TÉCNICO LISBOA

UNIVERSIDADE DE LISBOA

INSTITUTO SUPERIOR TÉCNICO

Toward Chromosome 6 Replacement in hPSCs by Editing Nucleases and Microcell Mediated Chromosome Transfer (MMCT)

João Manuel Coelho Carreira

Supervisor: Doctor Domingos Manuel Pinto Henrique

Co-Supervisor: Doctor Maria Margarida Fonseca Rodrigues Diogo

Thesis approved in public session to obtain the PhD Degree in

Bioengineering

Jury final classification: Pass with Distinction

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Chairperson: Doctor Joaquim Manuel Sampaio Cabral, Instituto Superior Técnico, Universidade de Lisboa.

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Doctor Domingos Manuel Pinto Henrique, Instituto de Histologia e Biologia do Desenvolvimento, Faculdade de Medicina, Universidade de Lisboa.

2022

ABSTRACT

Human Leukocyte Antigen (HLA) mismatch remains main obstacle for success in human allo-transplantation. HLA is the expression of Major Histocompatibility Complex (MHC). MHC is all inside both short arms in human chromosome 6 pair.

Human allo-transplantation relies on donor to recipient best found HLA-match (compatibility) whenever possible and immunosuppression to decrease remaining HLA-mismatch consequences. Those consequences are Graft-versus-Host Disease (GVHD) in bone Marrow transplantation and Transplant rejection by the Host in cell, tissue, or organ allo-transplantation. Immunosuppression has also relevant nefarious complications.

Donor's chromosome 6 pair replacement in Human Stem Cells by Host's chromosome 6 pair, will allow for full or needed HLA-match in Human Stem Cell-based allo-transplantation.

Chromosome 6 transfer has been several times accomplished in human cells and inverted Cre-LoxP has been successful in specific entire chromosome deletion as well as CRISPR/Cas9. As Human Pluripotent and Multipotent Stem Cells have been successfully cultured in feeder-free, serum-free, and xeno-free conditions allowing for compliance with Regulatory Authorities' requirements, author proposes a method directed to Donor/indigenous' chromosome 6 pair replacement by Host/recipient's chromosome 6 pair, followed by expansion and later differentiation of the new Allo-Auto-Stem Cells. This new method will enable any needed cell, tissue, or organ allo-transplantation to be successful by avoiding rejection syndromes.

Success in proposed Human Stem Cell chromosome 6 pair replacement, may have important prognostic, economic and QoL consequences in present and future indications for Human Stem Cell-based allo-transplants.

Experimental work is indicative that the proposed solution is plausible, and following experts'recommendations, a step toward the creation of an Off-the-Shelf product is being pursued. A Startup, (TWINORE), is in creation, resulting from the Lab2Market@IST 2020 program,to ease the Translation Medicine process, and the interest from Hovione Capital, (todayBionova Capital), a Venture Capital company toward our work, and a plan to obtainfinancing was designed.

Keywords: Chromosome 6 replacement, Editing Nucleases, Stem Cell, allo-Transplantation, Allo-Transplant rejection.

RESUMO

A incompatibilidade HLA (Human Leukocyte Antigen) mantem-se como o maior obstáculo ao sucesso na alo-transplantação humana. O HLA é a expressão do Complexo de Histocompatibilidade Maior (MHC). O MHC reside em ambos os braços curtos do cromossoma 6 humano.

A alo-transplantação humana depende da melhor coincidência, (compatibilidade), possível entre o HLA do dador e o do hospedeiro e de imunossupressão para diminuir as consequências da incompatibilidade restante. Essas consequências são: Doença do Enxerto contra Hospedeiro (GVHD), nos transplantes de Medula Óssea e a Rejeição do alo-transplante pelo hospedeiro no caso de células, tecidos ou órgãos. A imunossupressão é acompanhada de complicações graves.

A substituição do par de cromossomas 6 do dador pelo par de cromossomas 6 do hospedeiro permitirá a completa ou necessária compatibilidade HLA na alo-transplantação baseada em Células Estaminais Humanas.

A transferência do cromossoma 6 já foi obtida em células humanas e a deleção completa do cromossoma 6 foi obtida com recurso ao sistema Cre-LoxP bem como CRISPR/Cas9. É possível cultivar Células Estaminais Humanas Pluripotentes e Multipotentes, na ausência de substâncias ou células animais, cumprindo exigências das Autoridades Reguladoras. Propõe-se um método para a obtenção da substituição do par de cromossomas 6 indígena pelo par de cromossomas 6 do hospedeiro, seguida da expansão e diferenciação das Alo-Auto-Células Estaminais Humanas. O objetivo é criar, células, tecidos ou órgãos para alo-transplantação evitando as habituais síndromas de rejeição.

O sucesso da presente proposta de substituição do par de cromossomas 6, trará importantes benefícios prognósticos, económicos e da Qualidade de Vida, nas presentes e futuras indicações de alo-transplantação baseada em Células Estaminais humanas.

A experimentação indica que a proposta é plausível, e a criação de um produto pronto-a-usar, formaliza-se.

Frutos do programa Lab2Market@IST 2020 são: desenvolvimento da start-up TWINORE, e o interesse da HOVIONE CAPITAL, em relação ao financiamento do nosso projeto.

Palavras Chave: Alo-transplantação, Células Estaminais, Nucleases de Edição, Rejeição de transplante, Substituição do Cromossoma 6 humano.

ACKNOWLEGEMENTS

For All of YOU…

For your unacceptance of the word “impossible”!

For your believe in the eternal cycle of changes, replacements, and evolution…

Artists my gratitude!

Inventors my gratitude!

Philosophers my gratitude!

Scientists my gratitude!

By the roads and avenues, you taught me, through the deepest ocean valleys to the picks of Himalaya mountains and beyond, I am dreaming no roads, no avenues!

Exactly as you! All!

No roads, no avenues!

No obstacles in our ways!

Impossible has always been the only impossible!

So few know the secrets as You do…!

Life wishes more, Life is more, Life has more than You all taught me!

Wonder if we could gather!

Have a forum! In one only place and eternity!

Transcend forever our conditions! Be companions of God!

Be companions in God!

There, could I feel All Life, reinvent All Life, live All Life!

Each one in the place and all of us in all places! No place at all!

No Time no Space! All!

No Energy no Matter! All!

No Dark no Bright! All!

No Silence no Music! All!

No Start no End! All!

No Quarks no Universes! All!

In One single eternal moment…

only Wisdom! All!

To **Professor Carmo Fonseca** (IMM) and **Professor Joaquim Cabral** (IST, iBB-SCERG), my gratitude for constant and permanent support.

Special gratitude also for my Supervisors, **Professor Domingos Henrique** and **Professor Margarida Diogo** for so much you taught me!

Also special gratitude for **Professor Edgar Gomes** and Doctors **Carlos Rodrigues**, **Cláudia Miranda**, **Cláudio Franco**, **Leonor Saúde**, **Lars Jansen** (IGC), **Musa Mhlanga** (CapeTown Univ., South Africa), **Evguenia Bekman**, **Simão Rocha**, **Miguel Casanova**, **Teresa Silva, Mariana Branco**, **Ana Rita Carreira Gomes, Jaqueline Quaresma**, **Inês Milagre**, **Diogo Nogueira.** My gratitude to my Lab Colleagues: Dr **André Branco**, Dr **Carina Maranga**, Dr **João Cotovio**. And to **Sara Ferreira.**

Also for **PI/IST** and **TT/IST**; **Everis Foundation**; **I-deals** and BIONOVA CAPITAL for health

ALL PROFESSORS AND ALL COLLEAGUES IN BIOENGINEERING DEPARTMENT at IST and at IMM – João Lobo Antunes.

Gratitude toward my parents **Luzia** and **Cipriano**.

For the Morality I took from you both! For the unbreakable strength of your Perseverance!

Main guidance in my actions.

The harder the work the better the achievement. Everywhere, every time, no matter the aims!

I am grateful to you both.

To my sisters, **Helena**, and **Maria de Lourdes**, for the dreams we have always dreamed together. Each one of us in each dream, together! You were always so supportive!

In each one's achievements, together!

To my brothers-in-law, **Helder** and **Armando**. Permanently supportive!

