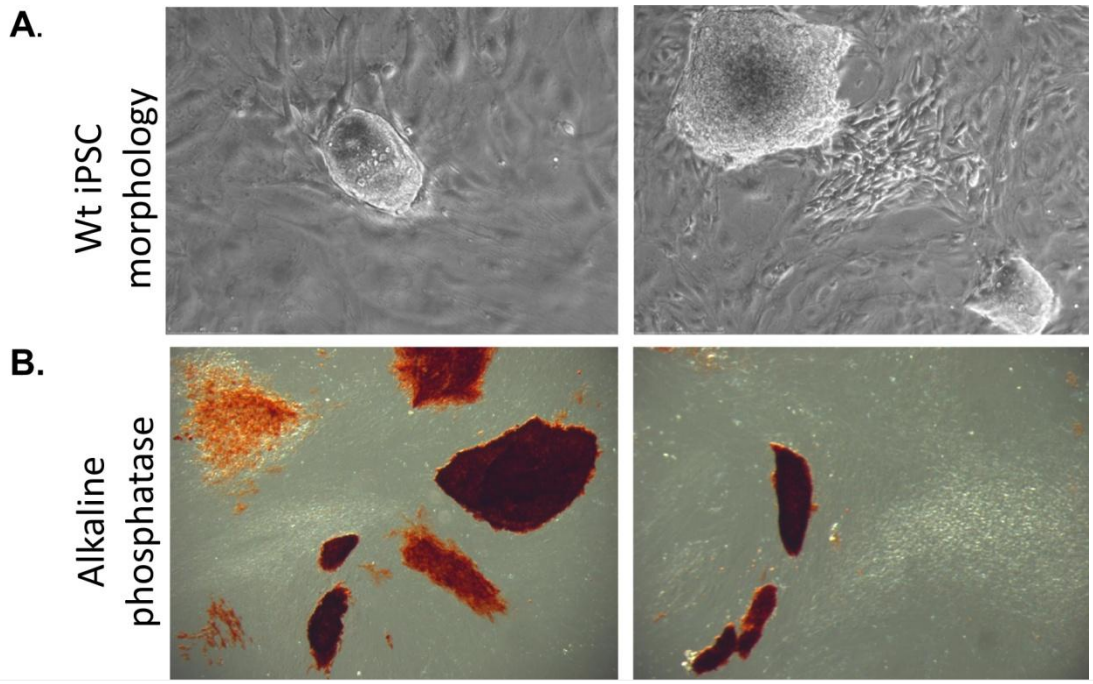


Figure 34. Representative images of wt iPSCs-like colonies generated with γ-RVs. **A)** Images obtained in bright field of mESC-like morphology of the colonies cultured on irradiated MEFs feeder layer: round/tear 3D shape forming syncitia and supportive stroma **B)** Images of alkaline phosphatase activity (AP) in iPSC like colonies captured at 4x.

Our results showed that the iPSC clones had a mESC-like morphology as can be observed in representative images of iPSC colonies in the upper part of Figure 34, where the characteristic 3D and compact shape of mESCs was observed. The lower part of Figure 34 shows the alkaline AP activity (red colour) that we obtained in the iPSC colonies where a high AP enzymatic activity was observed.

From the three experiments performed, seven iPSC clones were selected and characterized by AP activity detection. The proof of concept that this protocol allowed the reprogramming of wt adult fibroblasts let us standardize a reprogramming protocol for future experiments in FA deficient cells.

1.1.2 CELL REPROGRAMMING IN *Fanca*$^{-/-}$ AND *Brca2*$^{\Delta 27/\Delta 27}$ DEFICIENT CELLS

To investigate the comparative role that *Fanc*a and *Brca2* play in cell reprogramming process, MAFs from *Fanca*$^{-/-}$ (Cheng et al., 2000) and *Brca2*$^{\Delta 27/\Delta 27}$ *(Fancd1/Brca2)* (McAllister et al., 2002b) mice were reprogrammed with γ-RVs carrying the four reprogramming factors (*Oct3/4*, *Sox2*, *Klf4* and *c-Myc*).

To compare the reprogramming efficiency in both FA models with respect to the corresponding complemented cells, two days before reprogramming, *Fanca*$^{-/-}$ and *Brca2*$^{\Delta 27/\Delta 27}$ cells were transduced with the correspondent therapeutic lentiviral vector (PGK.FANCA and PCL1.BRCA2, respectively).

In the case of *Fanca*$^{-/-}$ MAFs, a total number of 45 iPSC-like starting colonies could be generated while only one iPSC colony could be isolated and maintained. In the *Brca2*$^{\Delta 27/\Delta 27}$ condition, eight iPSC-like colonies appeared, only in the condition in which the cells had been corrected with the therapeutic gene, while in the uncorrected cells one single colony was obtained. This colony could only be maintained with good mESC morphology for a few passages and was used as a control in different experiments. Significantly, iPSC colonies from corrected *Brca2*$^{\Delta 27/\Delta 27}$ cells appeared in the culture at day 13 post-transduction, while in the case of corrected *Fanca*$^{-/-}$ cells, iPSC-like colonies appeared two days earlier.

1.2 CHARACTERIZATION OF CORRECTED *Fanca*$^{-/-}$ AND *Brca2*$^{\Delta 27/\Delta 27}$ iPSCs

iPSCs from these two models were characterized according to well established criteria that assured the generation of "*bona fide*" iPSCs.

- **Morphology**

The first sign of having iPSCs was obtained by selecting colonies with a mESC-like morphology. These mESC-like features were a round/tear shape with defined borders, more than one nucleus forming syncytia and the formation of a surrounding stroma that fed the colony with required factors.

Our results showed the typical morphology of mESC colonies used as the reference cell population, to compare with the iPSCs generated (Figure 35). Based on this morphological criteria, four clones from *Fanca*$^{-/-}$ corrected cells (C1, C8, C8*, C11, C15, C23 and C35) and five from the *Brca2*$^{\Delta 27/\Delta 27}$corrected cells (C1, C2, C11, C14, C15, C20) were selected. The representative pictures in Figure 35 correspond to some of the iPSC clones with mESC-like morphology. The representative colony of uncorrected *Fanca*$^{-/-}$ iPSC-like cells, shown in the figure did not show such a 3D shape, demonstrating a limitation in the complete reprogramming of the cells.

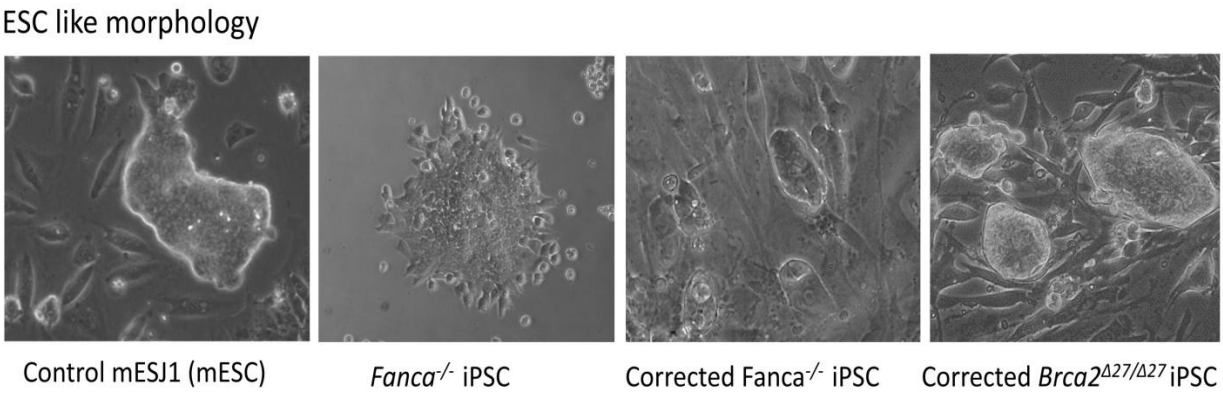


Figure 35. Representative images of the cell morphology observed in the iPSCs-like colonies generated from *Fanca*$^{-/-}$ deficient ear adult fibroblasts, genetically corrected *Fanca*$^{-/-}$ and corrected *Brca2*$^{\Delta 27/\Delta 27}$ iPSCs generated with the four reprogramming γ-RVs. Images obtained at 40x in bright field.

- **Immunofluorescence and FACS analysis**

Next we studied the AP enzymatic activity in the iPSC clones that were selected. All iPSC-like clones selected by morphology criteria were positive for this staining, although different levels of AP activity could be observed among them, being slightly lower in uncorrected *Fanca*$^{-/-}$ and *cBrca2*$^{\Delta 27/\Delta 27}$ iPSC-like clones (Figure 36).

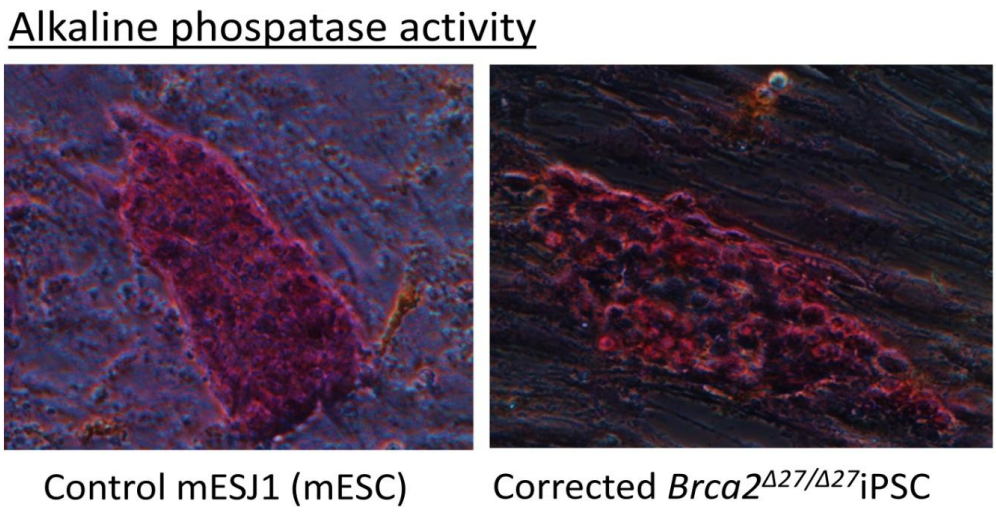


Figure 36. Representative images of AP activity in iPSC-like colonies generated from gene corrected *Brca2*$^{\Delta 27/\Delta 27}$ mouse adult fibroblasts (MAFs).

Once the AP activity was determined, the expression of the pluripotency markers SSEA1 and OCT3/4 was analyzed in corrected *Fanca*$^{-/-}$ and *Brca2*$^{\Delta 27/\Delta 27}$ iPSCs, either by FACS or by immunofluorescence, compared with mESC, used as control.

In Figure 37, the percentage of SSEA1 expression by FACS analysis is represented. In *Fanca*$^{-/-}$ corrected iPSC-like clones, it reached an average level of 10 %. These expression levels were not as high as the ones observed in the control population of mESC (60 %). The level of SSEA1 expression in *Brca2*$^{\Delta 27/\Delta 27}$ corrected iPSCs was even lower than that of *Fanca*$^{-/-}$ corrected cells, with an average of 2 % of cells positive for this marker.

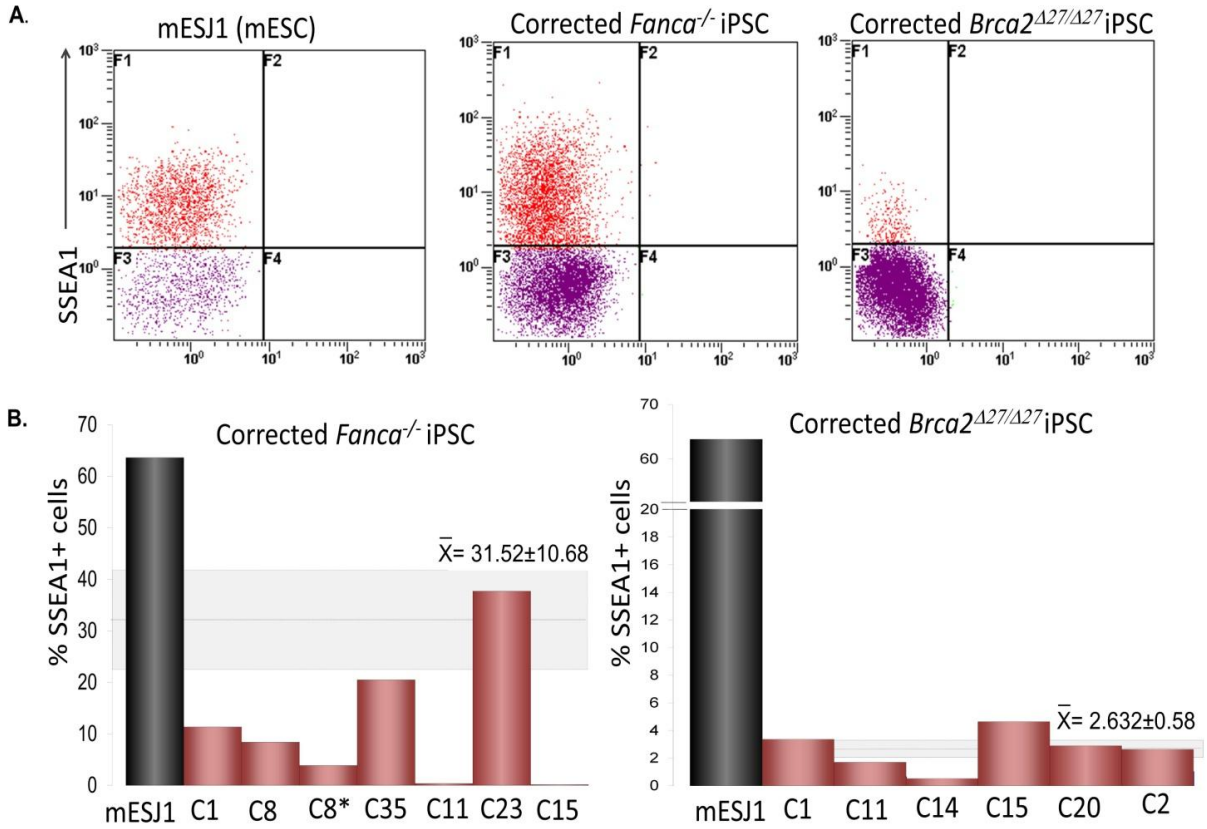


Figure 37. A) Representative dot-plots of the flow cytometry analysis of the percentage of cells positive for SSEA1 expression in *Fanca*$^{-/-}$ corrected iPSCs and in gene corrected *Brca2*$^{\Delta 27/\Delta 27}$ iPSCs, compared with the same study in mESC. **B)** Corresponds to the SSEA1 expression obtained in all the iPSC clones analyzed, both in *Fanca*$^{-/-}$ corrected iPSCs and gene corrected *Brca2*$^{\Delta 27/\Delta 27}$ iPSCs.

The OCT3/4 expression in *Fanca*$^{-/-}$ corrected iPSCs was also determined by FACS. As shown in Figure 38, higher percentages of cells positive for this pluripotency marker were observed, as compared to SSEA1 in *Brca2*$^{\Delta 27/\Delta 27}$ corrected cells. All selected clones were positive for this marker at equivalent levels to the ones obtained in mESCs.

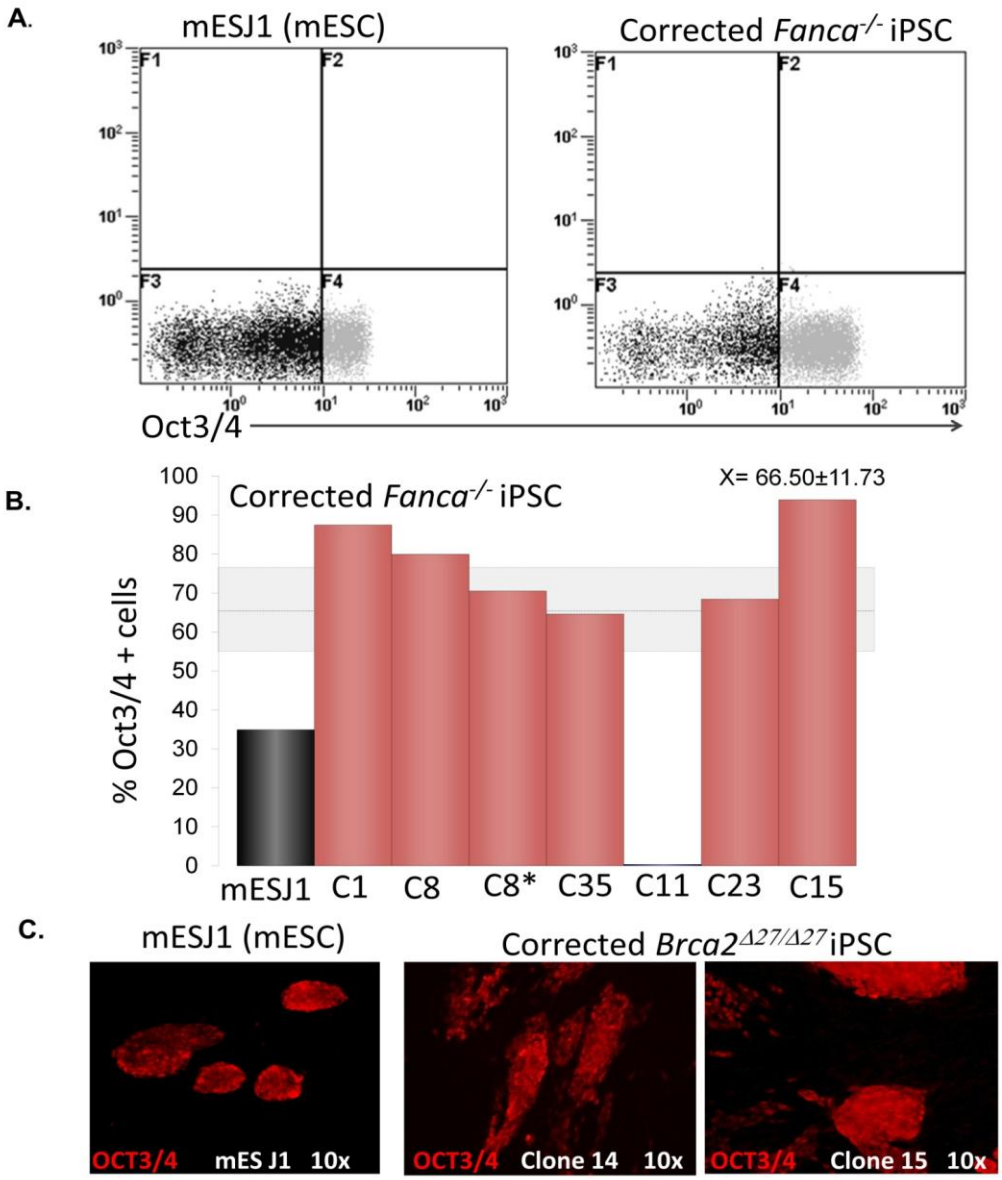


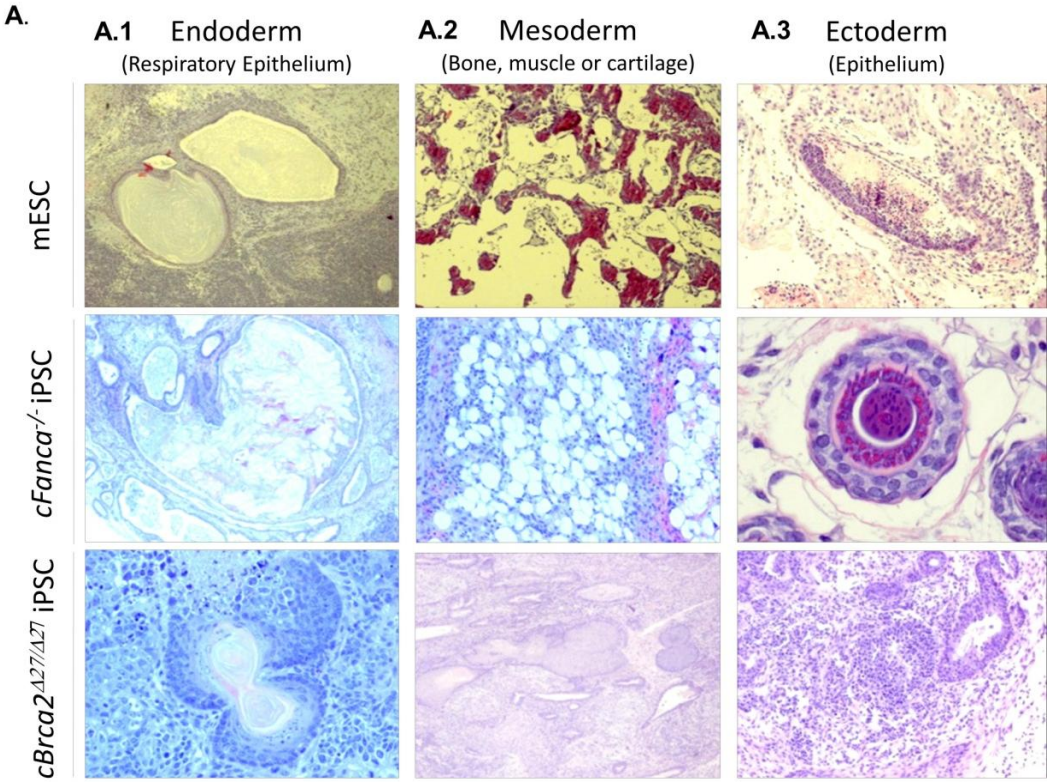
Figure 38. A) Representative dot-plots of the FACS analysis of the percentage of cells positive for the OCT3/4 expression in *Fanca*$^{-/-}$ corrected iPSCs compared with the same study in mESC. **B)** Corresponds to the OCT3/4 expression obtained from all the *Fanca*$^{-/-}$ corrected iPSCs clones analyzed, compared with the expression of this marker in mESC. **C)** Immunofluorescence of representative clones for the expression of OCT3/4 pluripotency marker in gene corrected *Brca2*$^{\Delta 27/\Delta 27}$ iPSCs.

- **Teratoma formation**

The capacity to form teratomas is a functional assay that is considered one of the essential tests in the characterization of iPSCs in terms of pluripotency. For this assay, 1 x 10^6 cells from each iPSCs selected clone were subcutaneously injected into NOD/SCID γc^{null} mice.

One month after injection of the iPSCs, a tumour mass could be observed in mice flanks. After dissection, histological analyses were performed to detect the presence of cells from the three germ layers. The histological samples were first stained with H&E. As shown in Figure 39, cells

belonging to the endoderm, mesoderm and ectoderm layers were detected, both in corrected *Fanca*$^{-/-}$ and *Brca2* $^{27/\Delta27}$ iPSC clones, similar to the structures observed in the control mESCs. Additionally, in the four c*Brca2*$^{\Delta27/\Delta27}$clones obtained, immunofluorescence staining with specific markers of the three germ layers showed that all the clones were positive for the alpha fetoprotein (AFP) endodermal marker (Figure 39). Furthermore, 3 out of 4 analyzed teratomas resulted positive for the FOXA2 marker. The specific mesodermal markers analyzed were BRACHYURY and αASMA. The expression of αASMA was detected, confirming the presence of cells of mesodermal origin. Finally, the staining with TUJ1 and GFAP ectodermal specific markers resulted positive in the clones that were analyzed, demonstrating that these iPSC clones were pluripotent.



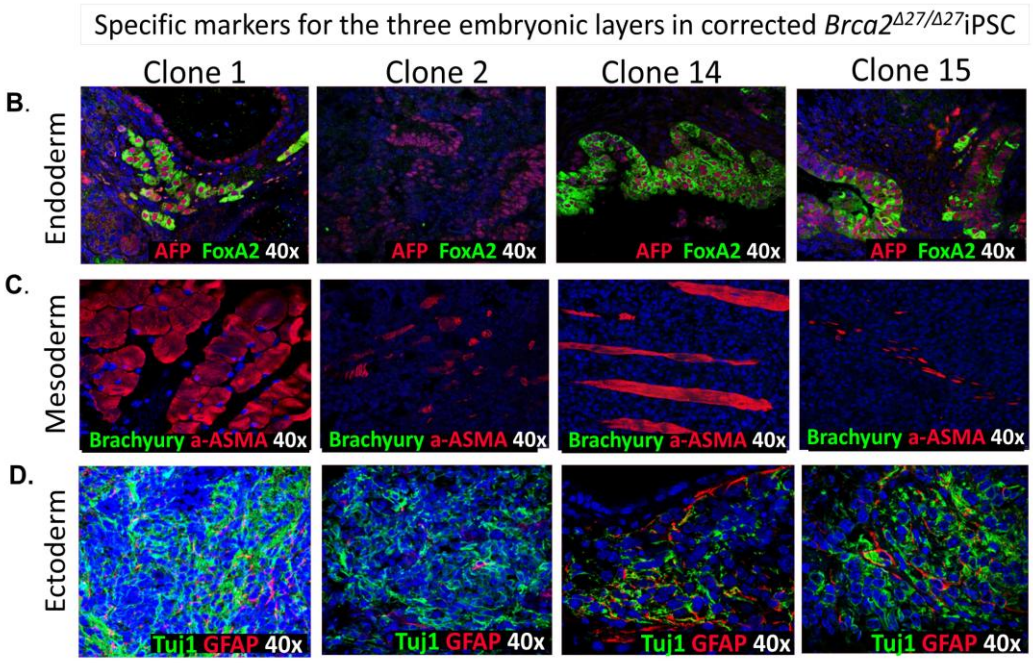


Figure 39. A) Representative images of teratoma formation in iPSCs derived from *Brca2*$^{\Delta 27/\Delta 27}$ MAFs and mESC control line. Images obtained at 40x and details at 200x **A.1)** Endodermal cell types corresponding to respiratory epithelium. **A.2)** Mesodermal cells of different tissues such as bone, cartilage or muscle. **A.3)** Epithelial cells corresponding to ectoderm. **B, C and D)** Representative images of specific immunofluorescence assays of each of the three germ layers, performed in the teratoma derived from the *Brca2*$^{\Delta 27/\Delta 27}$derived iPSC teratoma. **B)** Endodermal markers AFP (alpha feto protein) in green and FOXA2 in red. **C)** Specific mesodermal markers BRACHYURY in green and α–ASMA in red. **D)** Ectodermal markers Tuj1 in green and GFAP in red.

- Expression of endogenous pluripotency genes

In the c*Brca2*$^{\Delta 27/\Delta 27}$ iPSC clones that were selected to characterize, the expression of the endogenous pluripotency genes was analyzed, to verify if these cells had been effectively reprogrammed. In Figure 40 we can observe that the expression of the endogenous pluripotency genes was nearly undetectable when compared with the high pluripotency gene expression detected in mESCs.

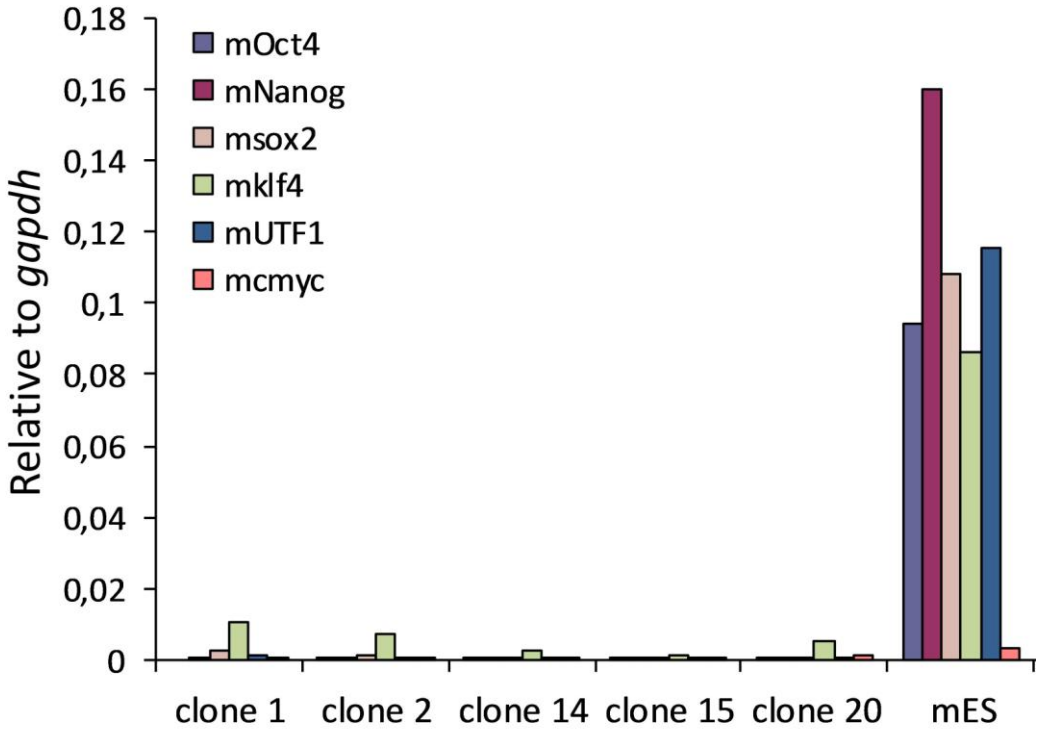


Figure 40. RT-qPCR analysis of endogenous pluripotency gene expression relative to mouse *Gapdh* in c*Brca2*$^{\Delta 27/\Delta 27}$ iPS clones. Purple colour corresponds to the expression of *Oct3/4* gene, aubergine colour bars represent the expression of *Nanog*, light pink corresponds to *Sox2* expression. The expression of *Klf4* is represented in green colour, *Utf1* in dark blue and *c-Myc* is represented in dark pink.

- Expression of exogenous pluripotency genes

We also analyzed, by RT-qPCR, the exogenous pluripotency genes *Oct3/4*, *Klf4*, *Sox2* and *c-Myc*, using specific primers for these exogenous genes.

Our results showed that clones 2, 14 and 15 were the ones with higher expression of these exogenous pluripotency genes, while in the others we could only observed a modest expression of these genes (Figure 41).

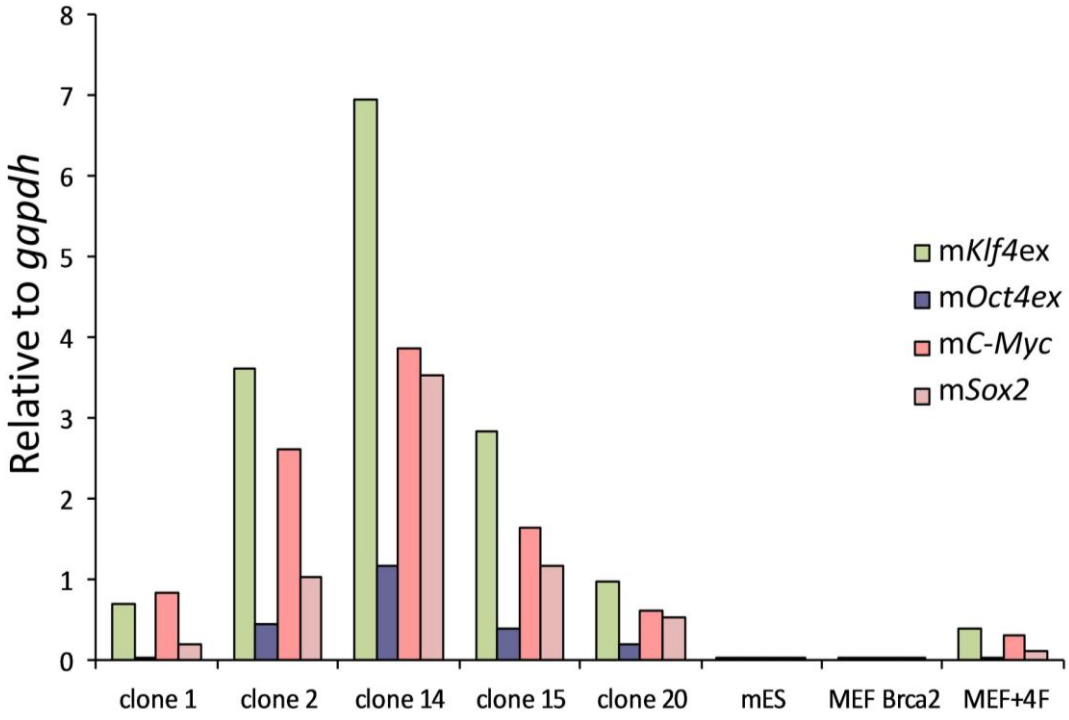


Figure 41. Graph representing the RT-qPCR analysis of exogenous pluripotency gene expression in *cBrca2*$^{\Delta 27/\Delta 27}$ iPS clones. Green colour corresponds to the expression of *Klf4* gene, purple bars represent the expression of *Oct3/4*, dark pink corresponds to *c-Myc* expression and the expression of *Sox2* is represented in light pink.

- The reprogramming of uncorrected *Fanca*$^{-/-}$ and *Brca2*$^{\Delta 27/\Delta 27}$ cells, showed that these two genes play important roles in cell reprogramming. However, compared to *Fanca, Brca2* was more critical, as no activation of the endogenous pluripotency genes could be reproducibly detected in *Brca2*$^{\Delta 27/\Delta 27}$cells.

- The reprogramming of gene-corrected *Fanca*$^{-/-}$ and *Brca2*$^{\Delta 27/\Delta 27}$ cells with γ-RVs carrying four reprogramming genes generated iPSCs-like cells that did not fulfill all the criteria of *"bona fide"* iPSCs.

2. GENERATION OF CORRECTED iPSCs USING POLYCISTRONIC EXCISABLE LENTIVIRAL VECTORS

2.1 RELEVANCE OF BRCA2 IN CELL REPROGRAMMING WITH POLYCISTRONIC EXCISABLE LENTIVIRAL VECTORS

To improve the efficiency and safety associated to reprogramming of corrected *Brca2*$^{\Delta 27/\Delta 27}$ cells, in the next set of experiments, MEFs, instead of MAFs, were used. Additionally, an excisable polycistronic lentiviral vector carrying the four reprogramming factors under the control of the EF1α promoter and a LoxP site to remove the reprogramming cassette, once the cells were reprogrammed, was used instead of the four reprogramming γ-RVs. In this experiment *Brca2*$^{\Delta 27/\Delta 27}$ deficient MEFs were transduced with the reprogramming vector, either after 48 hours, or simultaneously to transduction with the therapeutic vector (Figure 42).

MEFs from each condition were transduced with the polycistronic reprogramming LV in two rounds of infection. Four days later (day 6), infected cells were seeded on irradiated MEF-coated plates, when mESC-like colonies arouse, between day 11 and 13, clones were individually selected and expanded.

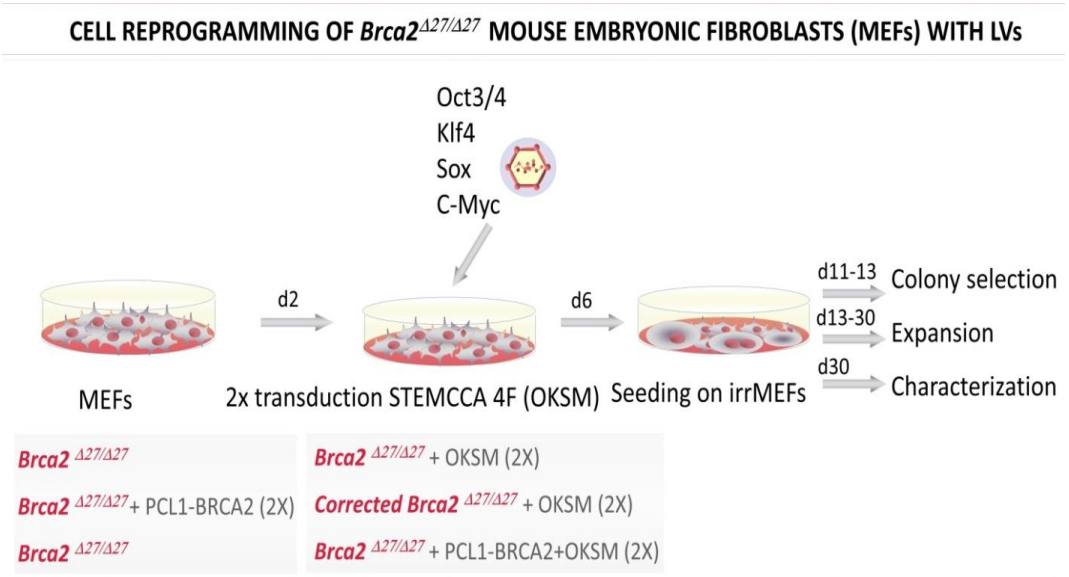


Figure 42. Cell reprogramming process in uncorrected and genetically corrected *Brca2*$^{\Delta 27/\Delta 27}$ MEFs with an excisable polycistronic lentiviral vector (STEMCCA-4F.OKSM). Selection, expansion and characterization once the iPSCs were established are also represented.

As shown in Figure 43, when *Brca2*$^{\Delta 27/\Delta 27}$ cells were not corrected from their genetic defect, no iPSC colonies could be obtained while in the two other conditions evident iPSC-like clones were generated and no significant differences in their respective numbers were observed.

The efficiency of cell reprogramming was evaluated in the three conditions, corresponding to uncorrected *Brca2*$^{\Delta 27/\Delta 27}$ cells, *Brca2*$^{\Delta 27/\Delta 27}$ corrected previous to cell reprogramming and *Brca2*$^{\Delta 27/\Delta 27}$ cells that were corrected simultaneously to the transduction with the reprogramming vector. Four different independent experiments were performed (mean value of 80 colonies in the condition in which the cells were first corrected and then reprogrammed and mean value of 150 colonies in the condition in which correction and reprogramming were performed simultaneously). In our hands, the efficiency of reprogramming was slightly higher, although not significant, in the condition in which *Brca2*$^{\Delta 27/\Delta 27}$ MEFs were gene complemented and reprogrammed at the same time (71.25 ± 31.75 and 197 ± 90.29, respectively).

From these experiments, a total number of 96 colonies were picked, and 26 colonies were further characterized.

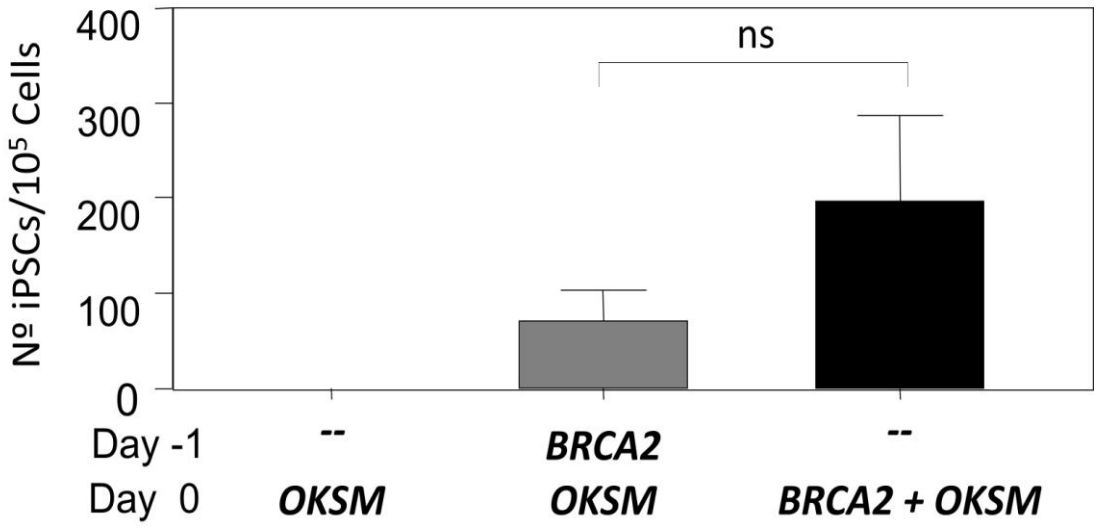


Figure 43. Analysis of the reprogramming efficiency of *Brca2*$^{\Delta 27/\Delta 27}$ mouse embryonic fibroblasts (MEFs), transduced with a polycistronic lentiviral vector harbouring the four reprogramming factors (*Oct3/4*, *Klf4*, *Sox2* and *c-Myc*). The graph corresponds to the three conditions studied: Uncorrected *Brca2*$^{\Delta 27/\Delta 27}$ cells; *Brca2*$^{\Delta 27/\Delta 27}$ cells corrected prior to or simultaneously to transduction with the reprogramming vector.

2.1.1 STUDY OF THE HOMOLOGY DIRECTED REPAIR PATHWAY DURING THE REPROGRAMMING OF COMPLEMENTED AND NOT COMPLEMENTED *Brca2*$^{\Delta 27/\Delta 27}$ CELLS

Due to the relevance of *Brca2* in cell reprogramming we wondered to which extent HDR could be playing a role during the process of cell reprogramming as described by Marion et.al. (Marion et al., 2009). The HDR during cell reprogramming was investigated and analyzed in uncorrected *Brca2*$^{\Delta 27/\Delta 27}$ cells, in genetically corrected *Brca2*$^{\Delta 27/\Delta 27}$ cells (c*Brca2*$^{\Delta 27/\Delta 27}$) and in their correspondent wt cells (Balb/c), as well as in *Fanca*$^{-/-}$ cells.

Our results showed that reprogramming of wt and genetically corrected cells *Brca2*$^{\Delta 27/\Delta 27}$ was associated with the generation of RAD51 *foci*. In *Brca2*$^{\Delta 27/\Delta 27}$ cells, although there was a basal percentage of RAD51 *foci* of around 5 % in non reprogrammed cells, the number of RAD51 *foci* during reprogramming was significantly lower than the basal ones (Figure 44).

This study confirms that HDR is activated during the process of cell reprogramming. However, in the case of *Brca2*$^{\Delta 27/\Delta 27}$ cells, this process was interfered, while gene complementation with BRCA2 restored the HDR process during cell reprogramming of these cells.

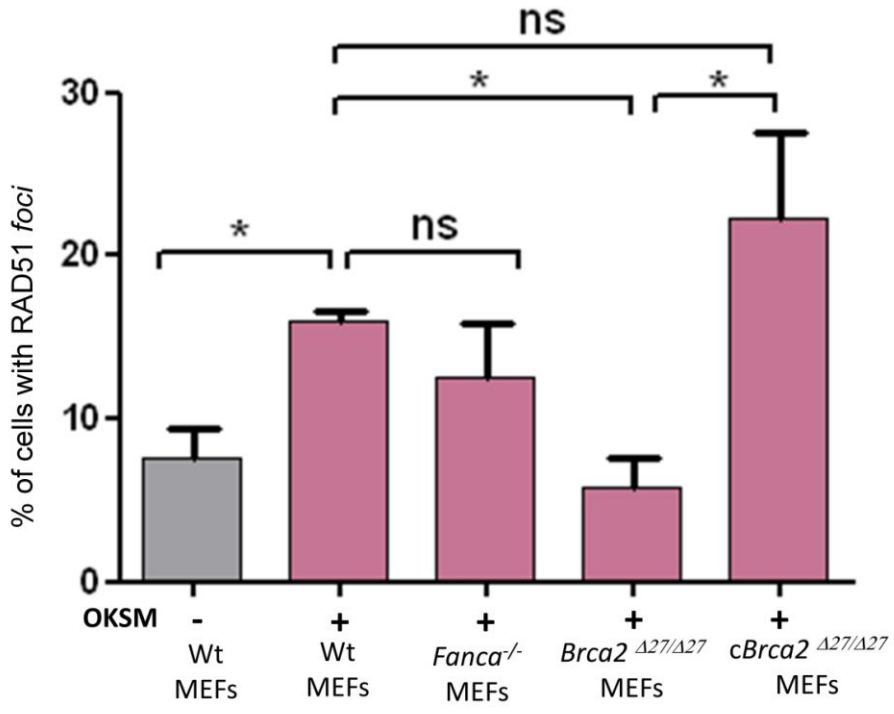


Figure 44. Study of the HDR during the cell reprogramming process by the analysis of the recruitment of RAD51 to DNA repair *foci*. The graph represents the percentage of cells with RAD51 *foci* in wt, in *Fanca*$^{-/-}$, *Brca2*$^{\Delta 27/\Delta 27}$ and in genetically corrected *Brca2*$^{\Delta 27/\Delta 27}$ MEFs (c*Brca2*). Grey bars correspond to the mock condition in which the cells were not transduced with the reprogramming vector. Pink bars represent cells transduced with the polycistronic reprogramming lentiviral vector. Data represent mean value of three independent experiments and standard

2.1.2 APOPTOSIS STUDIES DURING THE REPROGRAMMING OF CORRECTED AND UNCORRECTED *BRCA2*$^{\Delta 27/\Delta 27}$ CELLS

To study the reasons accounting for the extremely low reprogramming of *Brca2*$^{\Delta 27/\Delta 27}$cells, we analyzed the level of apoptosis in these cells during the process of cell reprogramming.

To ensure that the cells that we analyzed had been transduced with the reprogramming vector, in these three independent experiments, we used an excisable polycistronic lentiviral reprogramming vector harbouring three reprogramming factors (*Oct3/4*, *Klf4* and *Sox2*) and also the red fluorescent protein (RFP) as a reporter, to select the transduced population. At day 9 of reprogramming the parameters of viability, apoptosis and necrosis were analyzed by FACS.

Our results showed that the viability was reduced in reprogrammed cells both in wt and *Brca2*$^{\Delta 27/\Delta 27}$ with a percentage of dead cells that ranged from 50 to 70 %, in contrast with the 10 to 30 % observed in the cases in which reprogramming had not been induced. However, the results shown in Figure 45, demonstrated that there was an increase in the percentage of early and late apoptosis during the reprogramming of *Brca2*$^{\Delta 27/\Delta 27}$ cells, in contrast to the lower number of apoptotic cells observed during the reprogramming of wt cells.