To my beloved sons **João Pedro**, **Dinis**, and to my beloved and sweet Twins, **Pedro** and **Inês**.

Inspiring me all the time(s) and all the way(s). Wish I could know how to better divide Time and Energy with the Matter we all are! You were every day inside my work! Even when our Matters were so far away, my Spirit and my Energy were always with You!

All the same to, **Mauro** and **Cecilia**, my nephews!

To my beloved spouse, **Paula Carreira**. Physically so fragile as You seem, and so incredibly Strong as You are! Permanently! Constantly! Sweetest and Safest anchorage in all the storms of my life…!

Without you, nothing!

With you, everything!

Wish I could know how to live for You in eternity!

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ABBREVIATIONS

2pA - polyadenylation sequence

Δ**TK** - Thymidine Kinase gene

APC - Antigen Presenting Cell

ATMPs - Advanced Therapy Medicinal Products

B2M - β2-microglobulin

BACs - Bacteria Artificial Chromosomes

BAG 2 - chaperone regulator 2

BCL11A enhancer - BAF Chromatin Remodeling Complex Subunit **BCL11A** enhancer

BER - Base Excision repair

bGH pA - bovine growth Hormone polyadenylation sequence

BLESS-Seq - Direct in-situ Breaks Labeling, enrichment on Streptavidin and Next Generation Sequencing

BLISS-Seq - Breaks Labeling In Situ and Sequencing

CAGG – Chicken β-actin promoter coupled with Cytomegalovirus (CMV) earlier enhancer

CAR T Cell - Chimeric Artificial Receptor T Cell.

CHANGE-Seq - Circularization for High-throughput Analysis Nuclease Genome-wide Effects by Sequencing

CIRCLE-Seq - Circularization for In vitro Reporting of Cleavage Effects by sequencing

CHM - Complexo de Histocompatibilidade Major

CHO - Chinese Hamster Ovarian Cells

Chr 6 – human Chromosome 6

CIRCLE-Seq - Circularization for In vitro Reporting of Cleavage Effects by sequencing

ClassIITA - HLA Class II transactivators,

CMV - Cytomegalovirus immediate early promoter

c-Mic - multifunctional transcription factor for cell division

Cre-LoxP – Cycle recombinase - locus of X-over P1

CRISPR – Clustered Regularly Inter Spaced Palindromic Repeats.

CRISPR/Cas9 – Clustered Regularly Inter Spaced Palindromic Repeats, CRISPR-associated protein 9.

dCas9 - dead Cas9 (inactive Cas9)

DMEM - Dulbecco's Modified Eagle Medium

DNA - Deoxyribonucleic acid

DISCOVER-Seq - discovery of in situ Cas off-targets and verification by sequencing

DMSO - Dimethyl sulfoxide

DSBs - Double Strand Breaks

DSB-Seq - quantitative DSB sequencing

ECM - Extracellular Matrix

EDTA - Ethylenediamine tetraacetic acid

EF1α - Human elongation Factor 1**α** Promoter

EMA - European Medicaments Agency

END-Seq - "*method to quantitatively determine the DSB initiation landscape and end resection prior to DSB repair.*"

EOS pmt - Gene sox2 promoter

EPO - European Patent Office

EPRS - European Parliamentary Research Service, (European Union)

EtOH - ethanol

FACS - Fluorescence-Associated Cell Sorting

FBS - Fetal Bovine Serum

GFP - Green Fluorescent Protein

GMP - Good Manufacture Product

gRNA - guide RNA

GUIDE-Seq - Genome-wide, Unbiased Identification of DSBs Enabled by Sequencing

GVHD - Graft Versus Host Disease

GVL - Graft Versus Leukemia

GVT – Graft Versus Tumor

HA5' - Left Homology Arm

HA3' - Right Homology Arm

HACs - Human Artificial Chromosomes

HDR - Homology Directed Repair

hESC - human Embryonic Stem Cell

HEK 293 T - Human Embryonic Kidney cell line

hPSCs - human Pluripotent Stem Cells

hiPSC - human induced Pluripotent Stem Cell

HLA - Human Leukocyte Antigen

HLA I - Human Leukocyte Antigen Class I

HLA II - Human Leukocyte Antigen Class II

HLA-DR - Human Leukocyte Antigen Class II type DR

HLA–G - Human Leukocyte Antigen – type G

iBB - Institute for Bioengineering and Biosciences

IDLV - Integrative-Deficient Lentiviral Vectors

IDLV-capture Seq - Integrative-Deficient Lentiviral Vectors capture

IGC - Instituto Gulbenkian de Ciência

IL10 - Interleukin 10

IMM - Instituto de Medicina Molecular – João Lobo Antunes

INPI - Instituto Nacional da Propriedade Industrial

IPOFG-Lisboa - Instituto Português de Oncologia Franscisco Gentil - Lisboa

ISS - International Space Station

IST - Instituto Superior Técnico

ITR-Seq - Inverted Terminal Repeat Sequencing

IVF - *In Vitro Fertilization*

LB - lysogeny broth

LEO - Low Earth Orbit

LGSN - Lengsin, Lens Protein With Glutamine Synthetase Domain

mCherry- Red fluorescent Protein

MHC - Major Histocompatibility Complex

MiHC - Minor Histocompatibility

MIC - Modified Immune Cells

Klf4 - Kruppel-like factor 4

milliQ water/H2O - Ultrapure water

mL - milliliter

mM - milimolar

MMCT - Microcell Mediated Chromosome Transfer

MMR - mismatch repair

MS - Multiple Sclerosis

MSCs - Mesenchymal Stem Cells

N2 - Nitrogen

NeoR - amino 3'-glycosyl phosphotransferase (resistance gene)

NER - nucleotide excision repair

NGS - Next Generation Sequencing

NHEJ - Non-Homologous End Join

NK Cell - Natural Killer Cell

OMIM® - Online Mendelian Inheritance in Man

Oct4 - octamer-binding transcription factor 4, also known as POU5F1.

OS - Overall Survival

PAM - Protospacer Adjacent Motif

PBS - phosphate-buffered saline

PCR - Polymerase Chain Reaction

Pen/Strep - Penicillin/Streptomycin

pESCs - parthenote Embryonic Stem Cells

PGKpmt - Phosphoglycerate Kinase Promoter

PI/IST - Núcleo da Propriedade Intelectual in Instituto Superior Técnico

PuroR - Puromycin Transferase gene (resistance gene)

QA - Quality Assurance

QC - Quality Control

QoL - Quality of Life

Rh- - Rhesus antigen negative

RNA - Ribonucleic acid

ROS - reactive oxygen species

RT - Room Temperature

SCR7 - Ligase IV inhibitor (NHEJ), HDR Enhancer.

SCERG - Stem Cell Engineering Research Group

sgRNA - single-guide RNA

SITE-seq - Selective enrichment and identification of adapter-

tagged DNA ends by sequencing

SLE - Systemic Lupus Erithematosus Multiple Sclerosis (MS)

SNPs - Single Nucleotide Polymorphisms.

SNP seq - Single Nucleotide Polymorphism Sequencing

Sox2 - SRY-box 2 transcription factor.

SV40 pA - simian vacuolating virus 40 polyadenylation sequence

TAE - Tris-Acetato-EDTA buffer

TALEN - Transcription Activator-Like Effector Nuclease

TK - Tyrosine Kinase

TMO - Serviço de Transplante de Medula Óssea -IPOFG-Lisboa

T reg - regulatory T Cell

TT/IST - Núcleo de Transferência de Tecnologia in Instituto Superior Técnico

TWINORE - Transplantation With No Rejection

uL – micro Liter

UCB - Umbilical Cord Blood

UK - United Kingdom

UNESCO - United Nations Education, Science and Culture Organization

USA - United States of America

UTR - untranslated region

UV - Ultra violet light

WGS - Whole Genome Sequencing

WHO - World Health Organization

WMDA - World Marrow Donor Association

ZNF - Zinc Finger

INCIPIT PROLOGVS

“You must do a PhD in a difficult subject. Those PhD Programs are the most prone to bring Humanity real progress.”

Professor Robert S. Langer, MIT

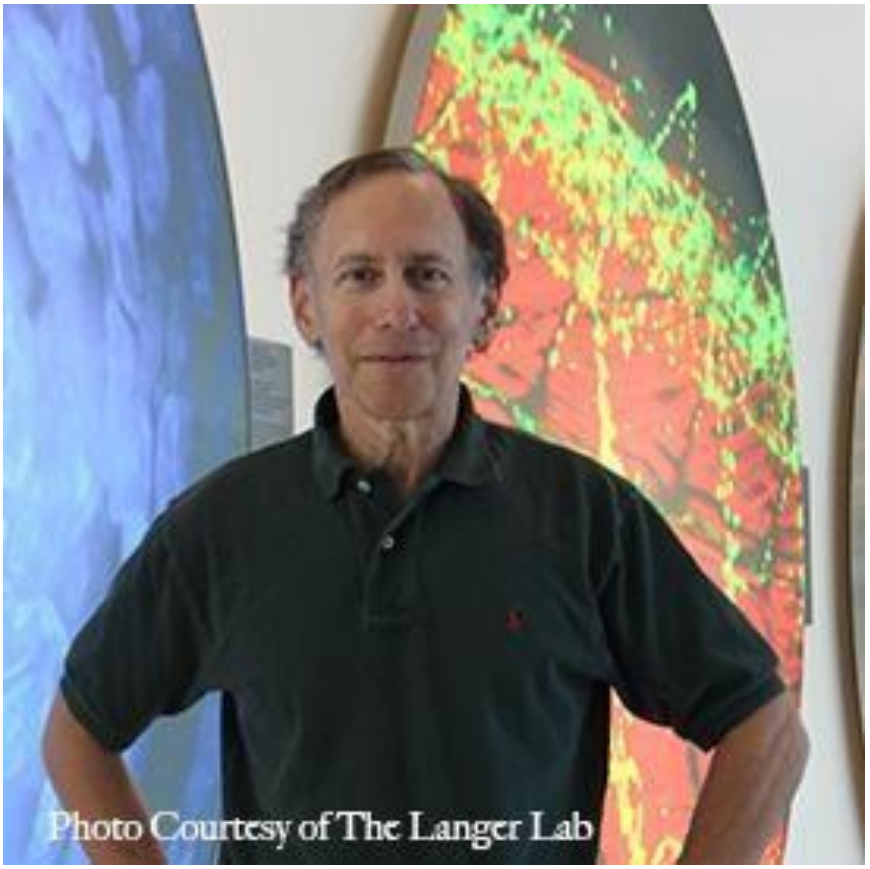
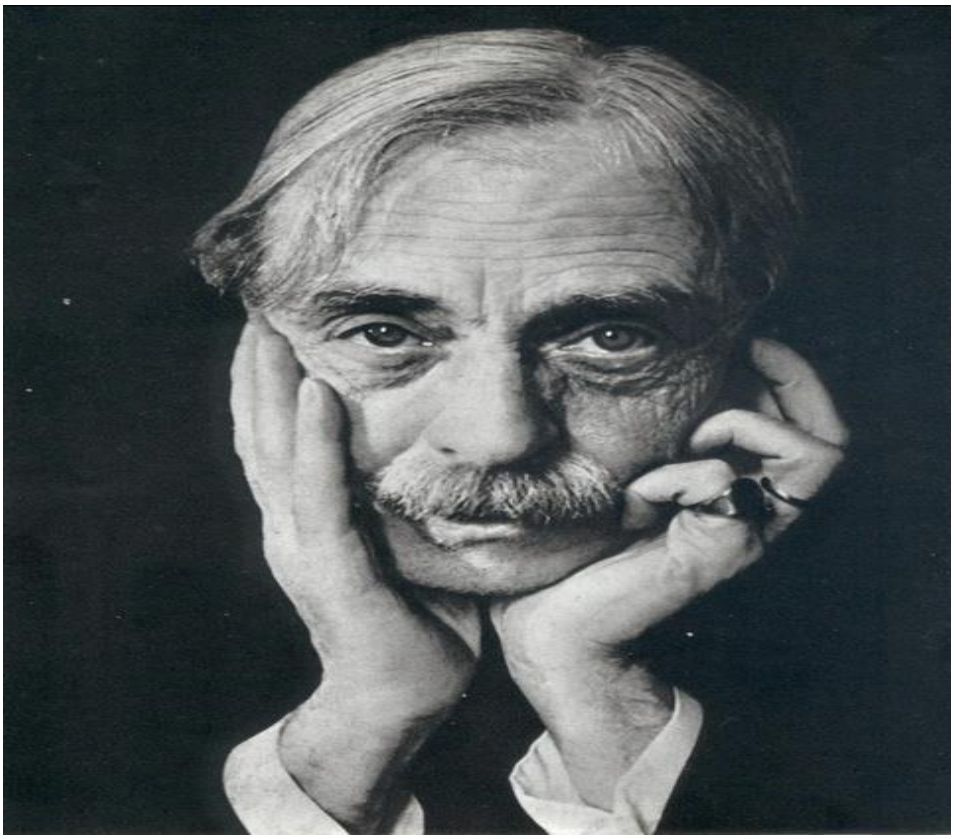


Photo reproduced with writen permition from Professor Robert Langer.

As quoted by Saeed Abbasalizadeh, PhD in is PhD Thesis defense, 19-01-2021, Instituto Superior Técnico, (IST) – Lisbon - Portugal

“A difficulty is light.

An irresolvable difficulty is the very sun.”



Ambroise-**Paul**-Toussaint-Jules **Valéry**

(30 October 1871 — Paris, 20 July 1945)

(https://www.google.com/search?q=Paul+Val%C3%A9ry&sxsrf=ALeKk01cUd6QnZhLDnp-eBobIWdHEPqpcg:1613387311733&tbm=isch&source=iu&ictx=1&fir=X0HTBlC8hyBa2M%252C5vUj1pRcHFxMIM%252C%252Fm%252F05vy1&vet=1&usg=AI4_-kTf5dDuD2MdBeM0DPzwvbBSvj462Q&sa=X&ved=2ahUKEwjmi-eM4OvuAhXiMVkFHX2vCKUQ_B16BAglEAE#imgrc=rTDPiB8TrmDSyM

PREFACE

"The dream of complete tolerance without need for immunosuppressant drugs seems like a mirage in the desert, that recedes as you seem to approach it."

This was the utopia and the reality Professor Jean Hamburger dreamed about and mentioned in the preface of a book entitled "Research in organ transplantation and tissue grafting" in 1996 [1].

Could the Path to Professor Jean Hamburger's dream become reality and the oasis be unveiled?

Since the days of my graduation in Medicine, I was very enthusiastic about Immunology. Immunology can bring hints in fields so diverse as cancer, infections, infestations, tolerance, atopy, military strategy and tactics, weaponry, computer programming, computer construction, Internet, Artificial Intelligence (AI), and many more naturally including from the simplest to the most complex phenomena related to human transplantation.

Everything started at that Winter 2013. It was a cold but sunny day in Instituto de Histocompatibilidade do Sul, Hospital Pulido Valente, Lisbon.

I finished my six months internship in Hematology, Hospital dos Capuchos, Lisbon, a curricular requisite for Imunohemoterapia specialization (Transfusion and Transplant Medicine), as well as another six months Bone Marrow Transplantation internship in Serviço de Transplante de Medula Óssea (TMO) at Instituto Português de Oncologia Francisco Gentil (IPOFG), Lisbon. Now I was in the middle of a new, six month-long internships in Histocompatibility and Transplantation.

Taking care of our patients with leukemias and lymphomas at Hematology Service (Serviço de Hematologia do Hospital dos Capuchos, Centro Hospitalar de Lisboa Central, Director Doctor Aida Botelho de Sousa, Lisbon) and trying to provide them with the best possible Bone Marrow Transplant, (Serviço de Transplante de Medula Óssea (TMO) – IPOFG-Lisbon, Director Professor Doctor Manuel Abecasis), I realized the huge difficulties all of us have in achieving cure for our patients, as well as the daily huge suffering conditions patients, doctors, nurses, and all caring staff are submitted to.

Main obstacle related to Bone Marrow Transplantation, also known as Hematopoietic Progenitor and Stem Cell (HPSC) transplantation, originates in the search for the best

Human Leukocyte Antigen (HLA)-match between donor and host, aiming to avoid the rejection syndromes related to allo-Bone Marrow transplantation known as Graft-Versus-Host-Disease (GVHD). At that time, the worldwide inscriptions for Bone Marrow donations included around two million candidates.