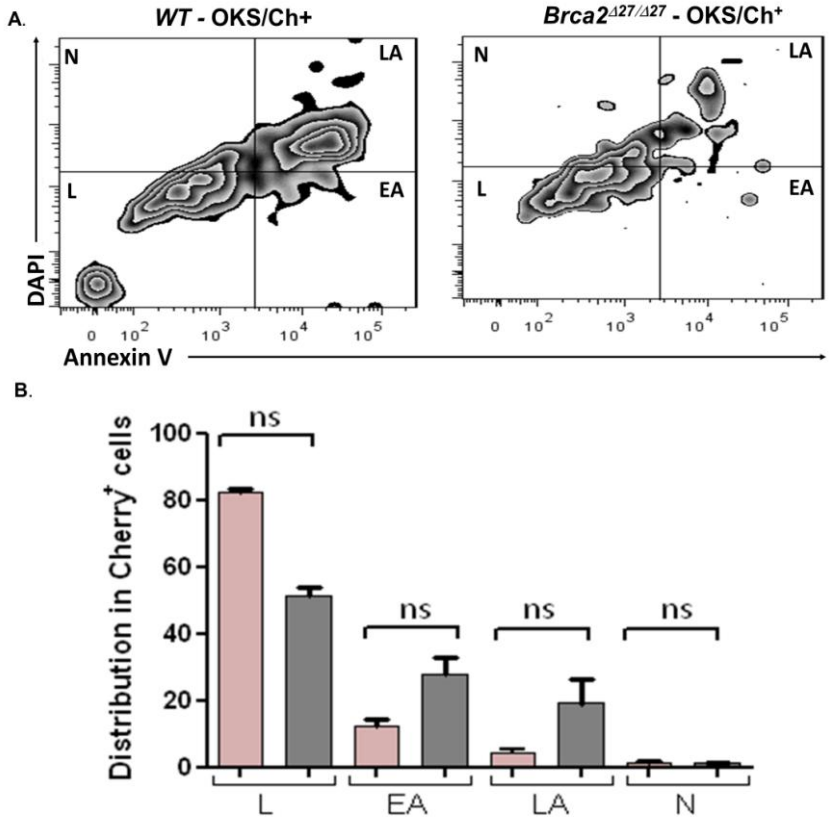


Figure 45. A) FACS analysiss of apoptosis in cherry positive cells by Annexin V assay. Annexin V binds to phosphatidylserine (PS) residues that are exposed in the cell membrane in the first steps of apoptosis. **L:** living cells (Annexin V negative); **EA:** Early apoptosis (Annexin V positive, DAPI negative); **LA:** Late apoptosis (Annexin V positive, DAPI positive); **N:** Necrosis (Annexin V negative, DAPI positive). Left panel corresponds to reprogrammed wt cells, while right panel represents reprogrammend *Brca2*$^{\Delta 27/\Delta 27}$ cells. **B)** Graph representing the distribution of the different populations analyzed in cherry positive cells. Pink bars correspond to wt cells during reprogramming and grey bars correspond to *Brca2*$^{\Delta 27/\Delta 27}$ cells during the process of cell reprogramming.

- The reprogramming of uncorrected and corrected *Brca2*$^{\Delta 27/\Delta 27}$ MEFs by transduction with a polycistronic lentiviral excisable vector confirmed that BRCA2 is essential for pluripotency induction.

- Studies of homology directed repair (HRD) demonstrated that this DNA repair mechanism is activated during the reprogramming of wild type or FA-A cells. However, this mechanism is not activated during the reprogramming of *Brca2*$^{\Delta 27/\Delta 27}$ cells. After correction of the genetic defect, the HDR capacity of *Brca2*$^{\Delta 27/\Delta 27}$ cells is restored.

- Apoptotic studies performed during the reprogramming of *Brca2*$^{\Delta 27/\Delta 27}$ cells revealed an increase in the early and late apoptosis of these cells, accounting – at least in part - for their extremely low efficiency of reprogramming.

2.2 CHARACTERIZATION OF COMPLEMENTED *Brca2*$^{\Delta 27/\Delta 27}$ iPSCs GENERATED FROM MOUSE EMBRYONIC FIBROBLASTS BY TRANSDUCTION WITH PLOYCISTRONIC EXCISABLE REPROGRAMMING LVs

The characterization of the iPSC from *Brca2*$^{\Delta 27/\Delta 27}$ corrected cells was done in two blocks, the first one corresponded to reprogrammed cells prior to the excision of the reprogramming cassette, and the other one was performed after the excision of the reprogramming vector.

- Morphology

The characterization of *Brca2*$^{\Delta 27/\Delta 27}$ iPSCs generated with the polycistronic lentiviral vector was first performed by the evaluation of their mESC-like morphology. When *Brca2*$^{\Delta 27/\Delta 27}$ cells were not genetically corrected, no iPSC colonies could be isolated.

Figure 46 shows representative images of the 26 clones that were selected by a morphology that resembled the morphology of mESCs.

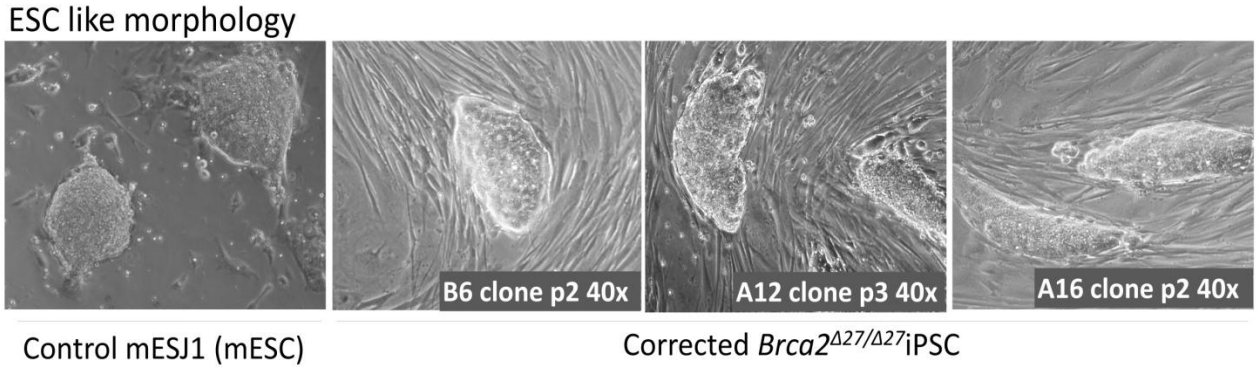


Figure 46. Representative images obtained at 40x in bright field, of iPSCs-like colonies generated from gene corrected *Brca2*$^{\Delta 27/\Delta 27}$ MEFs after transduction with the excisable reprogramming LV (STEMCCA-4F.OKSM). The mouse ESC line J1 was used as a control.

- Immunofluorescence and FACS analysis of pluripotency markers

The AP staining showed that the number of colonies positive for AP had increased in the reprogramming with the polycistronic lentiviral vector (Figure 47).

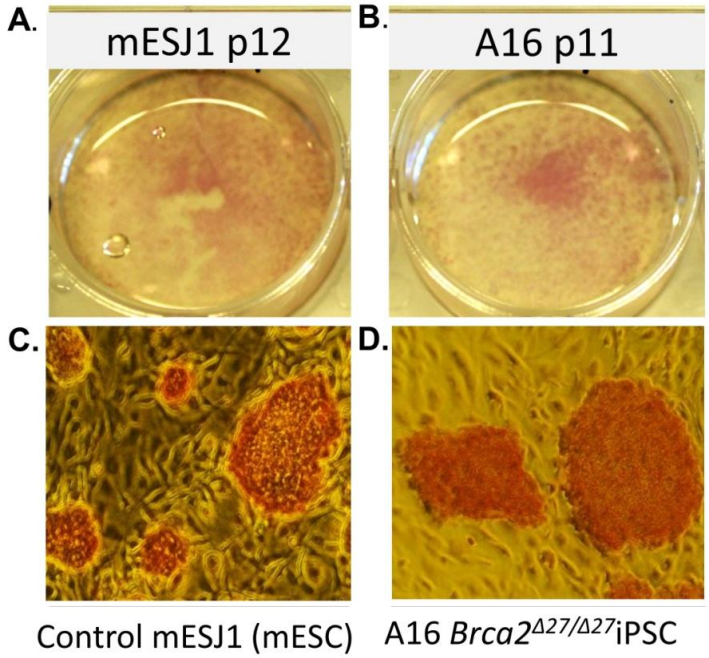


Figure 47. Images of AP staining of a representative c*Brca2*$^{\Delta 27/\Delta 27}$ iPSC clone generated by STEMCCA-4F.OKSM, cultured over a feeder layer of irradiated MEFs. **A** and **B** correspond to images of the cultured plates obtained after fixation and staining. **A)** Control mESC line **B)** A16 c*Brca2*$^{\Delta 27/\Delta 27}$ iPSC clone. **C** and **D** correspond to 40x images obtained in bright field obtained under a magnifying glass. **C)** Control mESC line. **D)** A16 c*Brca2*$^{\Delta 27/\Delta 27}$ iPSC clone.

The presence of SSEA1 in *Brca2*$^{\Delta 27/\Delta 27}$ corrected MEFs was determined by FACS. As it can be observed in Figure 48, all the clones expressed a very high proportion of SSEA1 (between 60 and 90%), equivalent to the percentage observed in the mESC used as a control. SSEA1 expression was confirmed by immunofluorescence analyses (Figure 49).

After the first screening of iPSC clones by AP activity and expression of the SSEA1 pluripotency marker, we analyzed the expression of other pluripotency markers, such as OCT3/4 and NANOG (Figure 49 and 50).

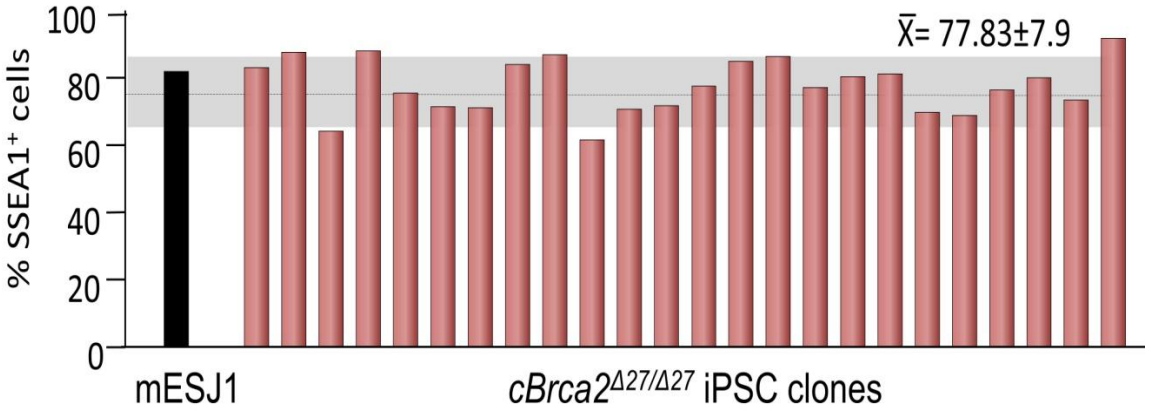


Figure 48. Graph representing the percentage of SSEA1^{+} cells analyzed by FACS in c*Brca2*$^{\Delta 27/\Delta 27}$ iPSC clones generated with STEMCCA-4F.OKSM. Mean values and standard error are represented by a grey bar.

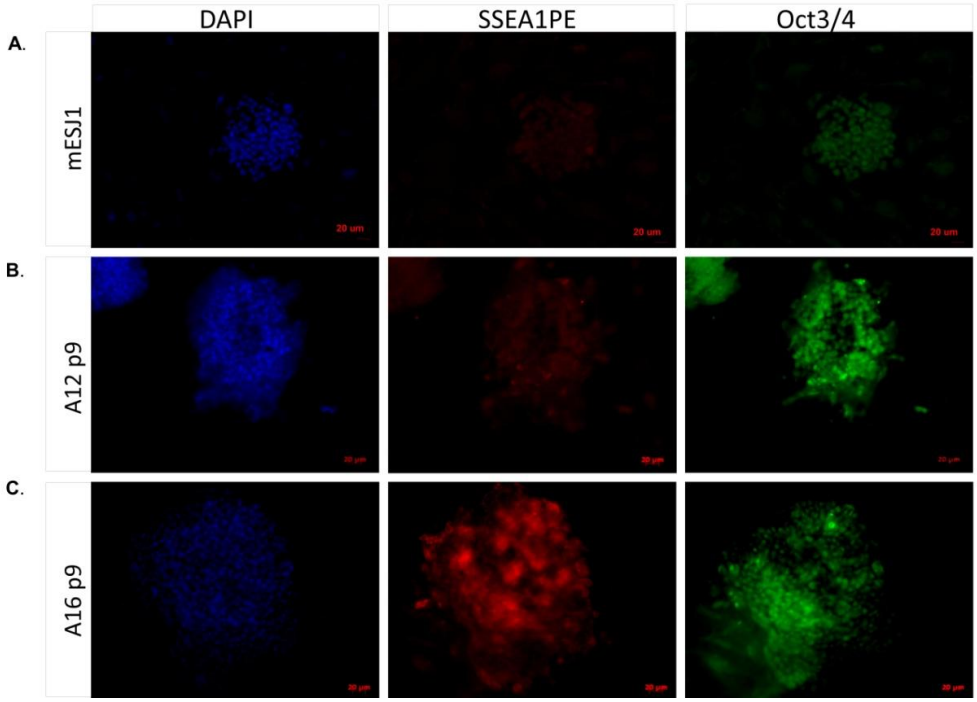


Figure 49. Immunofluorescence analysis of the pluripotency markers SSEA1 and OCT3/4 and the correspondent DAPI staining of the cellular nucleus in A12 and A16 *Brca2*$^{\Delta 27/\Delta 27}$ corrected clones and in control mESC line. **A)** DAPI staining, SSEA1 expression detected in texas red and OCT3/4 detected in FITC in the mESJ1 cell line. **B)** DAPI staining, SSEA1 expression detected in texas red and OCT3/4 detected in FITC in the A12 *Brca2*$^{\Delta 27/\Delta 27}$ corrected clone. C**)** DAPI staining, SSEA1 expression detected in texas red and OCT3/4 detected in FITC in the A16 *Brca2*$^{\Delta 27/\Delta 27}$ corrected clone.

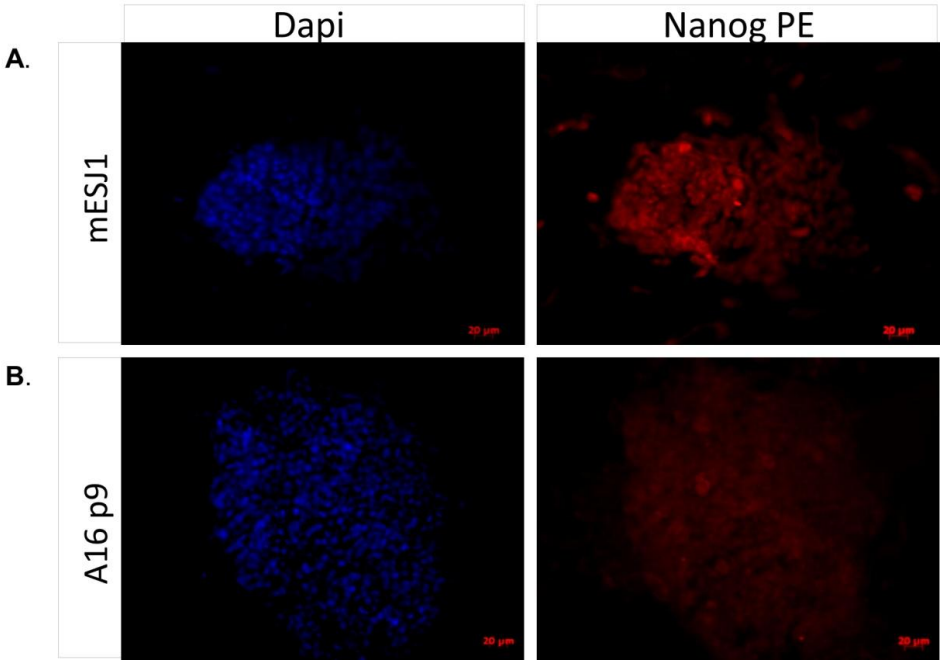


Figure 50. Images of the immunofluorescence analysis of the pluripotency marker NANOG in corrected A16 *Brca2*$^{\Delta 27/\Delta 27}$ iPSCs. **A)** DAPI staining and NANOG expression detected in texas red in the mESJ1 control line. **B)** DAPI staining and NANOG expression detected in texas red in the A16 *Brca2*$^{\Delta 27/\Delta 27}$ corrected clone.

- Cell cycle and karyotype analyses

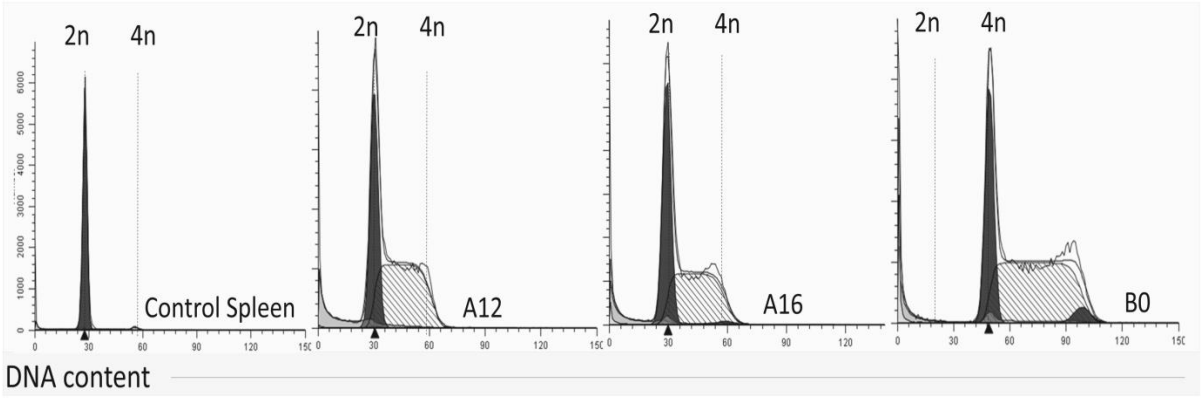


Figure 51. Cell cycle analysis of representative c*Brca2*$^{\Delta 27/\Delta 27}$ iPSC clones and the spleen cells used as a control. A12 and A16 show a diploid DNA content, while clone B0 was tetraploid.

Due to the chromosomal instability inherent to *Brca2*$^{\Delta 27/\Delta 27}$ cells and also to the iPSC generation process, we studied the cell cycle status of 26 clones (passage 17) using a reference population of diploid splenocytes. The results showed that at late passage number, only clones A12 and A16 were diploid, while all the other clones were aneuploid and polyploid (Figure 51).

Karyotype analyses of A12 and A16 clones were performed at passage 17, showing a normal karyotype in both cases (Figure 52A).

When karyotype analyses were performed in early *in vitro* passages, most of the iPSC clones had a normal karyotype, suggesting that many of the chromosomal abnormalities and aneuploidies took place during the *in vitro* growth of the iPSCs.

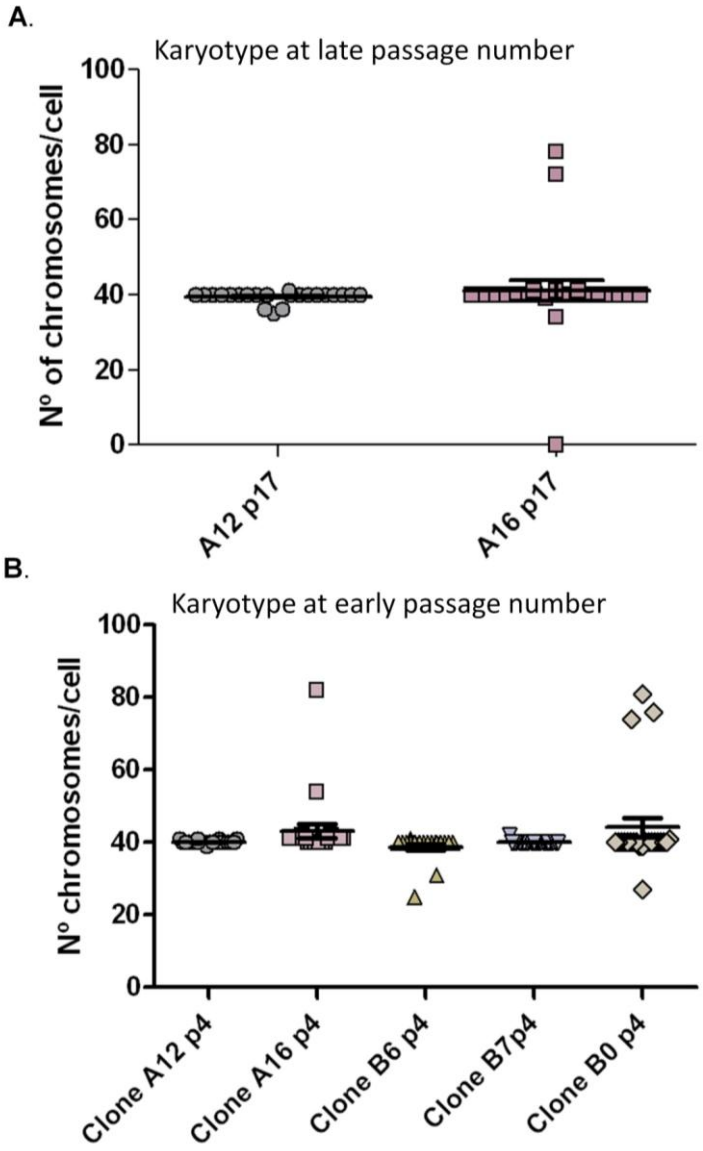


Figure 52. A) Karyotype analysis of two selected *cBrca2*$^{\Delta 27/\Delta 27}$ iPSC clones A12 and A16 at late passage number (p17). **B)** Karyotype analysis performed in these two clones and in other clones at an early passage number (p4).

- **Teratoma formation**

Selected iPSC clones, based on DNA content and karyotype analyses, were subcutaneously injected into NOD/SCID γc^{null} mice to analyze the capacity of the cells to generate teratomas, containing cells of the three embryonic germ layers. One million cells per mice were injected in the right flank and after one month the tumours generated were analyzed by expert pathologists.

These analyses showed that cells forming the tumoral masses did not contain cells of the three germ layers and they rather consisted of cells with an expansive growth (Figure 53). This

implied high mitosis rates and also necrotic and apoptotic cells due to the lack of blood supply to support the tumour growth.