In such adverse circumstances I took the challenge to start studying and thinking about solutions that could improve the results in allo-Bone Marrow transplantation or even in a broader allo-transplantation field.

In 25 March 2022, in the World Marrow Donor Association (WMDA), there were 39.859.535 candidates and umbilical cords in database from 55 countries, ready to provide for a HPSC transplant to save someone, no matter who, elsewhere on planet Earth. Even though, it is not enough to provide for safe HPSC transplants for everyone in need.

CHAPTER I – INTRODUCTION

CHAPTER I – INTRODUCTION

Main obstacle in Allo-transplantation is related to the differences between the Human Leukocyte Antigen (HLA) receptors present on the donor's cells as compared to the ones present on the host's cells. Those differences are designated HLA-mismatch or incompatibility. Those differences are responsible for the rejection syndromes, that makes allo-transplantation very hard to succeed.

In Bone Marrow Transplantation, also known as Hematopoietic Progenitor and Stem Cell (HPSC) transplantation difficulties arise in the search for the best HLA-match between donor and host, aiming to avoid the rejection syndromes related to allo-Bone Marrow transplantation known as Graft-Versus-Host-Disease (GVHD). A full coincidence between patient's and donor's HLA (full HLA-match) in allo-HPSC transplantation is an absolute rare achievement, because it only usually occurs when Bone Marrow donations (HPSCs) were collected from twins or in 25% of siblings[2]. More accurately the real probability in finding full-matched donors among siblings grow from 25% with a sibling, to 43,7% with two siblings, 57,8% with three siblings, 68,4% with four siblings, etc,[3]. In the industrialized world, the tendency is for couples to have only one or two natural children. The chances for a patient in need for a Bone Marrow transplantation to have one HLA-matched sibling is low. These facts conditioned all treatment, hampering overall survival (OS), as well as Quality of Life (QoL) parameters, and requiring immunosuppression protocols with serious nefarious secondary complications like infections, infestations, cancers, cardiotoxicity, Bone Marrow toxicity, gastrointestinal toxicity, and nephrotoxicity[4].

Something unknown but relevant that later would be called HLA-match was recognized from the very earlier stages of kidney allo-transplantation as the first patients where identical twins. In those days of the year 1954, doctors first tested rejection by performing small skin grafts between the twins, previously to kidney transplantation. Only after concluding that no skin rejection happened was the kidney transplantation performed. It was a transplantation success, despite the glomerulonephritis relapsed[5].

However, it was necessary to wait for the Human Leukocyte Antigen (HLA) discoveries made by Jean Dausset in 1958, as well as the work by Jon van Rood, Rose Payne and many others for humanity to be aware of the real reasons allo-transplantation was almost always unsuccessful.

HLA is expressed from Major Histocompatibility Complex (MHC) in one only human chromosome pair: the human Chromosome 6 pair[6] (**Figure 1**) and [7](**Figure 2**).

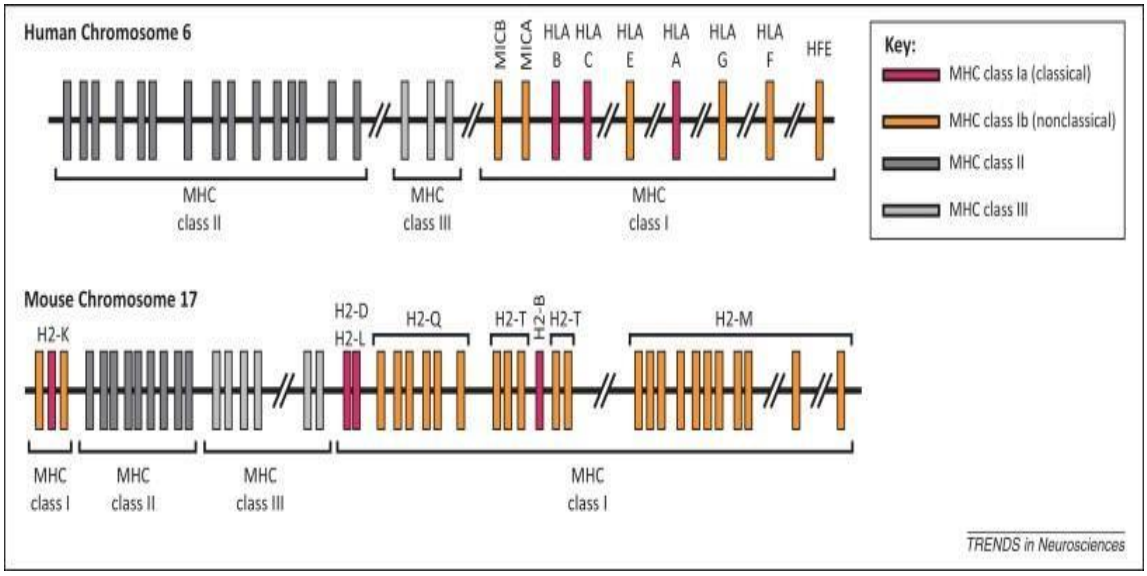


Figure 1 - Major Histocompatibility Complex (MHC). Human chromosome 6 and Mouse chromosome 17. **In:** Major histocompatibility complex Class I proteins in brain development and plasticity; Elmer B.M. & A. McAllister A.K. (2012)[6]. Permission in written was obtained from the authors and from the publisher.

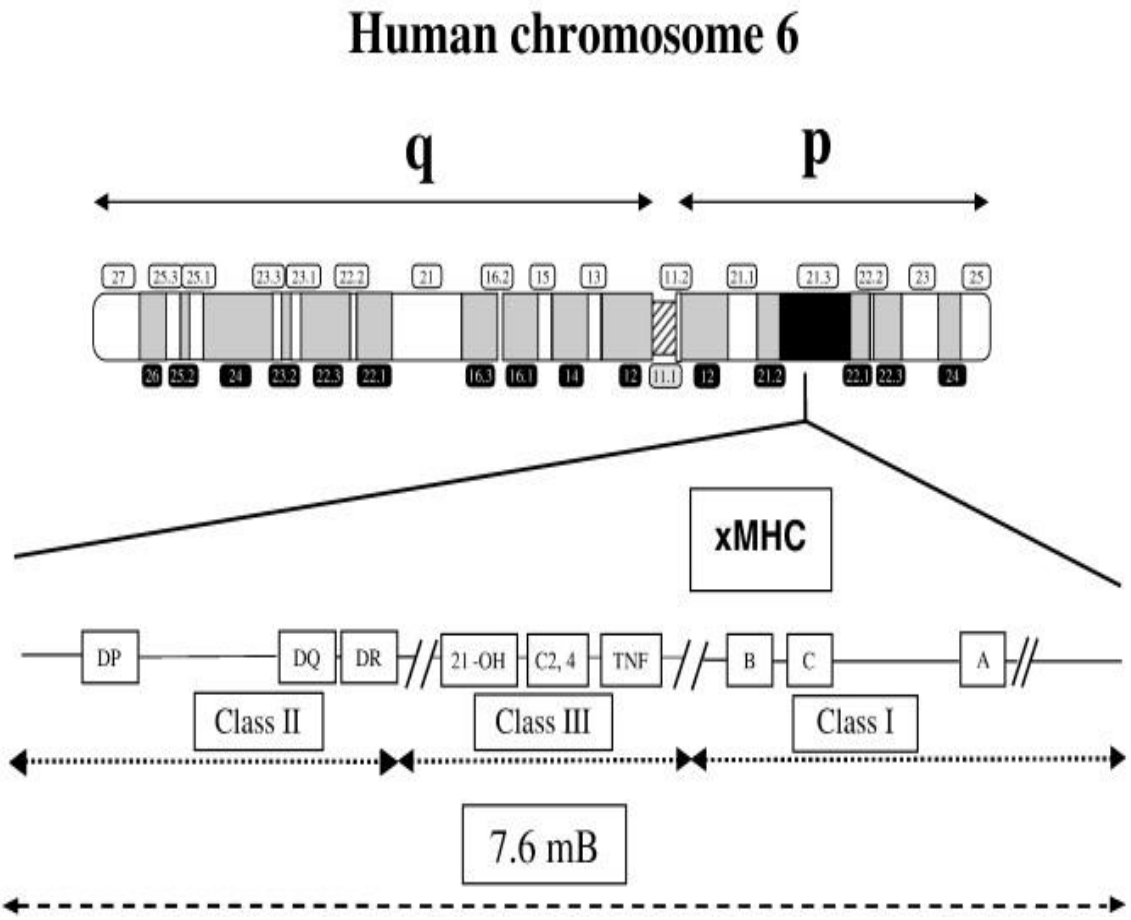


Figure 2 - Major Histocompatibility Complex in human chromosome 6. Dependent on the authors is 4.2Mb, Mungall A. J. *et al.* (2003)[7], to 7.6Mb wide, Figure reproduced from: Taylor M. (2009)[8], (Creative Commons)