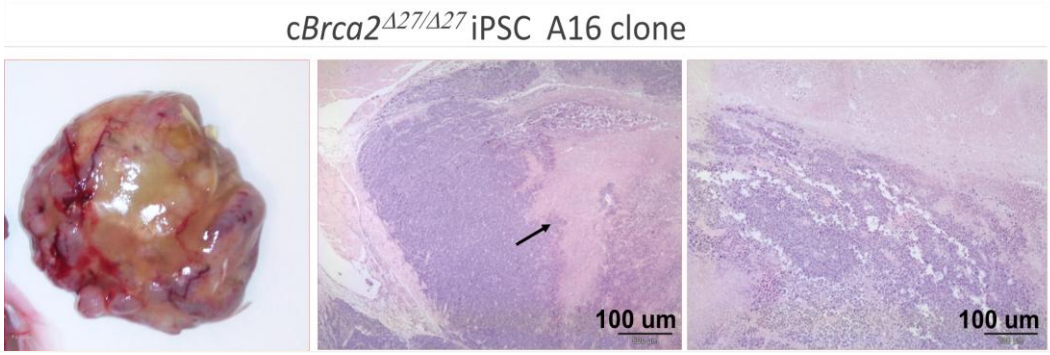


Figure 53. Image of an undifferentiated tumour mass obtained one month after subcutaneous injection of the A16 iPSC clone into NOD-SCID γc^{null} mice. Representative images 40x of H&E staining of undifferentiated cellular mass formation by c*Brca2*$^{\Delta 27/\Delta 27}$ A16 iPSC clone. Black arrow indicates a necrotic area with apoptotic cells.

2.2.1 MOLECULAR CHARACTERIZATION OF CORRECTED iPSC GENERATED WITH POLYCISTRONIC EXCISABLE LENTIVIRAL VECTORS

- Expression of endogenous *Nanog* pluripotency gene

All iPSC clones were analyzed for *Nanog* expression relative to the housekeeping gene m*Gapdh*. As shown in Figure 54 most of these clones showed a high endogenous expression of this gene and only A12 and A16 clones had a lower *Nanog* expression, similar to the one of mESCs. Significantly, these were the clones that best maintained the iPSC morphology and chromosomal stability.

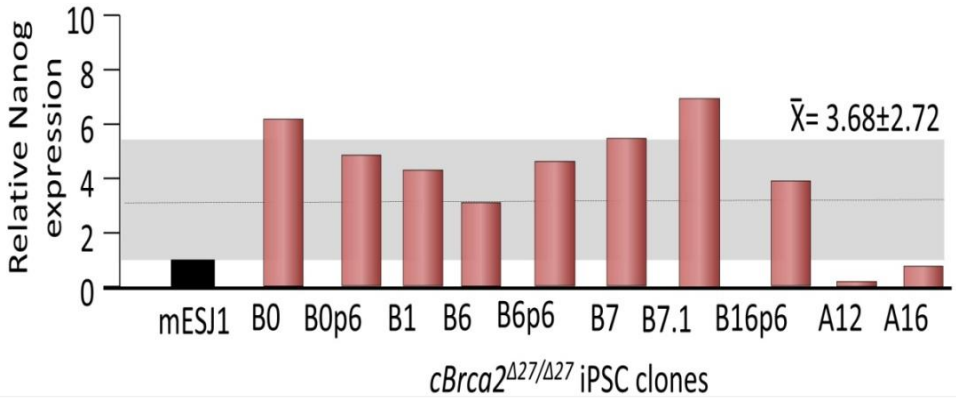


Figure 54. Graph representing the *Nanog* expression relative to *Gapdh* housekeeping gene in iPSCs determined by RT-qPCR. Standard error was determined and represented.

2.3 EXCISION OF THE REPROGRAMMING CASSETTE IN CORRECTED *Brca2*$^{\Delta 27/\Delta 27}$ iPSC CLONES

To prevent the unlimited expression of the reprogramming genes in corrected *Brca2*$^{\Delta 27/\Delta 27}$ iPSCs we decided to excise the reprogramming LV vector from iPSCs.

2.3.1 EXCISION WITH AN INTEGRATION DEFICIENT LENTIVIRAL VECTOR HARBOURING THE CRE RECOMBINASE (IDLV-CRE)

Taking advantage of the LoxP sequence contained in the polycistronic reprogramming LV (materials and methods) we excised the reprogramming cassette from A12 and A16 iPSC clones, using an integration deficient lentiviral vector vector carrying the Cre-recombinase.

To induce the excision process, 5 x 10^4 cells from each clone were transduced with the IDLV-Cre excision vector at a multiplicity of infection (MOI) of 100 in two rounds of infection. After 4 days, when iPSC colonies arose in the culture, individual colonies were picked, based on morphological criteria and were expanded in culture.

- Vector copy number determination

For the analysis of the reprogramming vector copy number (VCN) content we focused on A16 derived sub-clones, because they grew better in culture. We observed that in several A16 sub-clones the copy number of the reprogramming cassette, determined by RT-qPCR was less than one copy per cell (<0.4 copies/cell) (Figure 55). Among these sub-clones, A16Ex7 sub-clone was selected for further studies.

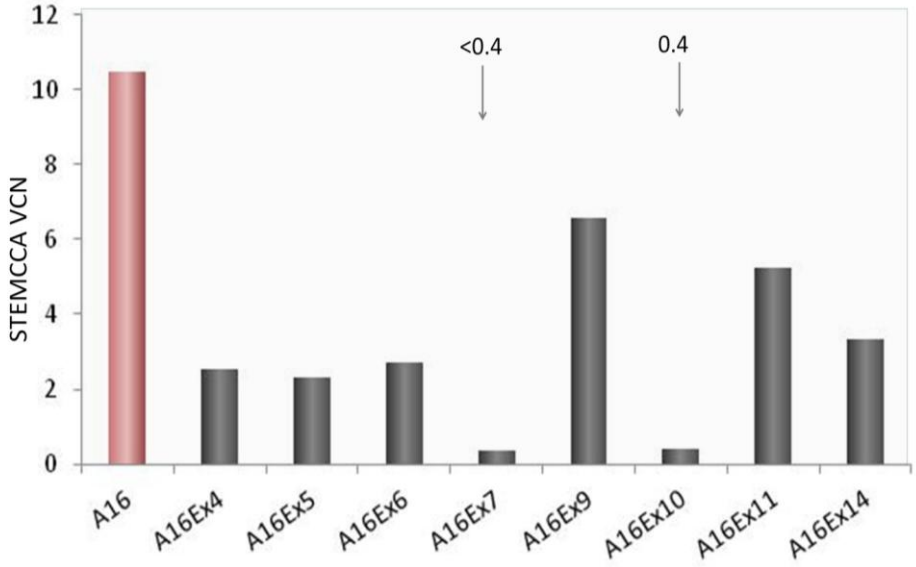


Figure 55. Analysis of the vector copy number after excision of the reprogramming cassette in A16 derived sub-clones that were selected after excision. Pink bar corresponds to the parental clone before the excision and grey bars represent the vector copy number analysis in the sub-clones obtained after excision of the reprogramming cassette with IDLV-Cre.

A16Ex7 cells were then analyzed by Southern Blot, using a mESC line, wt iPSCs and A16 iPSC clone as controls. Figure 56 shows that a band corresponding to the reprogramming vector was detected, with a size equivalent to the one observed in A16 clone, when hybridized with the wpre probe.

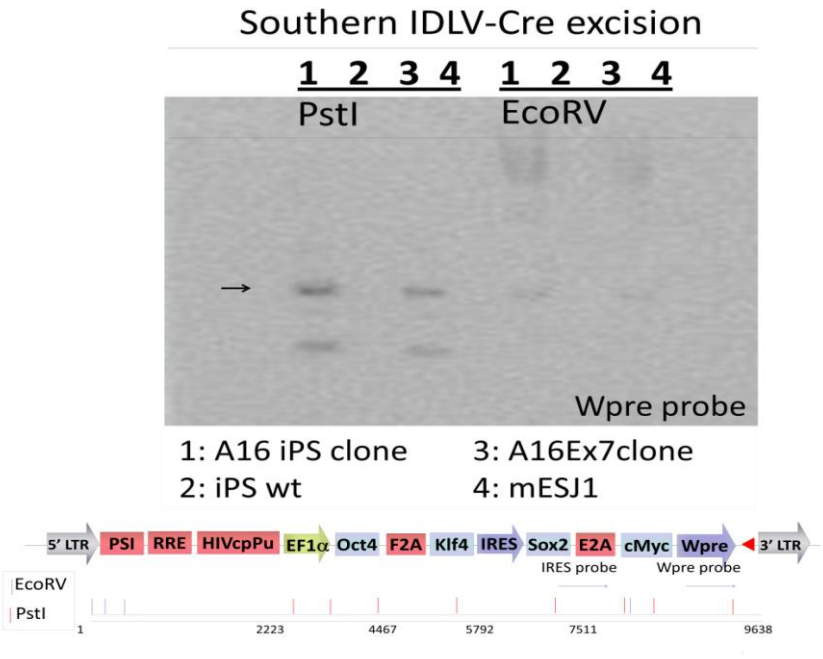


Figure 56. Southern Blot analysis of excised A16Ex7 iPSCs for the detection of inserted reprogramming provirus. **1)** Corresponds to the original A16 iPSC clone prior to excision that was used as control **2)** Wt iPSC cells. **3)** Excised A16Ex7 iPSC sub-clone **4)** corresponds to the mESC line of reference. The image corresponds to the hybridization with a wpre probe that recognizes the reprogramming provirus. The first four lanes represent the digestion with the restriction enzyme PstI and the second block of lanes corresponds to the digestion with the

Due to the limited efficacy of the reprogramming cassette excision with IDLV-Cre, these sub-clones were re-excised with a Ma.cre.prot system, based on the delivery of the Cre recombinase directly into the cellular nucleus (materials and methods). After excision with Ma.Cre.prot, the vector copy number was again determined by RT-qPCR. Now, no amplification of the reprogramming cassette was detected in A16Ex7 cells, implying that cells were free from the reprogramming cassette. Figure 57 represents the morphology of the cells after excision, showing that they still had the characterstic round/tear 3D shape, with defined borders and nucleus forming syncytia.

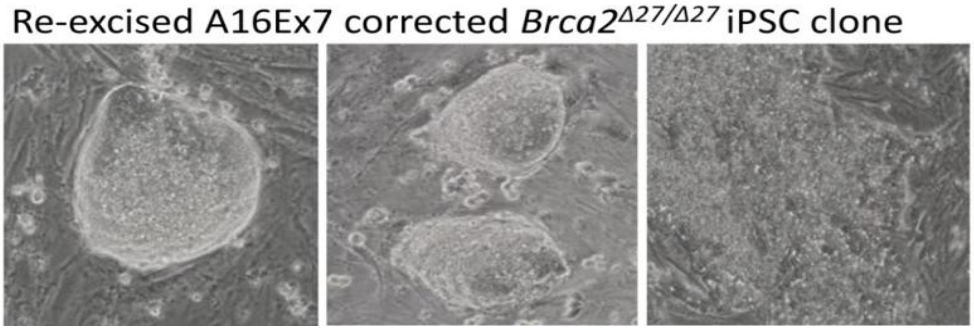


Figure 57. Representative images obtained at 40x in bright field, of re-excised A16Ex7 sub-clone obtained from c*Brca2*$^{\Delta 27/\Delta 27}$.

2.4 CHARACTERIZATION OF CORRECTED *Brca2*$^{\Delta 27/\Delta 27}$ iPSC AFTER EXCISION OF THE REPROGRAMMING CASSETTE

2.4.1 PHENOTYPIC CHARACTERIZATION STUDIES IN EXCISED iPSC CLONES

- **Morphology**

After excision of the reprogramming cassette, new sub-clones were individually selected and expanded for deeper characterization. Around 40 sub-clones were picked, although only the clones that maintained the morphological criteria of round/tear 3D colony shape and that grew properly in the plate were maintained in culture for further studies. Figure 58 shows the morphology of several sub-clones generated from A12 and A16 clones that resembled the characteristics of control mESCs.

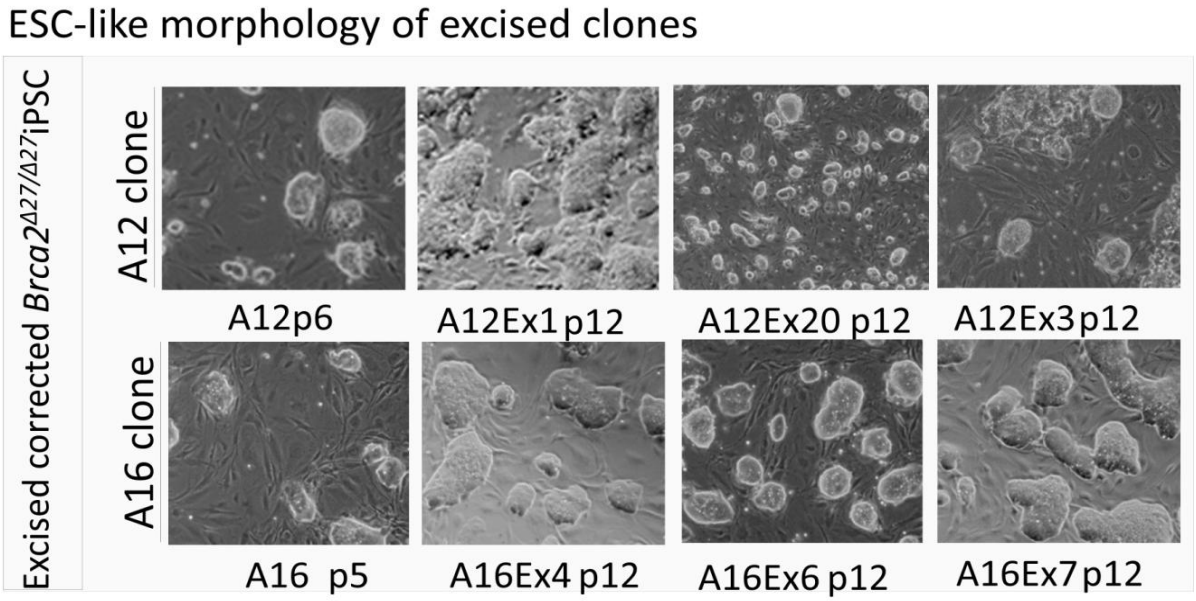


Figure 58. Representative images (40x) of the morphology of iPSC sub-clones obtained from A12 and A16 c*Brca2*$^{\Delta 27/\Delta 27}$ iPSC clones after the excision of the reprogramming cassette (STEMCCA-4F.OKSM).

- **Immunofluorescence of pluripotency gene expression**

In selected excised sub-clones, AP activity was measured and compared with the control mESC and with non-excised clones. The AP staining pictures of Figure 59 show a generalized increased AP activity in excised versus non-excised clones.

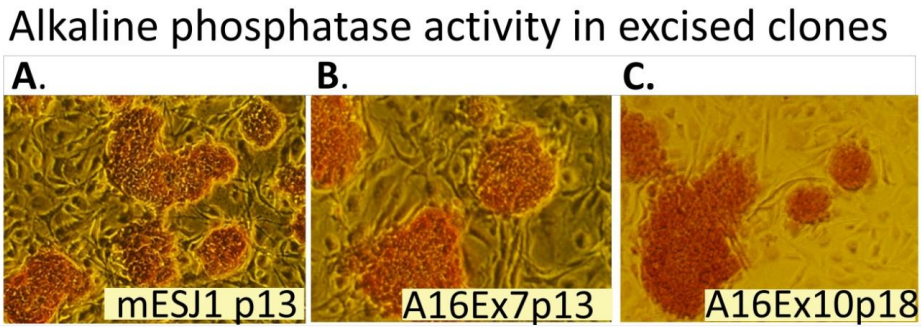


Figure 59. Images of AP staining of two representative excised c*Brca2*$^{\Delta 27/\Delta 27}$ iPSC clones, A16Ex7 and A16Ex10 obtained in bright field 40x. Cells were cultured over a feeder layer of irradiated MEFs. **A)** Control mESC line **B)** A16Ex7 c*Brca2*$^{\Delta 27/\Delta 27}$ iPSC clone. **C)** A16Ex10 c*Brca2*$^{\Delta 27/\Delta 27}$ iPSC clone.

In two A16 excised sub-clones that showed a higher AP activity, the expression of the pluripotency markers SSEA1, NANOG and OCT3/4 was analyzed by immunofluorescence. In panel A from Figures 60 and 61, the expression of these markers in the A16Ex7 sub-clone is shown. Panels B form these two figures correspond to the A16Ex 10 sub-clone, which was also positive for the three markers.

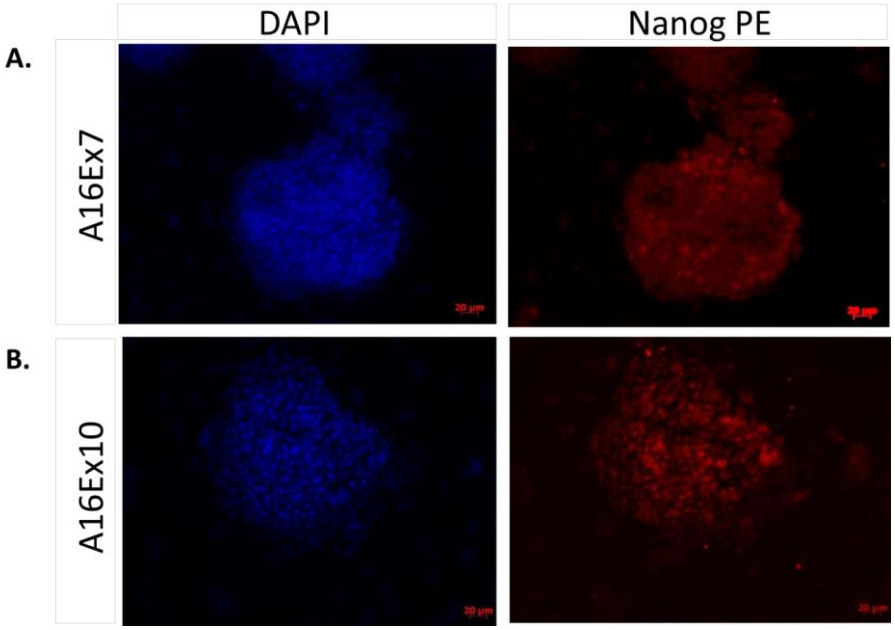


Figure 60. Representative images of the immunofluorescence analysis of the intracellular pluripotency marker, NANOG (in texas red) and the correspondent DAPI staining of the nucleus in two selected sub-clones derived from A16 c*Brca2*$^{\Delta 27/\Delta 27}$ iPSC. **A)** A16Ex7 sub-clone. **B)** A16Ex10 sub-clone.

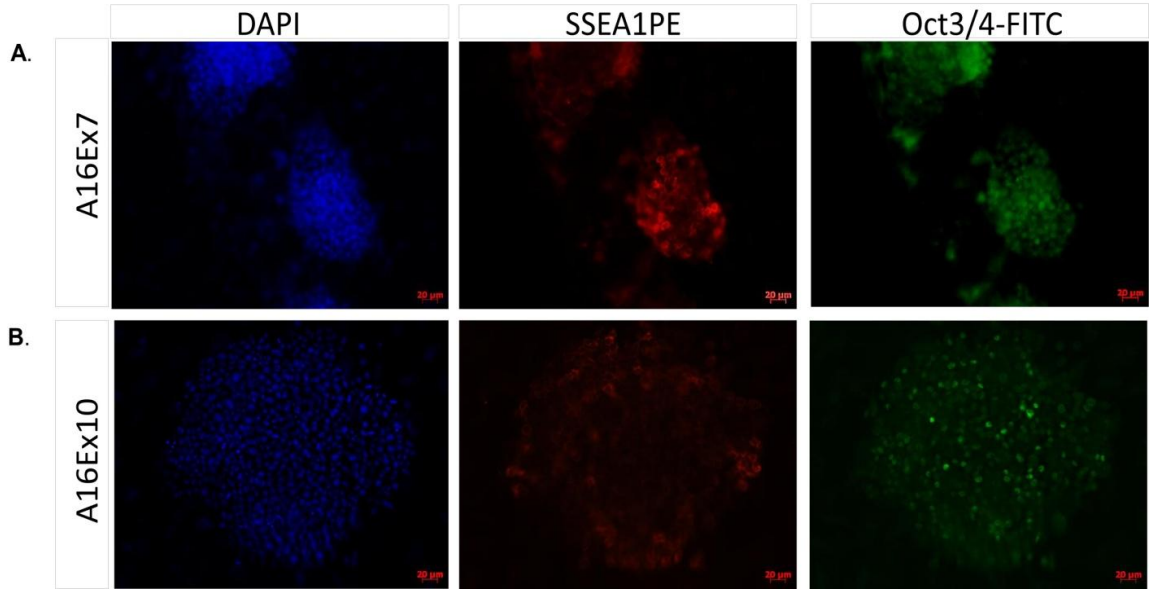


Figure 61. Representative images of the immunofluorescence analysis of the pluripotency markers SSEA1 (in texas red), OCT3/4 (in FITC) and the correspondent DAPI staining of the nucleus in two selected sub-clones derived from A16 c*Brca2*$^{\Delta 27/\Delta 27}$ iPSC. **A)** A16Ex7 sub-clone. **B)** A16Ex10 sub-clone.

- Teratoma formation

After excision, some of the selected sub-clones were subcutaneously injected into NOD/SCID γc^{null} mice to evaluate their ability to generate teratomas. As it happened with non-excised cells, tumours appeared one month after injection.

The results obtained showed that teratomas from the excised A16Ex7 sub-clone, contained cells of the three germ layers (Figure 62). Ectodermal structures, such as neuronal rosettes and keratinized epithelium, resembling the skin were found in these teratomas (panel A and B of Figure 62). Panels C and D in figure 62 correspond to cilial epithelium, either cuboidal or stratified with globet cells, similar to the ones present in the respiratory tract with an endodermal origin. Mesodermal cells detected in this tumour were a cartilaginous matrix with isogeneic groups with pale pink cells inside the matrix and are represented in panels E and F.