MHC is a huge gene complex located inside short arms of human Chromosome 6 pair and is the most polymorphic gene complex in the Human Genome[7]. Some authors refer MHC as being more than 4 Megabase pairs long[6], with more than 200 genes included. But other authors, admit extension toward 7 and 7,6 Megabase pairs[32,255], and more than 400 genes[9,10], (**Figure 2**). MHC gene expression is codominant, meaning that both maternal and paternal different sets of HLA receptors are expressed in every human being[11], (**Figure 3**).

Human Leukocyte Antigens (HLA) are the expression product of some of the genes inside the MHC[7], and from Chromosome 15 for β2-microglobulin in Class I HLA receptors[12,13].

Relevance of MHC maintains until today (2021/2022) as the main factor for rejection(s) in human allo-transplantations[14].

This has been, since long time now, unanimously considered the main obstacle to success in human allo-transplantation [9,10,14,15,16,17,18,19,20].

Codominant expression of HLA genes adds difficulties in the search for HLA-matched transplants[21](**Figure 3**).

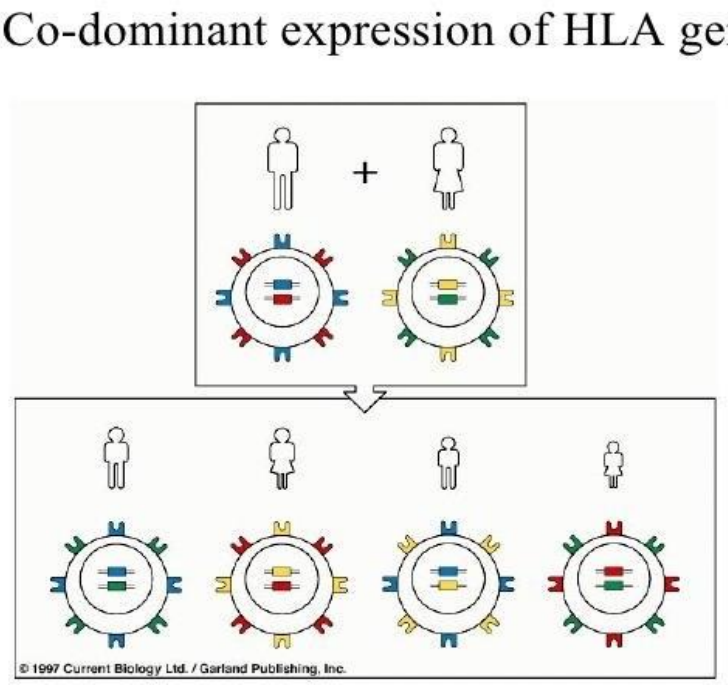


Figure 3 - MHC Gene expression is Codominant. **In:** https://www.slideshare.net/MUBOSScz/immunology-vii-hlaregulation

That is the explanation to why is so difficult to find full MHC coincidence (compatibility, or match), between donor and recipient (host), both in Solid Organ and in HPSC transplantation[21].

However, many authors are trying to overcome the problem of rejection syndromes in allo-transplantation by the use of the available tools to provide for safer transplants. One of the most promising raw materials are Human Stem Cells that constitute the foundation of Cell Therapies and Regenerative Medicine

I.1. - HUMAN STEM CELLS

Human Stem Cells are a special group of cells that by cell division have the capability to perpetuate its lineage, as well as giving rise to many types of cells, tissues, and organs a human body is composed of, and to "*interact with its environment maintaining a balance of quiescence, proliferation, and regeneration*"[22]. Those stem cells' capabilities are known as stemness.

Authors described two hypothesis a stem cell may be able to perpetuate itself and/or to give rise to other cells able to perform new specific tasks in the body, in a process known as differentiation.

1- One hypothesis admits that a stem cell, from the original pool of stem cells has the capability to divide into two stem cells identical to the original. This is known as symmetrical division. (**Figure 4, left**).

Any or both stem cells may thereafter acquire, a new fate allowing for the creation of the different cells, tissues, and organs in the human body (differentiation).

2- Another hypothesis admits that each dividing stem cell can originate two cells. One remains as stem as the original and the other acquires the capability to perform new tasks, (differentiation). This is designated as asymmetrical division. The daughter stem cell as stem as the original, maintains readiness to perpetuate the process, the other is a step forward in the differentiation process[23,24]. (**Figure 4, right**).

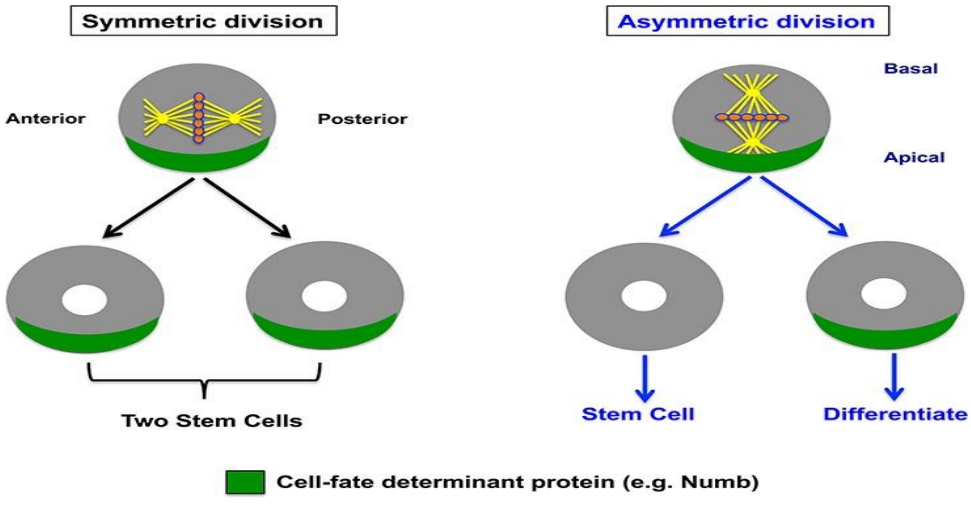


Figure 4 – Stemness conservation in stem cells explained by two different cell division possibilities, Symmetric and Asymmetric, Berika M. *et al.* (2014)[23]. Permission to reproduce the figure.

No matter which hypothesis is correct, stem cells by their intrinsic natural characteristics are of the utmost relevance with maximum potential in Cell Therapies and Regenerative Medicine. They are frequently considered the foundational elements to start each Cell Therapy and in Regenerative Medicine.