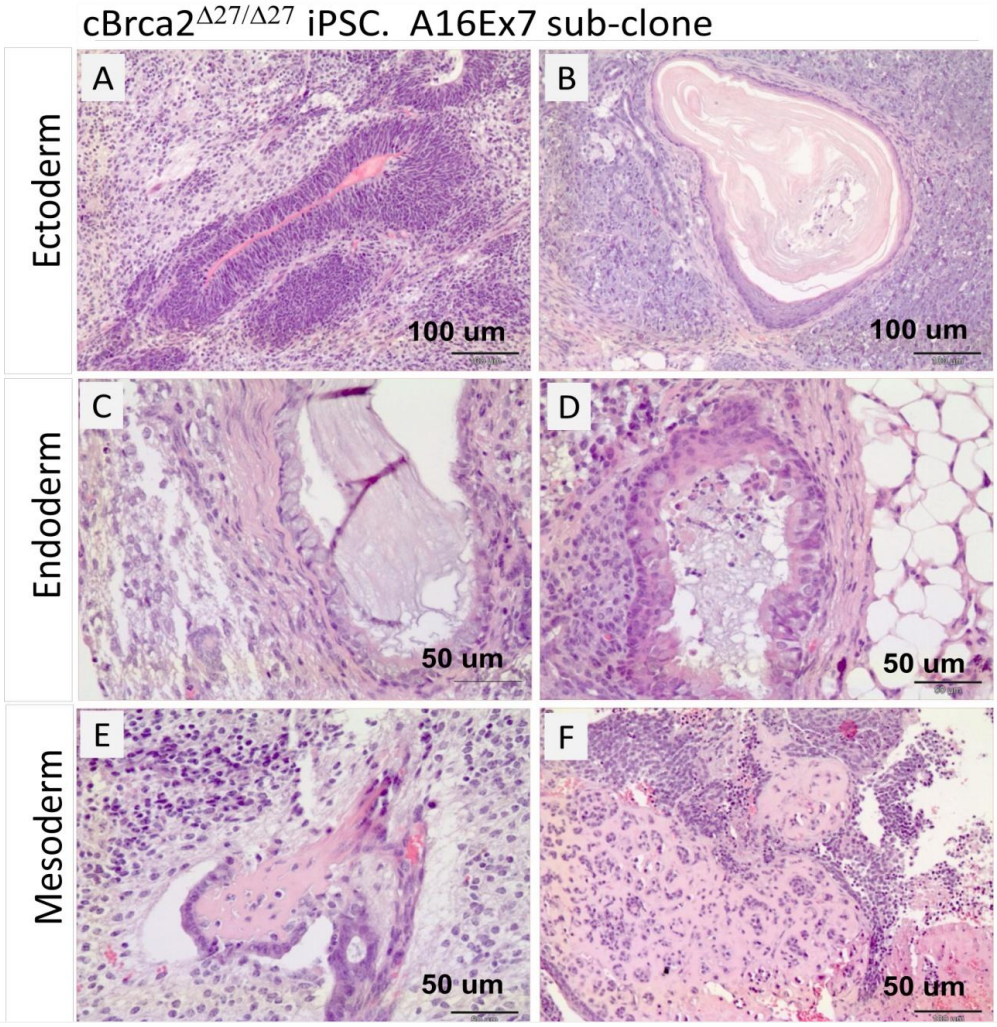


Figure 62. Analysis of histologic sections stained with H&E from teratomas generated by A16Ex7 excised sub-clone. A and B correspond to ectodermal structures. **A)** neuronal rosettes **B)** keratinized epithelium. **C)** and **D)** correspond to endodermal cell types **C)** Cuboidal cilial epithelium **D)** stratified cilial epithelium. **E** and **F** represent cartilaginous matrix that corresponds to a mesodermal structure.

2.4.2 MOLECULAR CHARACTERIZATION STUDIES IN EXCISED iPSC CLONES

Similarly to the characterization studies performed in iPSCs prior to the excision of the reprogramming cassette, we analyzed the expression of pluripotency marker genes, VCN and reversion of the disease phenotype in excised clones.

- Study of expression of endogenous pluripotency genes

After excision of the reprogramming cassette we analyzed the expression of endogenous pluripotency genes in A16Ex7 cells compared with a mESC line (mESJ1) as well as with the original *Brca2*$^{\Delta 27/\Delta 27}$ MEFs and the only iPSC-like clone that was generated without gene

correction. As shown in Figure 63, the excised A16Ex7 clone expressed elevated levels of *Oct3/4* that were even higher than the levels observed in the control mESC. With respect to the other pluripotency genes analyzed, all of them were expressed in these cells, meaning that these pluripotency genes had been activated.

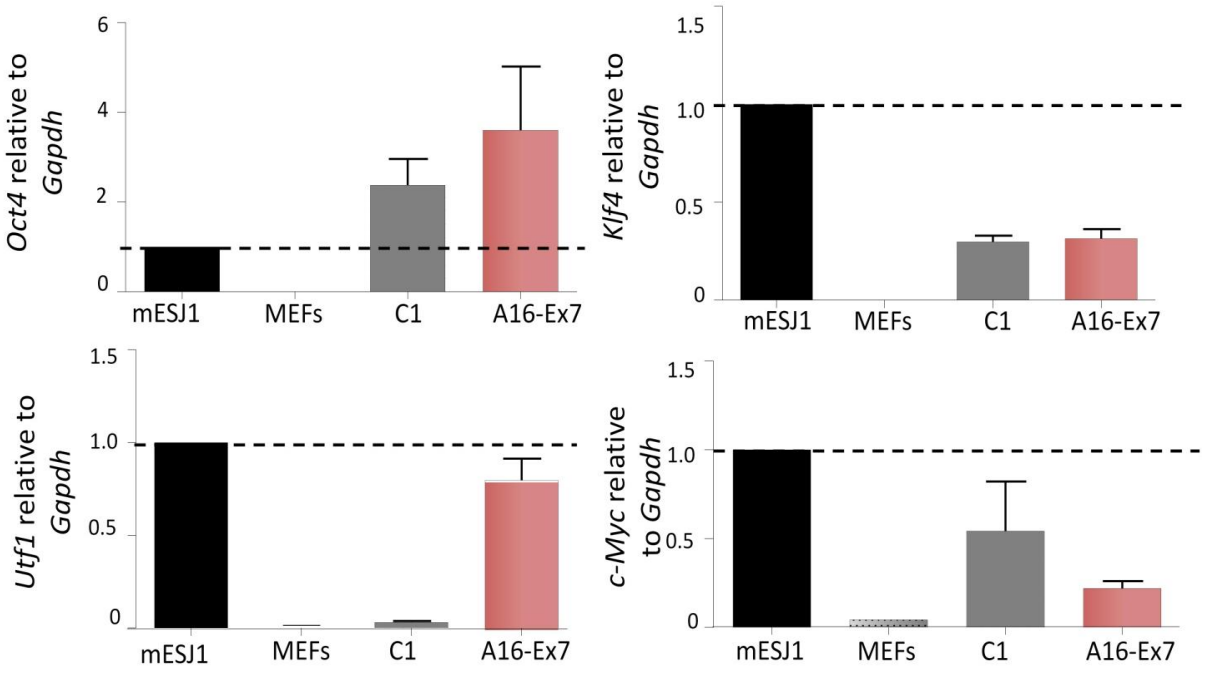


Figure 63. Endogenous expression of the pluripotency markers *Utf1, Sox2, c-Myc* and *Klf4* relative to *Gapdh* housekeeping gene expression, in excised A16 c*Brca2*$^{\Delta 27/\Delta 27}$ iPSC cells compared with the mouse ESC line mESJ1 and with the expression in the only uncorrected *Brca2*$^{\Delta 27/\Delta 27}$ iPSC that we could generate.

- Study of the hypomethylation of the pluripotency gene promoters

To verify that the excised iPSCs retained their pluripotency genes hypomethylated we carried out methylation analyses of the *Oct3/4* and *Nanog* promoters (as described in materials and methods) in A16Ex7 cells. As shown in Figure 64, both *Oct3/4* and *Nanog* promoters were hypomethylated in *cBrca2*$^{\Delta 27/\Delta 27}$ A16Ex7 iPS clone, as it happened in the positive mESC control and contrasting with the highly methylation of these promoters in the original *Brca2*$^{\Delta 27/\Delta 27}$ MEFs.

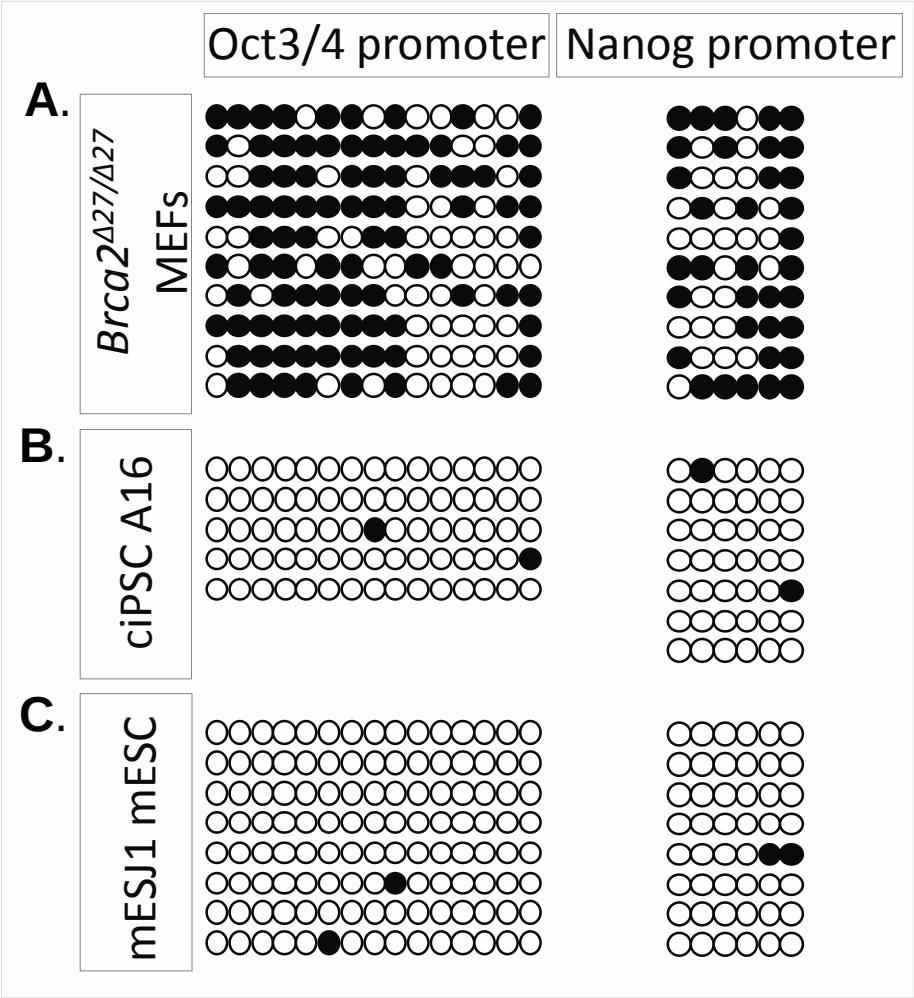


Figure 64. Analysis of the methylation status in c*Brca2*$^{\Delta 27/\Delta 27}$ iPSC clone A16 performed by the bisulfite method. **A)** Methylation status of the *Oct3/4* and *Nanog* promoters in the original *Brca2*$^{\Delta 27/\Delta 27}$ MEFs. **B)** Methylation status of the *Oct3/4* and *Nanog* promoters in the *cBrca2*$^{\Delta 27/\Delta 27}$ iPS clone A16Ex7 **C)** Corresponds to the methylation status observed in the control mESC line.

2.4.3 GENOMIC STABILITY STUDIES

Based on the chromosomal instability observed in non-excised clones we studied the ploidy and genomic stability in clones selected after excision.

- **Ploidy and karyotype analysis**

The analysis of the DNA content in A16Ex7 and A16Ex10 excised sub-clones showed a diploid content, similar to the one observed in the control mESC line to the diploid population of spleen cells and to the original clone A16.

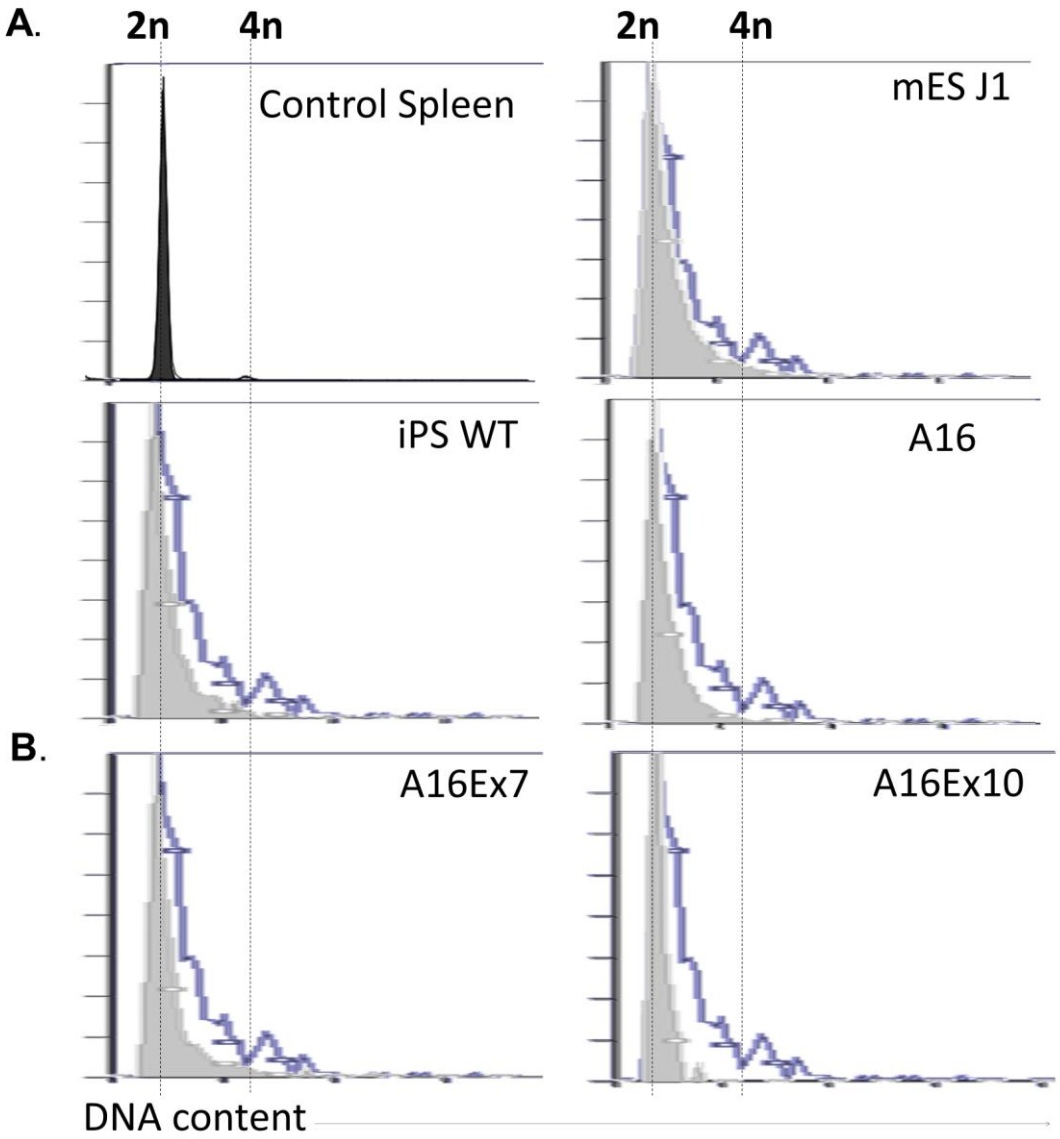


Figure 65. Analysis of the DNA content of two selected excised sub-clones obtained from A16 c*Brca2*$^{\Delta 27/\Delta 27}$ iPSCs (A16Ex7 and A16Ex10). Also analyses of the control cell populations are shown. **A)** Control cells consisting of diploid splenocytes; a mouse ESC line; wt iPS cells and the original A16 clone prior to excision. **B)** Represents the DNA content of the two excised sub-clones.

To investigate if the excision of the reprogramming cassette modified the chromosomal stability of the iPSCs, karyotype studies before and after excision, were represented. Figure 66 represents the chromosomal counts in the non-excised A16clone, as well as in excised A16Ex7 clone and in another clone with low copy number of the reprogramming vector (A16Ex10). In panel A of Figure 66, representative images of the karyotype before excision can be observed and Panel B represents chromosomal numbers per cell in A16 clone and in the two excised clones (A16Ex7 and A16Ex10). As shown in the figure, A16Ex7 cells showed a normal karyotype.

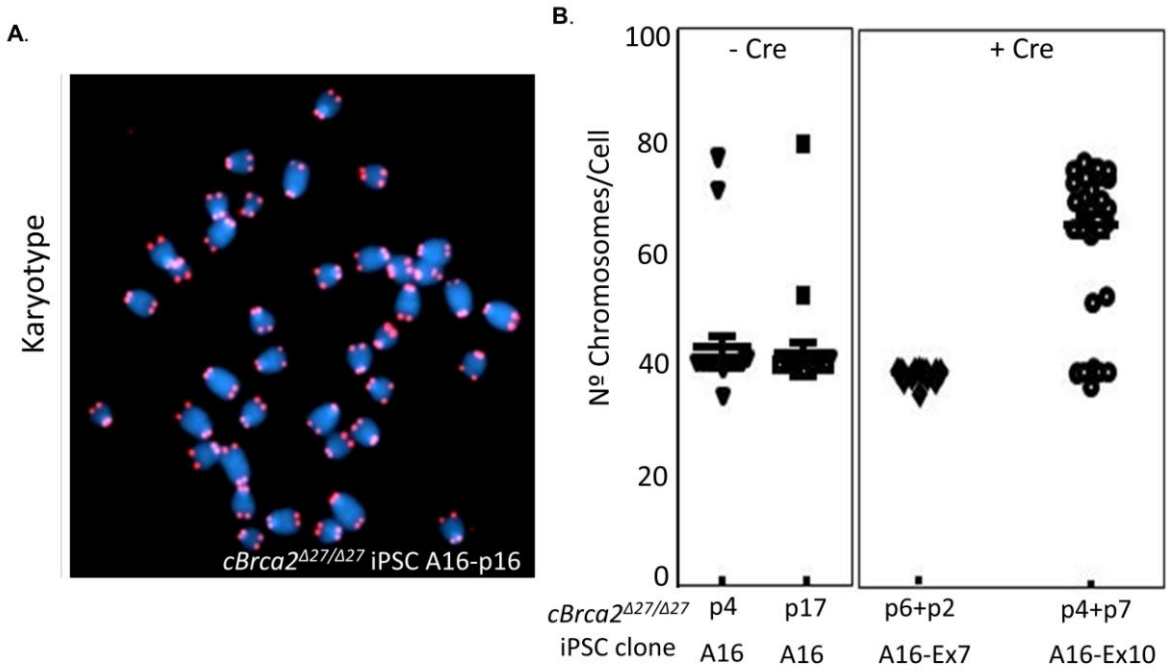


Figure 66. Karyotype study in the c*Brca2*$^{\Delta 27/\Delta 27}$ A16 iPSC clone, and in two excised clones, A16Ex7 and A16Ex10. Control populations were, the only uncorrected *Brca2*$^{\Delta 27/\Delta 27}$ iPSC colony, wt iPSCs and the mESC line mESJ1. **A)** Representative image of the karyotype of A16Ex7 iPSC cells. **B)** Respresents the quantification of chromosomes per methaphase.

- Comparative Genomic Hybridization array analysis (aCGH)

aCGH analyses were performed in wt iPSC and in the A16Ex7 sub-clone to study more in detail the chromosomal changes present in these cells.

Figure 67A represents the aCGH analysis in parental *Brca2*$^{\Delta 27/\Delta 27}$ MEFs, compared with genetically corrected *Brca2*$^{\Delta 27/\Delta 27}$ MEFs. These analyses revealed a duplication of 0.4 megabases, located in the cytobands 3qA3 at the genomic coordinates of chr3: 35052807-35453655. Also, a deletion of 0.46 megabases with 4 genes detected in cytobands 17qA3.3 at genomic coordinates of chr17:30586087-31049614. These alterations were observed both in *Brca2*$^{\Delta 27/\Delta 27}$ MEFs prior and also after correction of the genetic defect.

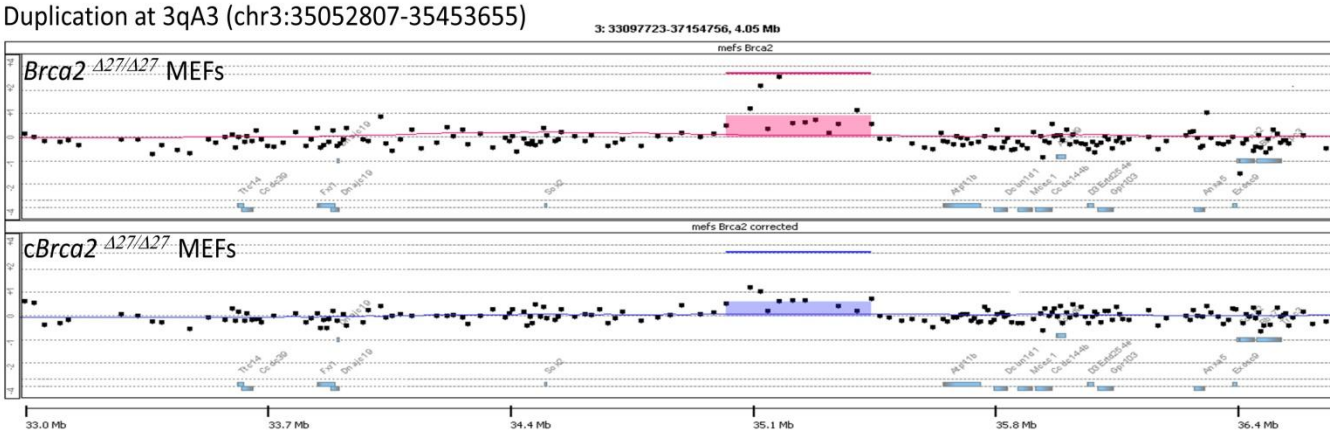
Figure 67B corresponds to aCGH results obtained in the A16Ex7 sub-clone, compared with genetically corrected *Brca2*$^{\Delta 27/\Delta 27}$ MEFs from which iPSCs were generated. In this case, several abnormalities could be observed, such as an amplification of 1.84 megabases detected in cytobands 4qD1 that affects chromosome 4 at coordinates 111725814-113561350. Another amplification of 0.12 megabases at cytoband 11qB4 at coordinates 70989662-71107244 was observed in chromosome 11. There was also a deletion of 0.06 megabases in cytoband 13qD1

at genomic coordinates of chr13:101149708-101211197. In cytoband 19qA, at genomic coordinates of chr19:9227197-9312496, a 0.46 megabase amplification was observed. Finally the same deletion previously observed both in uncorrected and corrected *Brca2*$^{\Delta 27/\Delta 27}$ MEFs was observed in chromosome 17 at cytobands 17qA3.3 at coordinates 30586087-31049614, indicative of the identity of this iPSC clone.