Human Stem Cells may be classified accordingly to the degree of **Potency for differentiation** they present, as: Totipotent, Pluripotent, Multipotent, Oligopotent, and Unipotent. Or accordingly to their **origin along timelapse after fertilization**: Human Morula Stem cells (Totipotent); Human Blastocyst Embryonic Stem Cells (Pluripotent); Human late Embryonic Stem Cells and Human Fetal Stem Cells (Multipotent, Oligopotent and Unipotent); Somatic Human Reprogrammed (induced) Pluripotent Stem Cells (hiPSCs) (Pluripotent), Cancer Stem Cells, and Human Somatic Adult Stem Cells, (Multipotent, Oligopotent and Unipotent). (**Figure 5** and **Figure 6**)

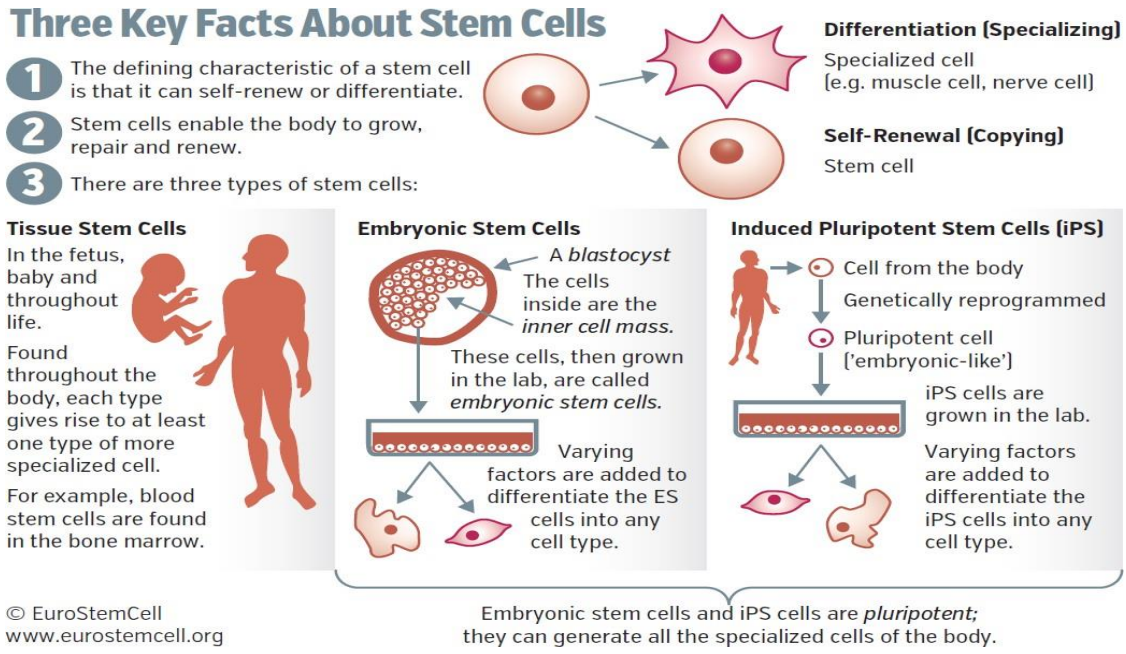


Figure 5 – Stem Cells by the origin. Not including Cancer Stem Cells. Human Embryonic Stem Cells (hESCs) and human induced Stem Cells (hiPSCs) may differentiate to any type of cells present in the 3 embryo layers: ectoderm, mesoderm, and endoderm. That is why they are designated as Human Pluripotent Stem Cells, (hPSCs). Tissue Stem Cells are considered multipotent, oligopotent, and unipotent. Cancer Stem Cells present as special Stem Cells with aberrant behavior. **Figure from:** www.EuroStemCell.org

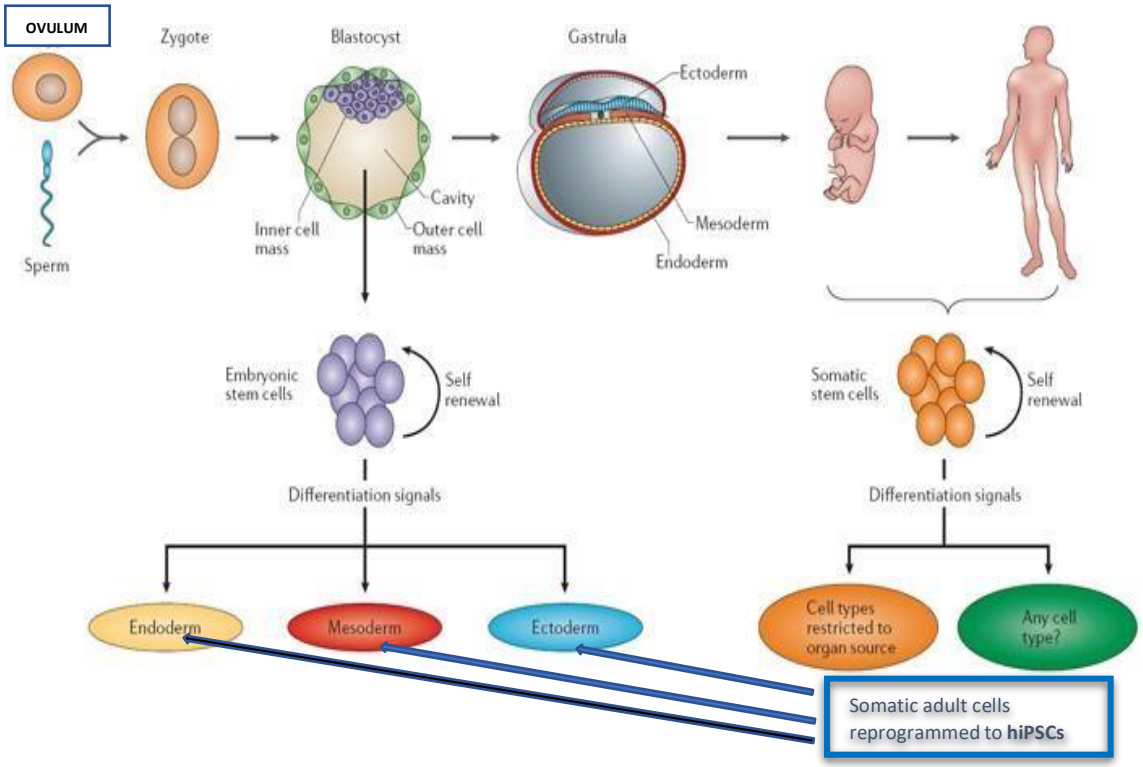


Figure 6 – Human Stem Cells. Not including Cancer Stem Cells. **Modified from** http://www.biosummary.com/types-of-stem-cells/

I.1.1. - HUMAN TOTIPOTENT STEM CELLS

The first totipotent Stem Cell that is created by/after fertilization is the egg. Egg cleavage creates the zygote and evolves to the morula, a compacted structure of cells that keeps cleaving until the stage of 32 cells along 5 cleaving steps (2-4-8-16, and 32 Cells). Blastomeres are created in a very early stage of zygotic life in a structure called morula (day 5 to day 12 in humans)[25]. Authors found that when compared to hESCs derived from intact blastocysts, cell lines derived from any single blastomere from 8- to 12-cell human morula, had a distinct transcriptome that was enriched in genes involved in trophoblast/ectoplacental core pathways, an indication that these cells retain trophoblast potential[25]. The blastomere-derived lines were also enriched for components of cholesterol metabolism, possibly *"underlining the relevance of rapid plasma membrane assembly during early embryo development"*[26]. In blastocyst-derived hPSC lines the expression of genes that are involved in a great number of morphogenic and developmental processes is upregulated when compared to blastomere-derived lines, suggesting a relatively more naïve state of the blastomere-derived cell lines[26]. Moreover, authors highlight the important fact that conventional hESCs (blastocyst-derived), have already initiated fate specification at this stage. This aligns with the concept that hESCs

derived from blastocysts contain cells in various primed states, biasing them for differentiation along certain lineages[26]. And different blastomeres from the same human embryo can present mosaicism as it is referred by Zuccaro *et al.* (2020) *"Embryos dissociated to single blastomeres showed mosaicism for multiple alleles*"[27].

I.1.2. - HUMAN EMBRYONIC STEM CELLS (PLURIPOTENT)

A few cell divisions later, cells from the morula give rise to the blastocyst. Blastocyst consists of two related structures composed of two different types of cells. The external cell layer in the blastocyst consists of trophectodermal cells, which will be responsible for the formation of the placenta. The internal part of the blastocyst contains the inner cell mass. Each cell in the inner cell mass has the potential to create all tissues of a human embryo (Pluripotency). At this exact stage the cells of the inner cell mass are classified as Pluripotent Stem Cells. As referred in the work of Graf T. & Stadtfeld M. (2008)[26], these human Pluripotent Stem Cells are primed, or priming for specific cell fates. That is why some hESC lines may be more prone to differentiate toward different fates, being not so naïve as blastomere derived cells.

Each hESC has still capability to create all three layers of the human embryo:

1- Endoderm. This layer originates all tissues in the pharynx, esophagus, stomach, intestines, liver, pancreas, bladder, lungs and epithelial parts in trachea and bronchi, and the thyroid and parathyroid.

2- Mesoderm. This layer forms all smooth and striated muscle, bones, cartilages, adipose tissue, circulatory system including the cells responsible for the hematopoiesis, lymphatic system, dermis, dentine in teeth, genitourinary system except bladder, serous membranes, spleen, and notochord from which to form the vertebral column and intervertebral discs.

3- Ectoderm. Forms from the embryo epiblast and will be composed by:

a- Surface ectoderm giving rise to the epidermis, hair, nails, lens in the eyes, sebaceous glands, cornea, teeth enamel, the epithelium in the mouth and nose.

b- Neural crest, responsible for the creation of the peripheral nervous system, adrenal medulla, melanocytes, and facial cartilage.

c- Neural tube, develops into the brain, spinal cord, posterior pituitary gland, motor neurons and retina.

I.1.3. - HUMAN INDUCED PLURIPOTENT STEM CELLS

By the work of Shynia Yamanaka's team[28], (Nobel prize in 2012 with John Gurdon), reprograming somatic adult human cells into human pluripotent stem cells was made possible. When artificially overexpressed in human somatic cells, the so called, Yamanaka Factors: Oct3/4, Sox2, c-Myc, and Klf4, can reprogram somatic cells into Pluripotent Stem Cells, known as human induced Pluripotent Stem Cells (hiPSCs)[28]. This was a major achievement as hiPSCs are very promising in the field of human auto-transplantation. Since that they can be reprogramed from adult somatic cells of any healthy or sick human being and as they are pluripotent, great hope was deposit that any cell, tissue, or organ a patient may be in need would be possible to be created, with the major advantage of not triggering any rejection after auto-transplantation. Also, hiPSCs could be of considerable relevance for "drug screening" paving the way for the creation of specific disease-related cells to be tested against several drugs, providing for the best to reverse/compensate disease phenotype.

Today several different protocols are available to reprogram human adult somatic cells. Different vectors may be used: Lentiviruses[29]; non-integrative rAdeno Associated Viruses (rAAV)[30]; non-integrative Sendai viruses[31]; mRNA[32]; Proteins[33], and small molecules[34]. Reprogramming can be obtained by overexpression of Yamanaka Factors (Oct3/4, Sox2, c-Myc, and Klf4) as well as by overexpression of: Oct3/4, Sox2, NANOG, and Lin28[35].

However, some hurdles must be overcome before this can be a reality in the clinical use of those cells. Main hurdles are related to genomic instability and the expression of oncogenic genes like Klf4 and c-Myc, that are responsible for the pluripotency in hiPSCs and that may still be expressed in differentiated products ready to be transplanted. Another concern relates to the epigenetics of hiPSCs because somatic epigenetics is never completely erased as compared to hESCs and mostly to Blastomeres or Human Naïve Embryonic Stem Cells. This results in that some hiPSC lines can be biased to differentiate upon certain fates in detriment of others, and the risk of some cells to de-differentiate within a differentiated tissue *in vivo* or *in vitro,* contaminating a transplant is not so low to be neglected. Genomic instability, epigenetics, oncogene expression, are some of the considerations raising concerns about safety in hiPSC-based transplantation[36,37,38]. A worrisome example of leukemic transformation of hiPSC-derived HSPCs is mentioned by Demirci S. et al. (2020)[39]: "*However, of those approaches, MLLAF4-engineered iPSCs displayed leukemic transformation during long-term follow-up [7], and*

dysregulation of the HOX pathway is strongly associated with leukemia progression [8]". Based on these arguments, authors' option was to perform a hESC-based HSPC differentiation protocol instead of hiPSCs-based[39].

Other interesting applications of hiPSCs are as disease models in congenital diseases as well as for "drug screening" mainly related to same congenital diseases and for the treatment of other non-genetic diseases[40].

However, everybody knew from the very beginning that reprograming of somatic cells does not directly solve any genetic disease a patient may suffer. Possibly after reprograming, but before cells, tissues or organs can be produced from hiPSCs a stage of Gene Therapy must be performed to correct the genetic mutation/s that is/are responsible for disease/s.

To obtain hiPSCs epigenetically "erased", several protocols are in place to bring them to so called "naïve" state[41,42]. Those naïve hiPSCs seem to have a more stable differentiation potential. Based on their lower levels of genomic methylation, naïve hiPSCs are even easier to genome editing than the other more committed hPSC lines[43,44,45]. Please see also **I.4.13.**

I.1.4. - HUMAN CANCER STEM CELLS.

Authors claim that in cancer, malignancy may be related to the presence of one or several clones of Malignant Stem Cells, responsible for perpetuation of tumors, relapses, and metastasis[22,46,47,48]. No one knows for sure if those Cancer Stem cells represent remnants from embryonic or fetal development, or if they acquired this "cancer stemness", later by various environmental influences[49].

I.1.5. - HUMAN SOMATIC ADULT STEM CELLS

Inside any adult tissue or organ is possible to find cells that have the capability to reconstitute or at least repair tissue or organ from normal wear and tear, various types of aggressions, or disease/s. Referred populations of Adult Stem cells are usually quiescent (not in division but prepared to initiate it), being only activated when appropriate stimulus or stimuli become present. A characteristic presented by these Adult Stem cells is that they have a higher-than-expected aneuploidy, mainly polyploidy[50,51,52].

I.1.5.1. - HUMAN MULTIPOTENT STEM CELLS

Hematopoietic Progenitor and Stem Cells (HPSCs), Mesenchymal Stem Cells[53], and Bulge Region Stem Cells[99] are examples of multipotent stem cells. They have the capability to self-renew and to differentiate into cells of many lineages. For instance, HPSCs have the capability to differentiate into the red blood cell (erythroblast), lymphoid, myeloid and megakaryoblast lineages. Mesenchymal Stem Cells (MSCs) may give rise to adipose tissue as well as bone, cartilage, and others. Bulge Region stem cells are important in the provision for cells to regenerate skin structures[54].

I.1.5.2. - HUMAN OLIGOPOTENT STEM CELLS

As an example, Myeloid Stem Cells (Myeloblasts) are considered oligopotent stem cells in the sense that they develop many lineages but only inside the myeloid trunk: neutrophils, macrophages, monocytes, eosinophils, mastocytes, basophils. Lymphoid Stem Cells (Lymphoblasts) are oligopotent in the sense they are responsible for the creation of T and B lymphocytes.

I.1.5.3. - HUMAN UNIPOTENT STEM CELLS

Unipotent stem cells give rise to cells of their own type, but only along a unique lineage. Epidermal Stem Cells[55], that give rise to keratinocytes as well as Hair follicle stem cells, both derived from the Bulge Region multipotent stem cells[54], are examples of unipotent stem cells. Satellite Stem Cells are the unipotent muscle stem cells, responsible for muscle functioning and regeneration[56]. Oocyte Progenitors/oogonia Stem Cells are another example of one however paradoxically haploid unipotent stem cell which is preparing to become a Totipotent Stem Cell[57,58]by fertilization. The complementary counterpart of Oocyte Progenitor/oogonia stem cells are Sperm Stem Cells, the other typeof haploid unipotent Stem Cell aiming for totipotency by fertilization[59].

I.2. - HUMAN CELL THERAPIES AND REGENERATIVE MEDICINE

A Cell Therapy is a medicinal product containing cells, that is transplanted to a patient, nowadays, prevalently by endo-venous injection as is the case of HPSC transplantation, and of transfusions of Red Blood Cell concentrates, and of Platelet concentrates. It can be originated from other human being (allo-transplant) or from the same human being that is transplanted (auto-transplant).

In European Medicaments Agency (EMA) definition, " *Advanced Therapy Medicinal Products (ATMPs) are medicines for human use that are based on genes, tissues or cells. They offer groundbreaking new opportunities for the treatment of disease and injury*". (Advanced ATMPs - European Commission DG Health and Food Safety and European Medicines Agency Action Plan on ATMPs) by the work of Eichler H.G. *et al*. (2021)[60]. That is why most new Cell Therapies may be considered as ATPMs.