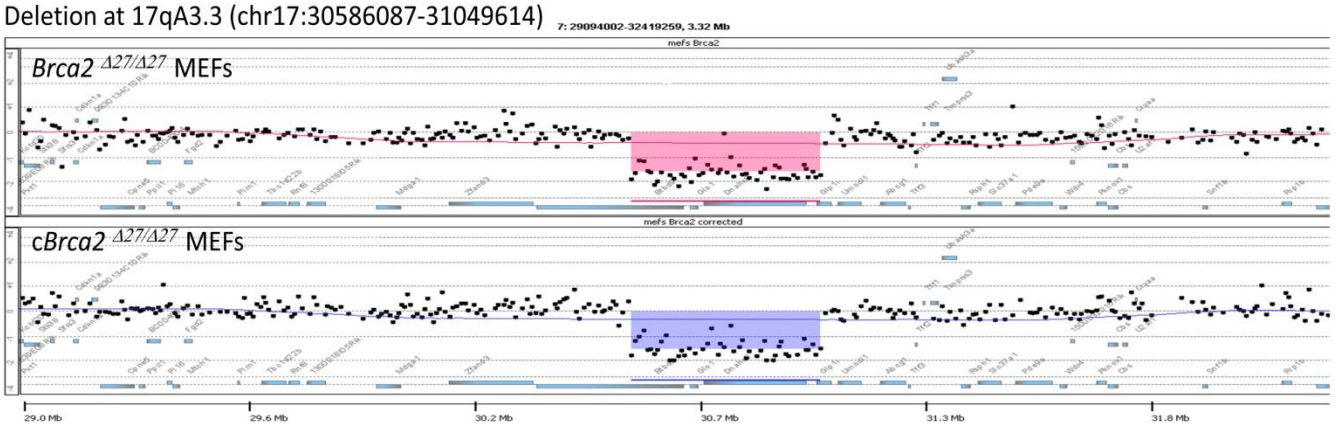
Importantly, the results obtained in a wt excised also showed significant numbers of genomic alterations, such as a duplication 16.6 megabase at cytobands 1qG3qH3 and coordinates chr1:156776790-173369079; a 23.7 megabase deletion located at 1qH3qH6 of chr1:173405837-197141847; and a duplication of 43.9 megabases at cytoband 12qC3qF2 of chromosome 12 at cordinates of 77339996-121241789.

A.

Duplication at 3qA3 (chr3:35052807-35453655)

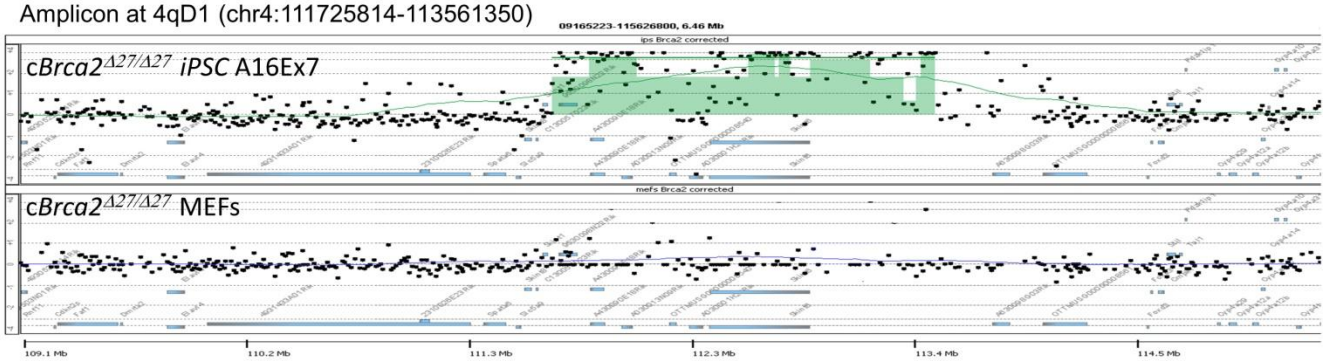


Deletion at 17qA3.3 (chr17:30586087-31049614)

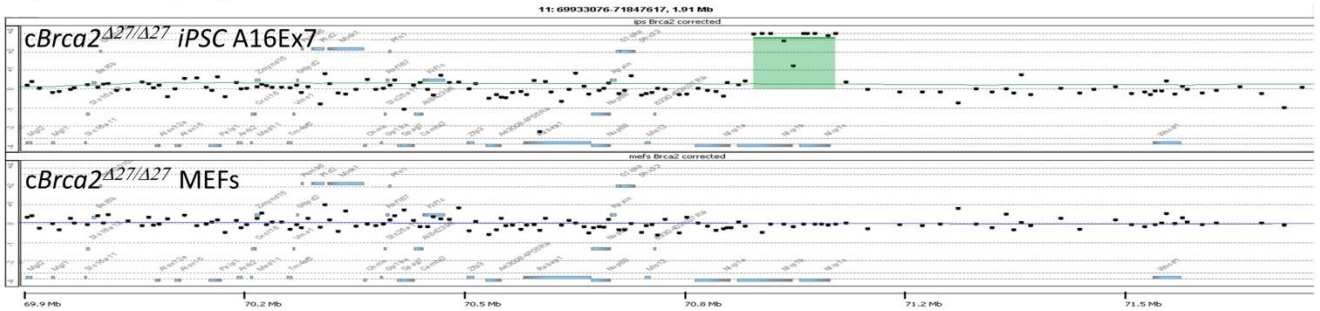


B.

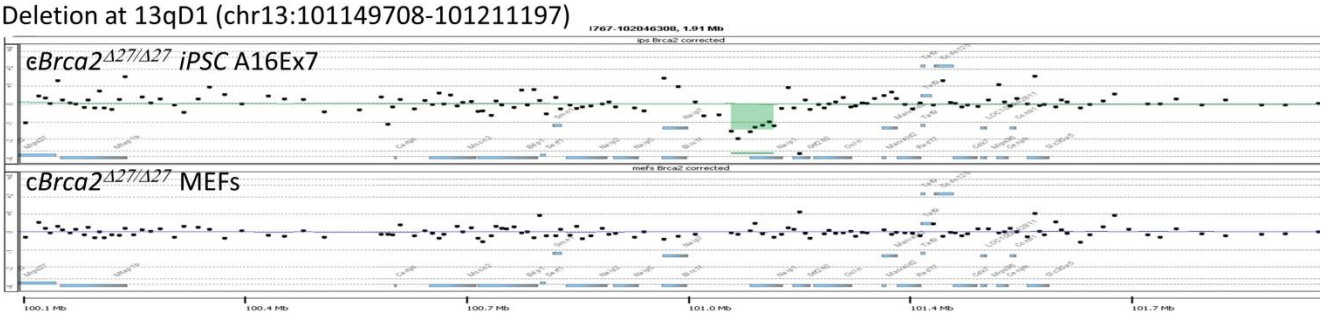
Amplicon at 4qD1 (chr4:111725814-113561350)



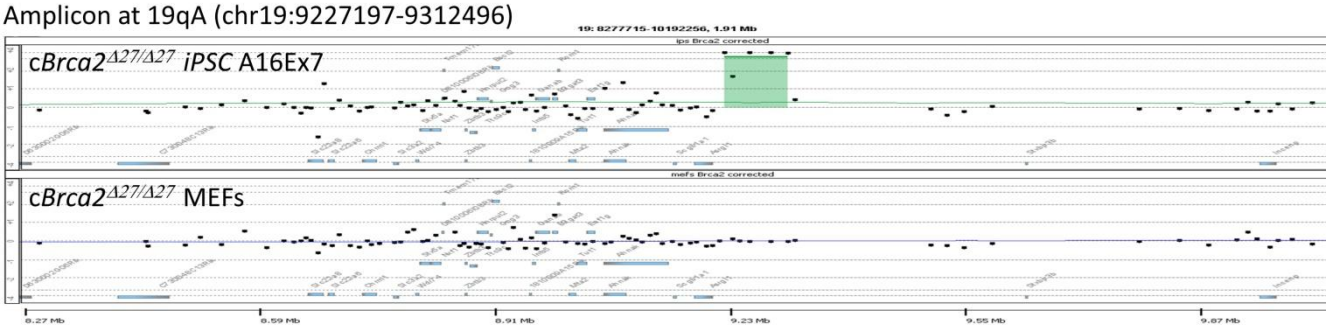
Amplicon at 11qB4 (chr11:70989662-71107244)



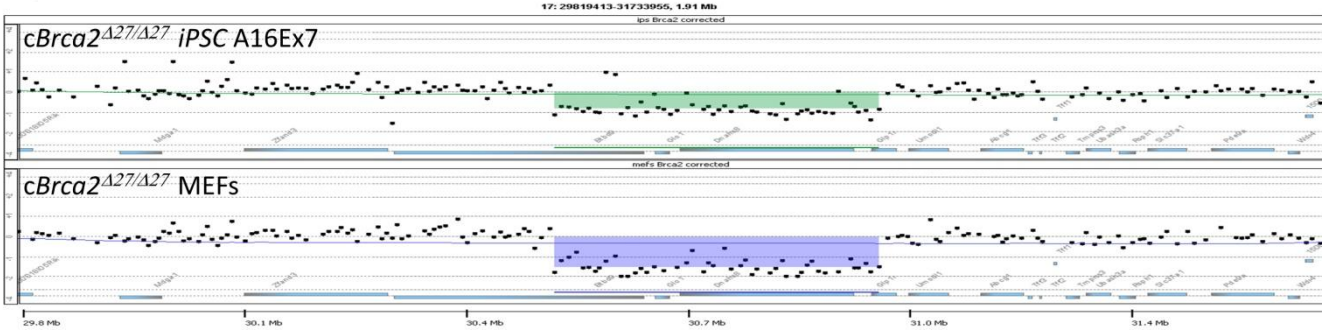
Deletion at 13qD1 (chr13:101149708-101211197)



Amplicon at 19qA (chr19:9227197-9312496)



Deletion at 17qA3.3 (chr17:30586087-31049614)



C.

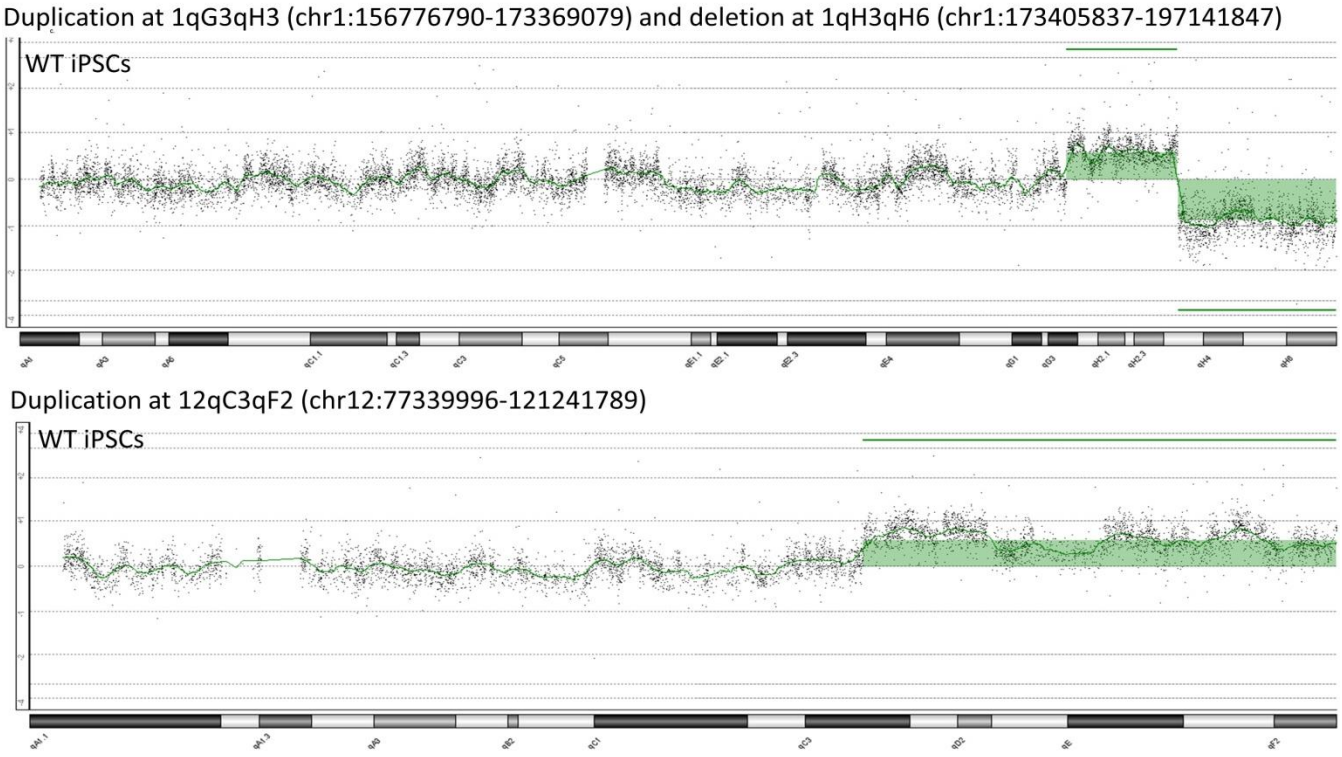


Figure 67. Comparative whole genome gene hybridization in uncorrected and gene corrected *Brca2*$^{\Delta 27/\Delta 27}$ MEFs and corrected *Brca2*$^{\Delta 27/\Delta 27}$ iPSCs compared to wt MEFS, considered as a reference population. **A)** Similar genetic aGCH footprint in uncorrected and gene-corrected *Brca2*$^{\Delta 27/\Delta 27}$ MEFs. **B)** Additional genetic alterations in c*Brca2*$^{\Delta 27/\Delta 27}$ iPSCs with respect to parental c*Brca2*$^{\Delta 27/\Delta 27}$ MEFs. **C)** Genetic alterations in wt cells.

2.5 CHARACTERIZATION OF THE FANCONI ANEMIA PHENOTYPE REVERSION IN c*Brca2* $^{\Delta 27/\Delta 27}$ iPSC

Additional phenotype analyses of gene corrected *Brca2*$^{\Delta 27/\Delta 27}$ iPSCs were investigated to verify that the FA phenotype characteristic of *Brca2*$^{\Delta 27/\Delta 27}$ cells had been reverted after gene therapy and cell reprogramming.

- BRCA2 expression

To analyze if *Brca2*$^{\Delta 27/\Delta 27}$ MEFs that had been transduced with the therapeutic BRCA2 LV had reverted the FA phenotype, we analyzed the expression of BRCA2 protein by RTq-PCR. Additionally, BRCA2 expression was investigated in corrected *Brca2*$^{\Delta 27/\Delta 27}$ iPSCs, prior and after excision of the reprogramming cassette. Our results showed that, in contrast to the null hBRCA2 expression observed in the original uncorrected MEFS, hBRCA2 expression could be detected both in corrected MEFs and in derived iPSCs.

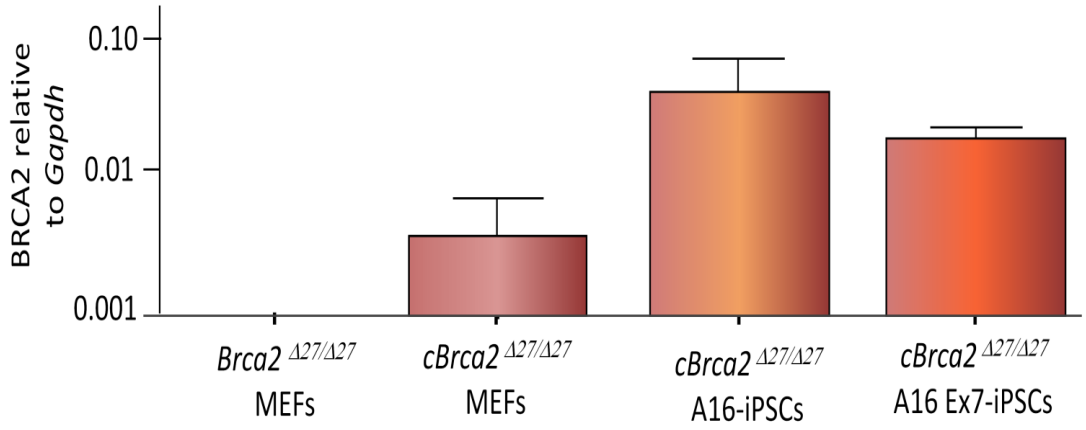


Figure 68. RT-qPCR analysis of hBRCA2 expression relative to *Gapdh*, in c*Brca2*$^{\Delta 27/\Delta 27}$ MEFs and c*Brca2*$^{\Delta 27/\Delta 27}$ iPSCs, before and after excision (A16 and A16Ex7 respectively). Uncorrected *Brca2*$^{\Delta 27/\Delta 27}$ MEFs were used as a control cell population.

- Re-establishment of the capacity to generate RAD51 *foci* after DNA damage

To determine if BRCA2 was functional in corrected *Brca2*$^{\Delta 27/\Delta 27}$ MEFS, the capacity of these cells to recruit RAD51 to DNA repair *foci* after DNA damage, induced by mitomycin C (MMC) was investigated. Figure 69 shows representative images of RAD51 *foci* formation revealing that, while uncorrected *Brca2*$^{\Delta 27/\Delta 27}$ MEFs were not able to form RAD51 *foci* (only a basal percentage of 5 % was detected), corrected cells showed a restored capacity to recruit RAD51 to the DNA repair *foci*. In genetically corrected MEFs and iPSCs, 60 % of the cells generated RAD51 *foci*

after MMC exposure. This value was similar to the percentage of wt cells with RAD51 *foci*, confirming the functionality of BRCA2 both in corrected *Brca2*$^{\Delta 27/\Delta 27}$ MEFS and in corrected iPSCs.

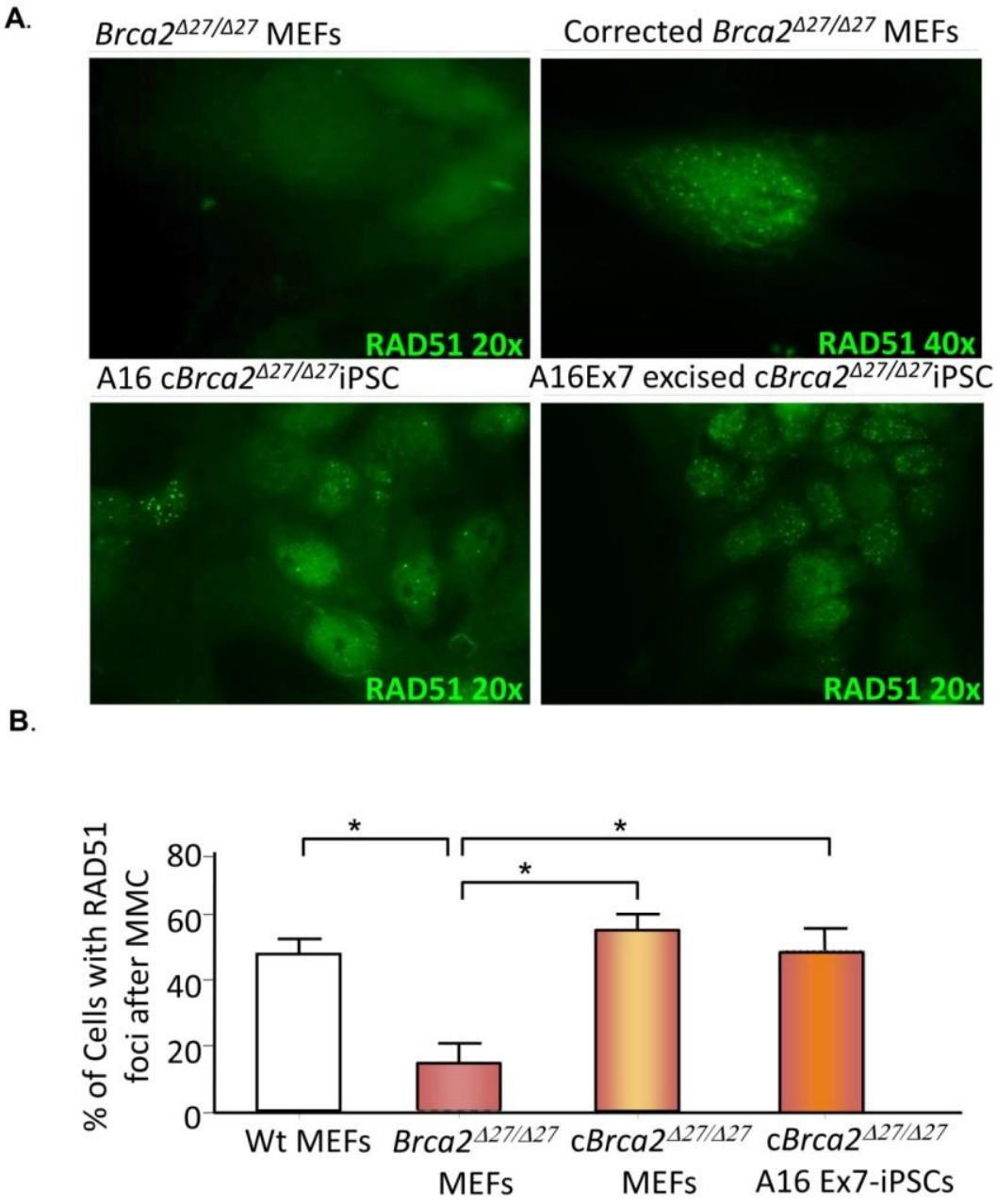


Figure 69. RAD51 *foci* in cells exposed to 40nM MMC treatment. The white bar corresponds to wt MEFs. Coloured bars correspond to uncorrected and corrected MEFs and to corrected *Brca2*$^{\Delta 27/\Delta 27}$ iPSCs, prior to and after excision of the reprogramming provirus.

- **Analysis of the chromosomal stability of corrected *Brca2*$^{\Delta 27/\Delta 27}$ iPSCs to diepoxibutane**

In these experiments we wanted to evaluate if corrected *Brca2*$^{\Delta 27/\Delta 27}$ iPSCs had reverted the chromosomal instability generated by DNA ICL drugs, a characteristic phenotype of FA cells.

Chromatidic breakages, chromosomal fragmentations as well as radial fragmentations were quantified in the uncorrected iPSCs-like clone that was generated (positive control) and in the excised c*Brca2*$^{\Delta 27/\Delta 27}$ A16Ex7 iPSC clone. Figures 70 A and C show that excised c*Brca2*$^{\Delta 27/\Delta 27}$ A16Ex7 iPSC clone had less chromatidic breakages and radial fragmentations than uncorrected iPSCs. The number of chromosomal fragmentations detected in the excised clone was also lower than the one observed in uncorrected iPSCs (Figure 70 B). These results show that the chromosomal instability of A16Ex7 iPSCs was significantly reduced in corrected versus uncorrected iPSCs, thus demonstrating the reversion of the FA phenotype, characteristic of c*Brca2*$^{\Delta 27/\Delta 27}$ cells.

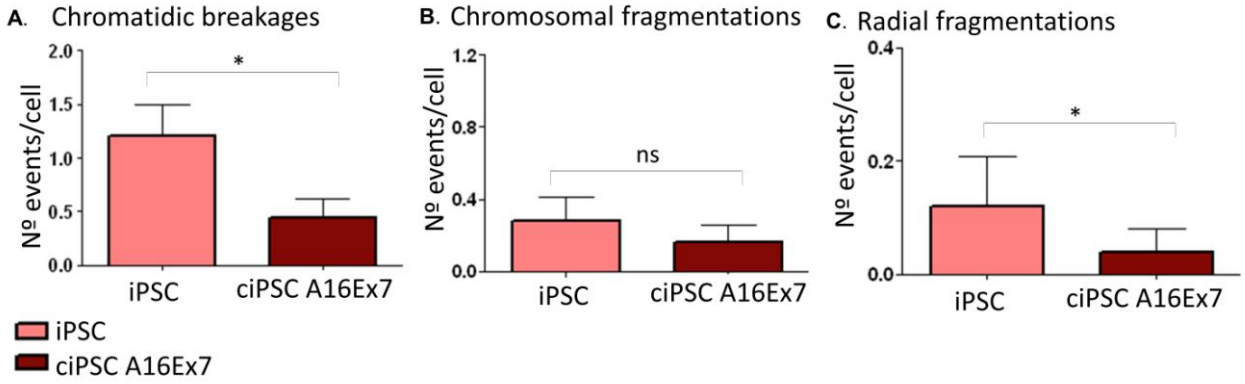


Figure 70. Chromosomal stability upon exposure to DEB in excised *Brca2*$^{\Delta 27/\Delta 27}$ A16Ex7 iPSCs compared with uncorrected *Brca2*$^{\Delta 27/\Delta 27}$ iPSCs. **A)** Chromatidic breakages **B)** Chromosomal fragmentations and **C)** Radial fragmentations. Error bars correspond to standard error and * to p< 0.05.

- The reprogramming of genetically corrected *Brca2*$^{\Delta 27/\Delta 27}$ MEFs with a polycistronic excisable lentiviral reprogramming vector restored the FA/BRCA pathway and generated *bona fide* iPSCs after the complete excision of the reprogramming cassette.

- At late passage numbers only one clone maintained a diploid karyotype. Even in this clone, several chromosomal anomalies were revealed by CGH analysis. Thus, high chromosomal instability was associated to the obtainment of *cBrca2*$^{\Delta 27/\Delta 27}$ iPSCs which could be due either to the reprogramming process, to the in vitro culture of the iPSCs, or to the excision of the reprogramming cassette.

- Gene corrected and excised *bona fide Brca2*$^{\Delta 27/\Delta 27}$ iPSCs were characterized by a FA reverted phenotype.

3. ESTABLISHMENT OF HEMATOPOIETIC DIFFERENTIATION PROTOCOLS FROM c*Brca2*$^{\Delta 27/\Delta 27}$ CELLS

One of the objectives proposed in this work was to investigate different sources of HSCs once we obtained phenotypically corrected *Brca2*$^{\Delta 27/\Delta 27}$ iPSCs. For this reason we focused on the differentiation of these disease-free pluripotent cells to restore the hematopoietic system of sub-lethally irradiated *Brca2*$^{\Delta 27/\Delta 27}$ mice.

Previous to the performance of hematopoietic differentiation protocols in iPSCs, different protocols were established using mESCs. These protocols allowed the determination of the optimal conditions and timing for the different steps needed to go through during the *in vitro* hematopoietic differentiation (MOLEIRO, 2010). The *in vitro* hematopoietic differentiation protocols developed, tried to reproduce protocols already established by other groups (Lesinski et al., 2012) (Ledran et al., 2008), introducing modifications to adapt them to the cells that we were using. These protocols were based on the use of cytokine cocktails and co-culture over hematopoietic derived stromas.

First, we evaluated in mESCs the effect of the cell culture medium and cytokine addition on the hematopoietic differentiation of the cells, the best method for EB and the kinetics of expression of a higher percentage of mesodermal markers, to establish the time point at which they had to be dissociated to continue with the differentiation (MOLEIRO (2010).

Once we observed that we could obtain specific hematopoietic markers after the differentiation of mESCs, we carried out *in vitro* and hematopoietic differentiation protocols in c*Brca2* iPSCs and we then evaluated the capacity of those *in vitro* differentiated cells to repopulate the hematopoietic system in an *in vivo* context.

3.1 *IN VITRO* HEMATOPOIETIC DIFFERENTIATION OF CORRECTED *Brca2*$^{\Delta 27/\Delta 27}$ iPSCs

Several attempts were performed trying to differentiate mESC and c*Brca2* iPSCs towards the hematopoietic lineage, based on the generation of EBs and the sequential addition of hematopoietic cytokines to the co-cultures on hematopoietic stromas. Stromas were either obtained from bone marrow (OP9 cells) or from the embryonic region aorta gonada mesonephros (AGM -AM20.4). Prior to co-culture, iPSCs were transduced with LIM homeobox

transcription factor *HOXB4* known to contribute to the *in vitro* and *in vivo* expansion of hematopoietic differentiating cells. In Table 10 the different hematopoietic differentiation methods used are described.

- **Hematopoietic differentiation of iPSCs based on the transduction with HOXB4 γ-RV and co-cultures with hematopoietic stromas (OP9 cells)**

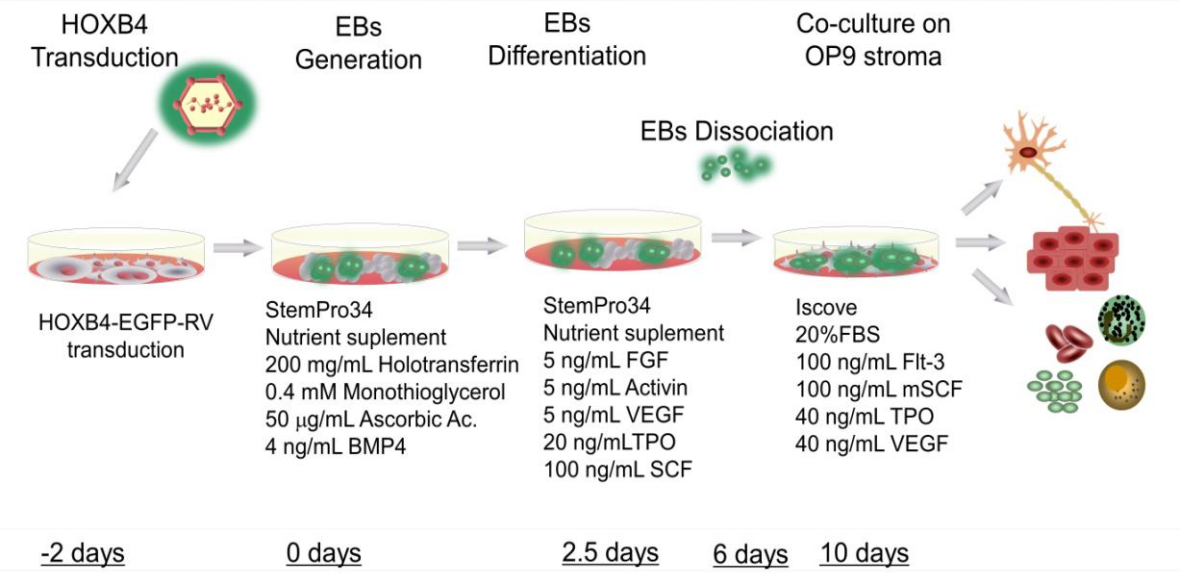


Figure 71. Schematic protocol to differentiate iPSCs towards the hematopoietic system based on the transduction with *HOXB4*, the generation of EBs and co-cultures on OP9 stromas.

The hematopoietic differentiation protocol shown in Figure 71 was performed in gene-corrected c*Brca2*$^{\Delta 27/\Delta 27}$ iPSCs (A16Ex7), control mESCs (mESJ1) and wt iPSCs. Cells transduced with *HOXB4.2aGFP*-RV were used for EBs generation. Thereafter, mesodermal differentiation was induced by the sequential addition of BMP4 for two days, and then to the hematopoietic lineage with activin A, VEGF, TPO and SCF. EBs were maintained for six days, after which they were dissociated and co-cultured on OP9 hematopoietic stromas for 10 days. During OP9 co-culture the same hematopoietic cytokines were added to the medium, only replacing activin A for Flt3 to induce a more differentiated stage of the cells.

After 10 days of hematopoietic differentiation, cells were analyzed by FACS for the expression of CD41, CD34, CD45 and c-Kit. As shown in the representative experiment of Figure 72, the

expression of hematopoietic markers in iPSCs, both wt and c*Brca2*$^{\Delta 27/\Delta 27}$ was more modest, than the expression obtained in mESJ1 cells. We also observed that, among the cells that expressed hematopoietic markers only a small fraction of them was double positive for HOXB4 and for hematopoietic markers. We can highlight the high proportion of CD41^{+} cells (47.61 %) obtained after the differentiation of mESCs from which 13.91 % corresponded to HOXB4^{+} cells. Within these cells we found cells positive for other markers such as CD45, c-kit and CD34 that ranged from 10 to 15 %. In the case of A16Ex7 iPSCs, a 5.5 % of these cells were CD41^{+}, most of which were negative for HOXB4. In the case of CD34 marker, a relatively high proportion of CD34^{+} cells (30 %) was observed. In wt iPSC a 3.9 % CD41^{+} cells was obtained, again most of them negative for HOXB4 and around 15 % c-kit^{+} and CD34^{+}.

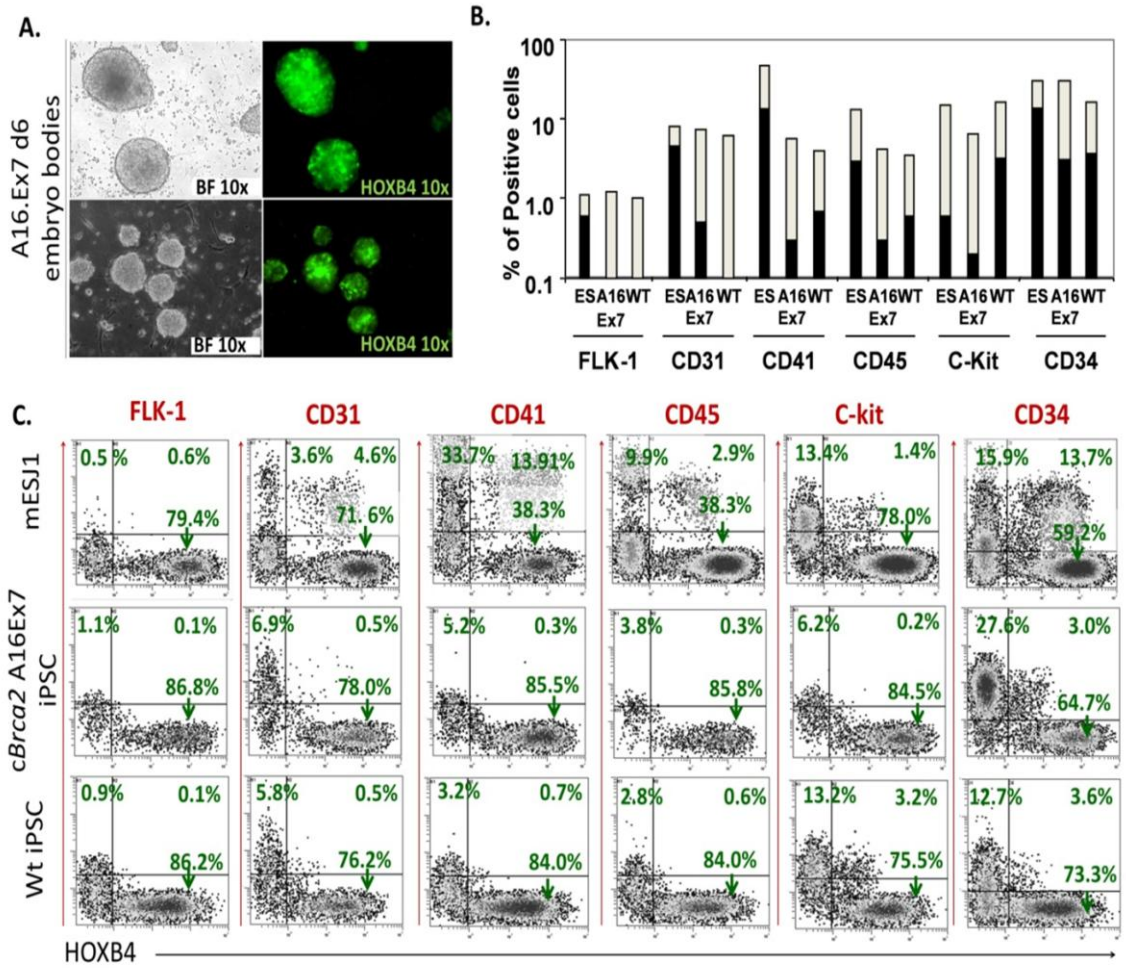


Figure 72. Representative hematopoietic differentiation experiment showing the expression of hematopoietic markers from iPSCs and mESC. Experiments were performed with mESJ1 cells, wt iPSCs and c*Brca2* iPSC (A16Ex7). **A.** Representative images of day 6 embryo bodies generated from A16Ex7 iPSCs expressing HOXB4. **B.** Percentage of cells positive for differentiation markers (white bars) and both for differentiation markers and HOXB4/EGFP (black bars) and **C.** Histograms of FACS analysis in which the expression of different hematopoietic differentiation markers was analyzed.

- **Hematopoietic differentiation of iPSCs by co-cultures on aorta-gonada mesonephros (AGM) stromas**

With the objective of improving the hematopoietic differentiation of the iPSCs we tested another protocol based on the co-culture of the cells with stromas derived from the aorta-gonada-mesonephros region (AGM), as described in materials and methods.

We performed this experiment in A16Ex7 iPSC clone and in the control cell line (mESJ1). EBs were generated by natural aggregation, and sequential addition of cytokines, to induce differentiation to mesoderm (BMP4) followed by hematopoietic cell induction with FGF, activin A and VEGF. After 6 days, EBs were dissociated and co-cultured on the AGM derived stromas (AM.20) cells.

During the differentiation process, different hematopoietic markers were analyzed at day 21 of differentiation. Representative data shown in Figure 73, illustrate that the marker expression was very low, both in mESJ1 (between 0-4%) and in A16Ex7 cells (between 0 and 2%).

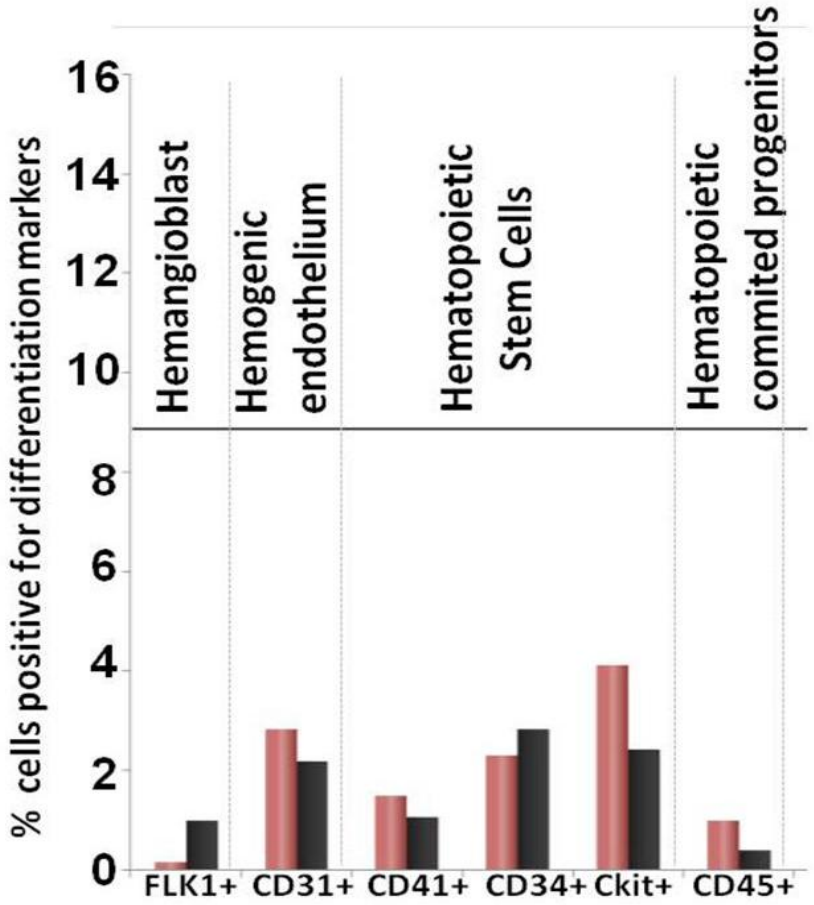


Figure 73. Flow cytometry analysis of *cBrca2* iPSC derived embryo bodies, co-cultured with aorta-gonada mesonephros (AGM) stromas at day 21 after embryo body generation.

3.2 *IN VIVO* HEMATOPOIETIC REPOPULATING ABILITY OF iPSC-DERIVED HEMATOPOIETIC PROGENITORS OBTAINED IN VITRO

To investigate if A16Ex7 c*Brca2*$^{\Delta 27/\Delta 27}$ derived cells could generate functional HSCs, able to reconstitute the hematopoiesis of *Brca2*$^{\Delta 27/\Delta 27}$ recipients, one million cells, derived from wt iPSC and from A16Ex7 c*Brca2*$^{\Delta 27/\Delta 27}$ iPSCs (day 10 of *in vitro* differentiation) were transplanted in each recipient mouse.

As shown in Figure 74, only very low levels of exogenous reconstitution could be detected at day 15 post-injection.

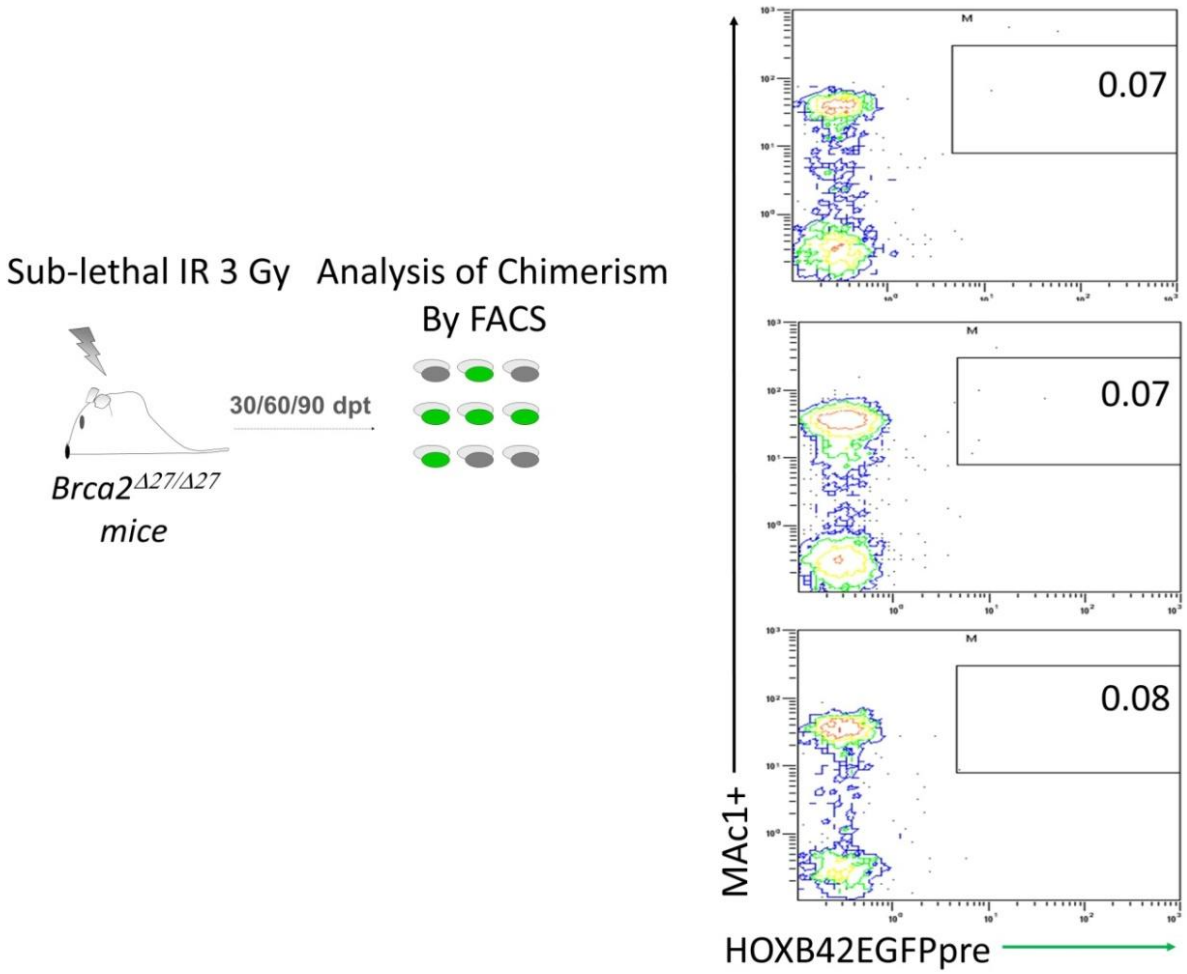


Figure 74. Representation of the percentage of chimerism observed in the *in vivo* differentiation protocol based on intravenous injection of EB-derived differentiated cells into sub-lethally irradiated *Brca2*$^{\Delta 27/\Delta 27}$ mice. The upper panel corresponds to the analysis performed in peripheral blood from the mice injected with wt iPSC derived cells and the two lower panels correspond to blood from the mice injected with A16Ex7 iPSC derived cells.

- These results show that either mESCs, wt iPSCs or c*Brca2*$^{\Delta 27/\Delta 27}$ iPSCs can be differentiated towards the generation of cells expressing characteristic hematopoietic markers. However, a more modest hematopoietic differentiation was observed in iPSC-derived cells, as compared with mESC-derived populations.

- The transplantation of c*Brca2*$^{\Delta 27/\Delta 27}$ iPSC-derived populations into irradiated *Brca2*$^{\Delta 27/\Delta 27}$ mice, did not result in significant hematopoietic engraftments, at least under the experimental conditions used in these studies.

The discovery made by Shinia Yamanaka and colleagues in 2006 in which pluripotency was induced in adult somatic cells, unwinding their genetic program towards an embryonic stem cell-like stage (Takahashi et al., 2007) (Yamanaka and Takahashi, 2006) (Yu et al., 2007), opened a new horizon that is facilitating the understanding of the physiological processes involved in the differentiation, of healthy cells as well as in different disease models. Cell reprogramming is also helping to develop new potential therapeutic approaches based on gene and cell therapy (Daley, 2007) (Tiscornia et al., 2011). Along these years, many advances have been achieved in which different pluripotency induction techniques were developed to increase the efficiency and the safety of cell reprogramming and to unveil the molecular processes underlying reprogramming (Banito et al., 2009) (Batista et al., 2011) (Fong et al., 2011) (Hong et al., 2009; Li et al., 2009; Marion et al., 2009; Muller et al., 2012a; Raya et al., 2009; Utikal et al., 2009; Yung et al., 2013).

In the field of FA, the FA pathway actively participates in DNA repair after the generation of deleterious lesions during replication and after DNA damage with genotoxic agents. As already exposed in the introduction, the principal function of the FA pathway is to maintain the integrity of the genome. In particular, members of the lower part of the FA pathway constitute the key players in HDR, while proteins of the core and I-D complex mainly participate at the initial steps of the DNA damage response.

The main limitation for the development of gene therapy protocols in FA is the scarcity of hematopoietic stem cells in the bone marrow of these patients, the target population to perform gene therapy. A previous collaborative work of our group with CMRB and the Autonoma University of Barcelona, showed for first time the possibility of generating disease-free hematopoietic progenitors from skin cells obtained from FA patients, of the FA-A and FA-D2 complementation groups (Raya et al., 2009). Other studies have also shown the reprogramming of human and mouse cells from the FA-A and FA-C complementation groups (Ledran et al., 2008; Tulpule et al., 2010).

However, the role of BRCA2 in cell reprogramming had not been investigated so far, despite its important participation in DNA repair by HDR. Thus, to understand the involvement of HDR in cell reprogramming we took advantage of the *Brca2*$^{\Delta 27/\Delta 27}$ mouse model, that is one of the models that better recapitulates the phenotype of FA patients (McAllister et al., 2002b; Navarro et al., 2006). This issue, together with the possibility of having an *in vivo* model of FA iPSC-derived cell and gene therapy, tempted us to explore the possibility of restoring the

hematopoietic system of these animals, by the transplantation of disease-free *Brca2*$^{\Delta 27/\Delta 27}$ iPSC-derived HSCs.

1. INVOLVEMENT OF FANCA AND BRCA2 IN CELL REPROGRAMMING

In a first instance we were interested in discerning the involvement of these two FA proteins, FANCA and BRCA2 in the process of somatic cell reprogramming to generate iPSCs.

As previously observed in human FA-A cells, our results showed that only after gene complementation, iPSCs could be generated and maintained (Raya et al., 2009). As we observed in a mouse model deficient in *Fanca* (Cheng et al., 2000), iPSC-like starting colonies arose without correction of the genetic defect, but only one iPSC colony could be maintained, which is in agreement with our previous studies in which FANCA was needed to preserve human FA-A iPSC colonies *in vitro* (Raya et al., 2009). With respect to the relevance of FANCA in cell reprogramming, Müller et al. described that FA-A cells were resistant, but not completely refractory to be reprogrammed (Muller et al., 2012a), since uncorrected iPSCs could be obtained using low oxygen concentrations. Lako et al. also confirmed the relevance of FA proteins from the FA core-complex in cell reprogramming (Ledran et al., 2008).

The stronger phenotype observed in FA-D1 patients when compared with patients corresponding to other complementation groups led us think that BRCA2 could have a more relevant implication in cell reprogramming as compared to other FA proteins.

The generation of iPSC clones from *Fanca*$^{-/-}$ and *Brca2*$^{\Delta 27/\Delta 27}$cells by transduction of the four γ-RVs described by Yamanaka (Takahashi et al., 2007), showed that only after cell correction with the therapeutic vectors (PGK-hFANCA-Wpre for *Fanca*$^{-/-}$ cells and PCL1-BRCA2 for *Brca2*$^{\Delta 27/\Delta 27}$ cells) iPSC-like colonies could be established.

Considering that the use of four independent vectors to reprogram FA cells might have collaborated to the low efficiency of reprogramming, we used embryonic fibroblasts and an excisable polycistronic vector (Sommer et al., 2012a) to increase the efficiency of the process. However, once again an extremely low reprogramming efficacy of Brca2$^{\Delta 27/\Delta 27}$ hypomorphic cells was observed, that prevented the generation of any iPSC colony. In contrast with this observation, a significant number of *Fanca*$^{-/-}$ iPSCs could be generated. The observation that *Brca2* is critical for the generation of iPSCs, together with publications showing the

involvement of *Brca2* with HDR and cell reprogramming (Banito et al., 2009; Blasco et al., 2011; Marion and Blasco, 2010; Marion et al., 2009) allowed us to propose the involvement of HDR in iPSC generation (Navarro et al., 2014).

The analysis of the capacity of *Fanca*$^{-/-}$ and *Brca2*$^{\Delta 27/\Delta 27}$ cells to recruit RAD51 to the DNA repair *foci* (Esashi et al., 2007) during cell reprogramming revealed that *Fanca* deficient cells were not impeded in their capacity to activate HDR, in contrast with the impossibility observed in *Brca2*$^{\Delta 27/\Delta 27}$ cells. Upon correction of the genetic defect, either simultaneously or previous to reprogramming, *Brca2*$^{\Delta 27/\Delta 27}$ cells had restored their ability to respond to the DNA damage caused by the reprogramming process. This functional test further supported that HDR was induced during the reprogramming process.

The correction of the genetic defect in *Brca2*$^{\Delta 27/\Delta 27}$ cells allowed us to efficiently generate disease-free iPSC colonies that had restored their capacity to recruit RAD51 protein to the DNA repair *foci*, supporting again that a correct HDR function is needed during cell reprogramming and that *Brca2* gene is essential in this process (Gonzalez et al., 2013).

This conclusion was highly consistent with the results obtained by other authors that published these data in parallel with our observations (Gonzalez et al., 2013). The important role of BRCA2 protein and its homologous protein from other organisms in cell reprogramming, oogenesis, telomere maintenance and response to genotoxic substances or pathogen infections (Badie et al., 2010; Dumont et al., 2011; Rodriguez-Mari et al., 2011; Singh et al., 2012; Wang et al., 2010; Wang and Dai, 2006), supports the importance of this highly conserved protein in stress and DNA damage response.

Several studies have stressed the involvement of cellular senescence episodes or *P53* overexpression, to be directly related with the low efficiency of reprogramming. Due to these observations we decided to study if the low efficiency of reprogramming observed in *Brca2*$^{\Delta 27/\Delta 27}$ cells could be due to increased apoptosis. The study of the level of apoptosis induction during the early stages of reprogramming revealed that there was an increase in early and late apoptosis in *Brca2*$^{\Delta 27/\Delta 27}$ cells. It is at these stages when the silencing of tissue specific genes takes place to activate their endogenous pluripotency genes (Brambrink et al., 2008), and it constitutes a period that is critical for the generation of iPSCs (Banito et al., 2009). Our observation of increased apoptosis during the reprogramming of *Brca2*$^{\Delta 27/\Delta 27}$ cells contrasts with the results obtained by Gonzalez who did not observe any increase in the apoptosis of the same cell type during cell reprogramming. In contrast to our study, these

authors analysed apoptosis induction at much later stages after cell reprogramming (Gonzalez et al., 2013).

The use of FA cells to study cell reprogramming made possible to understand how this DNA repair pathway influences the process of iPSC generation, being at least partially responsible for the low efficiency that characterizes this process in FA cells.

2. GENERATION AND CHARACTERIZATION OF *BONA FIDE* CORRECTED *Fanca*$^{-/-}$ AND *Brca2*$^{\Delta 27/\Delta 27}$ iPSC

Once we studied the importance of FANCA and BRCA2 proteins in cell reprogramming, our next interest was the generation of *bona fide* gene corrected *Brca2*$^{\Delta 27/\Delta 27}$ iPSCs. We explored different methods to increase the efficiency and safety of reprogramming.

The phenotypic characterization of the iPSCs cells generated by transduction with the four γ-RVs, revealed that iPSC-like cells generated from corrected *Fanca*$^{-/-}$ and *Brca2*$^{\Delta 27/\Delta 27}$ cells fulfilled most of the phenotypic criteria to be *bona fide* iPSCs, in terms of pluripotency marker expression and teratoma formation capacity. In *Fanca*$^{-/-}$ corrected cells we observed that the expression of the SSEA1 and OCT3/4 pluripotency markers was higher than that observed in corrected *Brca2*$^{\Delta 27/\Delta 27}$ iPSC, although both *Fanca*$^{-/-}$ and *Brca2*$^{\Delta 27/\Delta 27}$ corrected iPSCs generated teratomas (Takahashi et al., 2007).

Due to our interest in exploring the role of *Brca2* in reprogramming we decided to continue the molecular characterization of the iPSC clones generated from this model. The study of the expression of the endogenous pluripotency genes revealed that most of the clones expressed nearly undetectable levels of these markers, in contrast with the high endogenous expression observed in the control mouse ESC line. On the other hand, the exogenous expression of these genes was very high in the clones that were analyzed. These analyses showed that iPSC clones generated by γ-RV transduction were not fully reprogrammed, despite the generation of teratomas in immunodeficient mice. This phenomenon could be due to a persistent over-expression of the exogenous pluripotency factors that could be limiting the reactivation of the endogenous machinery (Ji et al., 2013). Another possibility could be that not all the cells had integrated the four reprogramming factors. The recently reported requirement of telomere reprogramming, simultaneous to the induction of pluripotency, to achieve a correct

maintenance of iPSCs (Ji et al., 2013) could be another cause of the incomplete reprogramming of the cells, although we did not study the telomere status in c*Brca2*$^{\Delta 27/\Delta 27}$ iPSCs.

The characterization of iPSC cells generated from c*Brca2*$^{\Delta 27/\Delta 27}$ mouse embryonic fibroblasts (MEFs) by transduction with an excisable polycistronic lentiviral vector (Sommer et al., 2012a) revealed that the selected clones fulfilled the criteria of iPSCs in terms of morphology and pluripotency (Takahashi et al., 2007). However, unless the reprogramming cassette was excised, teratomas were not generated, but only undifferentiated tumoral masses, probably generated due to the exacerbated and permanent expression of the reprogramming factors, such as *c-Myc* (Deb-Basu et al., 2006).

At early passages, many of the clones were diploid. However, most of them became polyploid at later passages, probably due to the acquisition of new copy number variations and other genetic abnormalities that led to changes in the genetic content of the cells, as also reported both in mouse (Gonzalez et al., 2013) and human iPSC lines (Mills et al., 2013).

Since the generation of iPSCs from genetically corrected *Brca2*$^{\Delta 27/\Delta 27}$ cells was based on the use of a reprogramming cassette that could be removed with the Cre-recombinase, (Sommer et al., 2012b), we excised it to prevent that a residual expression of the reprogramming genes could be affecting the epigenetic print of the pluripotency genes (Sommer et al., 2012b) and thus, the ability of non-excised iPSCs to generate teratomas (Ji et al., 2013).

RT-qPCR and Southern Blot studies showed that the excision of the reprogramming vector with an integrase deficient LV harbouring the Cre recombinase (IDLV) did not allow a complete removal of the reprogramming cassette (Banasik and McCray, 2010) (Sommer et al., 2012b). The re-excision based on the delivery of the Cre-recombinase enzyme directly into the target iPSCs, allowed us to remove the reprogramming vector to undetectable levels. After this re-excision, several clones of corrected *Brca2*$^{\Delta 27/\Delta 27}$ iPSCs showed morphological and phenotypic characteristics of *bona fide* iPSCs (Takahashi et al., 2007), conserved a stable karyotype and at this point they were able to form teratomas. These observations are consistent with the hypothesis that a residual activity of exogenous reprogramming genes could interfere with the expression of the endogenous pluripotency genes required for the complete reprogramming of the cells (Sommer et al., 2012b) (Ji et al., 2013). In this respect, since the expression of *c-Myc* is only required during the initial steps of cell reprogramming (Sridharan et al., 2009), the removal of this gene might have been critical to facilitate the generation of teratomas, instead of rapidly growing tumoral masses.

The excised clones that better fulfilled the *bona fide* iPSC criteria were the ones in which a lower *Nanog* expression had been observed before excision. This is consistent with the previously commented notion that the long-term expression of the exogenous pluripotency factors could interfere with the activation of the endogenous pluripotency machinery (Sommer et al., 2012b). In fact, our observations showed that once the reprogramming vector was excised, a high expression of the endogenous pluripotency genes *Oct3/4* and *Utf1*, as well as a significant expression of *Klf4* and *c-Myc*, was observed. In agreement with other observations that reported that the endogenous *Oct3/4* and *Nanog* promoters should be hypomethylated when the endogenous pluripotency machinery is active (Polo et al., 2012), we observed that these promoters were strongly hypomethylated in the A16Ex7 clone after excision, in contrast to the methylation status of these genes in parental mouse embryonic fibroblasts (Sommer et al., 2012b).

Karyotype studies performed after excision revealed that all the excised clones had an abnormal karyotype except c*Brca2*$^{\Delta 27/\Delta 27}$A16Ex7 iPSC clone that maintained a normal karyotype even after extensive passage numbers. In this clone we observed that after excision all the cells that were analyzed were diploid, while before excision a little deviation in ploidy was observed. The explanation to this observation could underlay in the possibility of the preferential survival of karyotypically normal cells as recently described by Bershteyn in human cells (Bershteyn et al., 2014).

In spite of the normal karyotype of the excised c*Brca2* $^{\Delta 27/\Delta 27}$iPSC clone, a deeper study of the genome of these cells, by Comparative Genomic Hybridization arrays (aCGH), showed chromosomal abnormalities that were not present either in parental *Brca2*$^{\Delta 27/\Delta 27}$MEFs or in corrected MEFs (c*Brca2*$^{\Delta 27/\Delta 27}$). We cannot discern whether these defects were due to the reprogramming process or to the preferential expansion of an abnormal starting cell population during iPSC culture, as other authors have described (Blasco et al., 2011; Hussein et al., 2011; Laurent et al., 2011; Lister et al., 2011; Mayshar et al., 2010; Pasi et al., 2011; Young et al., 2012). Additionally the excision process might also have had a role in these observations.

Despite these chromosomal abnormalities, the selected c*Brca2* $^{\Delta 27/\Delta 27}$iPSC clone did not show the characteristic FA phenotype (Rio et al., 2008). We observed that this clone efficiently expressed the human *BRCA2* gene, and also recovered the capacity to recruit RAD51 to the DNA repair *foci*, meaning that the FA pathway was functional and that homology directed repair worked properly in these cells.

An additional confirmation of the functional correction of c*Brca2* $^{\Delta 27/\Delta 27}$ iPSCs was deduced from the increased genomic stability of c*Brca2*$^{\Delta 27/\Delta 27}$ A16Ex7 iPSC clone after DNA damage with diepoxybutane (DEB).

3. EVALUATION OF HEMATOPOIETIC DIFFERENTIATION IN CORRECTED *Brca2* iPSC

The last part of our work consisted of the evaluation of the hematopoietic differentiation ability of corrected *Brca2*$^{\Delta 27/\Delta 27}$ iPSCs, with the final objective of exploring their potential therapeutic use in *Brca2*$^{\Delta 27/\Delta 27}$ mice.

Several hematopoietic differentiation protocols were performed in our studies. Most of the existing *in vitro* hematopoietic differentiation protocols are based on the transduction with hematopoietic progenitor cell-promoting transcription factors from the LIM homeobox family, such as *HOXB4* (Bonde et al., 2008) (Chan et al., 2008) (Hanna et al., 2007) (Pilat et al., 2005), *Cdx* (Wang et al., 2005) or *LHX2* (Kitajima et al., 2013). In these protocols, the hematopoietic differentiation is completed by co-culture over hematopoietic tissue-derived stromas, such as OP9 or aorta-gonada-mesonephros (AGM) stromas.

The hematopoietic differentiation of our c*Brca2*$^{\Delta 27/\Delta 27}$ iPSCs based on transduction with *HOXB4* (Bonde et al., 2008) (Chan et al., 2008; Hanna et al., 2007; Lesinski et al., 2012; Pilat et al., 2005) and followed by co-cultures with OP9 or AGM derived stromas, allowed us to obtain cells with a modest hematopoietic phenotype. We found that the hematopoietic differentiation observed in these co-cultures was not efficiently reproduced in the different experiments conducted in our laboratory.

In our hands, the protocol based on transduction of iPSCs with *HOXB4*, followed by embryo body generation and co-culture on OP9 stromas was one of the most efficient ones in promoting hematopoietic differentiation. This protocol showed that wt and c*Brca2*$^{\Delta 27/\Delta 27}$ A16Ex7 iPSCs became positive for hematopoietic markers of different stages of hematopoiesis. However, only a very small proportion of these cells were double positive for the hematopoietic markers and HOXB4 (Bonde et al., 2008; Chan et al., 2008; Pilat et al., 2005). In contrast, in mESC the percentage of cells that were double positive for both hematopoietic markers and HOXB4 was significantly higher. Some promoter interference could be occurring within the HOXB4 γ-RV, that did not allow the efficient expression of the second protein of the construction (eGFP) (Zhou et al., 1998) despite the efficient initial transduction of the cells. However, since the outcome of hematopoietic differentiation is highly dependent on the iPSC

clone, and given that we did not select a cell clone that stably expressed HOXB4 (Lesinski et al., 2012), we cannot assure that a vector silencing process had occurred in our cells.

The hematopoietic differentiation obtained in co-cultures with AGM stromas, both in mESC and in c*Brca2*$^{\Delta 27/\Delta 27}$ A16Ex7 iPSCs clone, showed percentages of expression of primitive hematopoietic markers under 4 %, either in mESC or in c*Brca2*$^{\Delta 27/\Delta 27}$ A16Ex7 iPSCs clone. In our hands, this procedure did not result to be an efficient hematopoietic differentiation system in mouse cells, as Ledran proposed for human embryonic stem cells (Gordon-Keylock et al., 2010; Ledran et al., 2008).

In humans, *in vitro* hematopoietic differentiation of ESC and iPSC has been shown to be more efficient and has advanced more rapidly. Nowadays, a relatively efficient production of hematopoietic cells from healthy donors and patient cells has been achieved (Ramos-Mejia et al., 2012; Schenke-Layland et al., 2008; Woods et al., 2011) (Jiang et al., 2012; Raya et al., 2009; Xu et al., 2012)(Kyba et al., 2002) (Amabile et al., 2013; Daley, 2007; Suzuki et al., 2013). However, the efficient engraftment of iPSC-derived HSCs into sub-lethally irradiated mice remains challenging (Kaufman, 2009), (Kennedy et al., 2007; Ledran et al., 2008) (Zambidis et al., 2008) (Klimchenko et al., 2009).

Our interest on evaluating the repopulating ability of *in vitro* differentiated c*Brca2*$^{\Delta 27/\Delta 27}$ iPSCs cells was based on the results obtained in a mouse model of sickle cell anemia (Hanna et al., 2007) (Kitajima et al., 2011). However, in contrast with the results obtained by these authors, the transplant of c*Brca2*$^{\Delta 27/\Delta 27}$ A16Ex7 iPSCs-derived cells into sub-lethally irradiated *Brca2*$^{\Delta 27/\Delta 27}$ mice did not result in any significant engraftment of the cells. More recently, Müller and collaborators generated chimeras in which contribution of the iPSC-derived cells to fetal liver was observed, showing the potential of these cells to reconstitute a hematopoietic system *in vivo* (Muller et al., 2012a). However, in this study, the long-term hematopoietic differentiation in the chimeric animals was not analyzed.

Several publications have recently arisen, in which the hematopoietic differentiation capacity of iPSCs was said to be conditioned by the expression or repression of factors involved in the regulation of the differentiation process. For example, it has been shown that BRD4 regulates *c-Myc* expression during hematopoietic differentiation (Rodriguez et al., 2014). Similarly, A20 was shown to be essential for hematopoietic development (Nagamachi et al., 2014Nagamachi et al., 2014). Some of these proteins that interact with related signalling pathways could account for the blockage of the differentiation process of iPSCs. The hematopoietic differentiation of mESCs or iPSCs seems to be highly dependent on the cellular line.

Additionally, the reprogramming process, together with the elevated genomic instability observed in iPCS cells currently limits the generation of iPSC clones for hematopoietic therapeutic purposes.

Our study in a relevant disease mouse model has shown that iPSC differentiation is an inefficient process in which the generation of hematopoietic stem cells (HSC) as a new source of cells, to be used for the hematopoietic reconstitution of FA recipients is still an objective to be achieved. More efficient *in vitro* culture systems that may mimic the physiological conditions should be developed (Park et al., 2011).

Taken together, our studies of gene therapy and cell reprogramming of *Brca2*$^{\Delta 27/\Delta 27}$ iPSCs have shown new achievements and also current limitations in the application of reprogramming approaches in hematopoietic stem cell therapies.

1. The FANCA/BRCA pathway plays an important role in the process of cell reprogramming, being BRCA2 critical for the generation of mouse iPSCs.

2. The process of cell reprogramming activates the homology directed DNA repair, measured by the recruitment of RAD51 to DNA repair *foci* in response to cell reprogramming.

3. Increased apoptosis during the first stages of cell reprogramming should account, at least in part, for the limited reprogramming of *Brca2*$^{\Delta 27/\Delta 27}$cells.

4. Gene complementation of *Brca2*$^{\Delta 27/\Delta 27}$ cells restores the capacity of these cells to recruit RAD51 to DNA repair *foci* and allows the reprogramming of these cells.

5. The transduction of genetically corrected *Brca2*$^{\Delta 27/\Delta 27}$ fibroblasts with four gamma-retroviral vectors carrying the *Oct4, Sox2, Klf4* and *c-myc* reprogramming genes was unable to generate *bona fide* iPSCs.

6. The transduction of genetically corrected *Brca2*$^{\Delta 27/\Delta 27}$ fibroblasts with a polycistronic lentiviral reprogramming vector efficiently generated iPSCs-like cells.

7. At late passage numbers only one clone maintained a diploid karyotype, although contained several chromosomal anomalies, as revealed by CGH analysis. Thus, a high chromosomal instability was associated to the generation of corrected *Brca2*$^{\Delta 27/\Delta 27}$ iPSCs.

8. The reprogramming of genetically corrected *Brca2*$^{\Delta 27/\Delta 27}$ fibroblasts with a polycistronic excisable lentiviral reprogramming vector, followed by the complete excision of the reprogramming cassette generated *bona fide* iPSCs with a disease-free phenotype.

9. *In vitro* hematopoietic differentiation protocols based on iPSC transduction with *HOXB4*, followed by embryo body generation and co-cultures over hematopoietic stromas, modestly differentiated c*Brca2*$^{\Delta 27/\Delta 27}$ iPSCs towards the hematopoietic system.

10. *In vitro* differentiated cells from c*Brca2*$^{\Delta 27/\Delta 27}$ iPSC were not able to engraft into irradiated *Brca2*$^{\Delta 27/\Delta 27}$ mice, indicating a deficient generation of hematopoietic repopulating cells in these cultures.

Our study in a model defective in HDR supports that:

1. Fanconi anemia/BRCA pathway actively participates in the process of cell reprogramming activating the homology directed repair mechanism through BRCA2.

2. Stem cell therapy applications based on combined gene therapy with cell reprogramming requires improvements to enhance the genetic stability and the *in vivo* repopulating properties of iPSC-derived cells.

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