The most prevalently used and forgotten to mention Cell Therapy are Red Blood Cell and Platelet concentrates for transfusions, saving thousands of patients daily all over the world. They are so well established for so long that we are not considering them as ATMPs anymore. Other examples are HPSCs and Mesenchymal Stem Cell-based Therapies. However, there are other proposals like: Cells producing anti-hemophiliac Factor VIII and Factor IX respectively for treatment of Hemophilia A (A Phase 1/2 Open-Label, Dose-Escalation, Safety, Tolerability, and Efficacy Study of SIG-001 in Adult Patients with Severe or Moderately-Severe Hemophilia A Without Inhibitors (SIG-001-121) and of Hemophilia B, or Pancreatic β-Cells for production of insulin to treat diabetes[61]. In those examples, so far, the cell product needs to be encapsulated aiming to separate allogeneic cellular product from host's immune system. By this technology what is intended is to make impossible for transplanted cells to be rejected, or at least to diminished or delay rejection[61].

More recently, Autologous or Allogeneic T-Cell-based therapies have been developed for cancer therapy, mainly hematologic cancers, like leukemias, lymphomas, and Multiple Myelomas. Use of Gene Therapy tools helped creation of modified and expanded T Cell pools, known as Chimeric Antigen Receptor (CAR) T-Cells, able to recognize and induce apoptosis of cancer cells hopefully expressing specific new non-self-antigens, that can be recognized by the new CAR presented by the modified T-Cells[62].

Despite great interest and enormous promise raised by Cell Therapies, there are at least three factors hindering faster development improvements.

Those factors are:

I.2.1. - Manufacturing challenges.

On the opposite to chemical molecules that can be produced, stored, and distributed in accordance with long-lasting manufacturing standards, cells are much more complex to standardize in all aspects one can consider.

I.2.2. - Affordability.

Cell therapies, by the manufacturing constrains involved and many other aspects, are still awfully expensive to manufacture, to comply with the Regulatory Authorities' requirements, from cGMP manufacturing to the transplantation day and beyond, and all the way down related to transportation, storage, and infusion or transplantation into patients. However, depending on the disease and transplantation efficacy, they may be economically and medically competitive since the very first day if compared to drug-based therapeutics.

As cells "know" better than regularly taken pills or injections, how to regulate molecule levels a patient is in need, and are much "portable" than devices, they have inherent relevant advantages when compared to medications. One can expect better disease control, and in some examples, long-term best cost-efficiency relationship in comparison to drug-based treatments.

I.2.3. - Compliance with Regulatory Authorities requirements.

Evolution in ATMPs have been grounded by Cell Therapy manufacturers and Regulatory Authorities, in favor of the highest interests of patients. Balancing risks and benefits have not been easy. Maximum safety and efficacy are on the interest of all parts involved[63].

I.2.4. - Other Issues in Cell Therapies.

For long time now, Human Stem Cells by their inherent qualities of stemness, are considered a relevant starting/raw material to be useful in Cell Therapies and Regenerative Medicine[109]. Any stem cell embodies the potential for a curative or at least a highly efficacious relieving Cell Therapy. However, for any Cell Therapy to be developed, commercial factors that include for major reason Investors and many others, enter the equation. Investors will not be interested in developing a system that will not guarantee reimbursements. This may trigger a vicious cycle, created by lack of any possibility for investments to be protected, for instance by Patents, turning Investors' choice to not apply and Patients to not get saved. Paradoxically, legislation that is put forward trying to protect human embryos, even those that already a decision was made not to be implanted and to be discarded, may be impairing millions of other human beings to be saved from, in many ways, horrific sufferings!

Today, use of totipotent human cells, both for research and for Cell Therapies, are not allowed, based on the erroneous assumption that pluripotent stem cells only can be obtained by human embryo destruction (*stricto sensu*). Also based on the same argument, patenting in hESCs, (an example of human Pluripotent Stem Cells), is also not possible

in many jurisdictions if cells were obtained resulting from destruction of human embryos[64]. There are attempts to overcome these prohibitions, as examples exist that human Embryonic Stem Cells may be obtained without destruction of human embryos[25,66,67,68]. One example could be totipotent cells and their derived hESCs obtained by parthenogenesis. Arguments come from non-human primate parthenotes that may exhibit 64% heterozygosity levels in the parthenote Embryonic Stem Cells (pESCs), with the 36% of remaining homozygosity being represented by near centromeric and near telomeric genes[54]. However, by parthenogenesis, homozygosity in pESCs will always be present. Homozygosity is not a desirable condition for Cell Therapies and Regenerative Medicine[54]. For example, a human male parthenote was described: "*The patient was diagnosed with mild developmental abnormalities, hemifacial microsomy, and signs of sex reversal. Genetic analysis detected chimerism in skin fibroblasts and peripheral blood leukocytes consisting of normal biparental (46,XY) and parthenogenetic (46,XX) cells*"[65]. Human pESCs may be useful to prove that the arguments for hESCs non-patentability may be challenged and prove that totipotent cells may also be created under a non-embryonic canonical (*stricto sensu*) environment. Both possibilities (parthenotes and clones), possibly being considered falling outside the arguments carried to the jurisdictional foundation of present legal prohibitions, that considers a human embryo as the result of human fertilization (*stricto senso*), may be useful to legally oppose them. However, in the Clinic, parthenote inherent homozygosity or at least, mosaic 36% of remnant homozygosity does not contribute for a safe use in Cell Therapies or Regenerative Medicine. That is why, in practice, artificially produced Human parthenotes, although never dependent for their creation on human embryos destruction, are not helpful both for their present specific patentability issues neither for Cell Therapies, but only for philosophical arguing against patentability prohibitions in the field of hESCs. And related to human clones, as it is not allowed, we also cannot rely on this argument. Nevertheless, in various publications different authors demonstrated that several pathways may be followed that could enable the creation of Human Pluripotent hESC lines without destruction of human embryos[25,66,67,68]. The real problem is to scientifically and legally prove that a hESC line is derived from one specific human embryo, that later gave rise to a healthy human being. This kind of experiment will not be allowed, because there are plenty of possibilities to create one human being without incurring in such level of risk. Today every existing hESC lines, both for research and/or cGMP-for-Cell Therapy, where produced from IVF surplus human embryos that were

sacrificed on the process. Not one single line of hESCs is available from a staying-alive human embryo, even if this is claimed technically possible! Could such a hESC line overcome the legal and moral restrictions are raised for its patentability? Probably not, because as it correctly happens in transfusion, HSPC, or solid organ transplantation, no commercialization is allowed. And this behavior is grounded in well stablished safety principles to defend both donors and the patients. However, anywhere those materials are processed, from collection until the transplantation, a price is calculated based on the overall technical procedure costs. On this basis one must reason, (because in real life it always happens), that any manufacturing procedures hESCs may be submitted to allow for the creation of a transplantable Cell Therapy, could have a price calculated. This being the *modus faciendis*, any patented process that would be necessary to apply to hESCs to allow for the creation/manufacturing of a Cell Therapy, could and should be included in the overall price of the final transplantable product. How those arguments could fit with the jurisdictional arguments that hESCs are not candidates for patentability, will be a material for discussion. However, hiPSCs are patentable on the basis that they are produced without human embryos' death. If hESC lines in the future can be also produced without embryos' death, could them become patentable?

This is a relevant field in medicine, and as soon as a solution for HLA-mismatch could be created, like the one we propose here, more and more pressure will be put on the legislators' shoulders to allow for hESC lines patentability. Mainly because huge investments are needed for hESC line creation, and no Investor will be interested in financing any project without having fair reimbursement expectation, this will ultimately impair patients' treatment. This vicious cycle, in some wise way must be broken, if extended development in Cell Therapies is to be created as is widely recognized as needed.

Cell Therapy and Regenerative Medicine solutions based on human Pluripotent Stem Cells (hPSCs) and in Human Multipotent Stem Cells have been created[70,71].Theoretically, for Cell Therapy and Regenerative Medicine applications in humans, the wider the Stem Cell Line potency the better. The wider the potency of the considered Stem Cell line the larger scope of Cell Therapies will be available for that Stem Cell Line,because the wider the potency the larger the diversity of different cells, tissues or organscan be differentiated from. But this extended stemness do not have only advantages, as the broader the potency the larger the capability to develop teratomas and eventually

teratocarcinomas. Human Pluripotent Stem Cells are the ones having the higher propensity to be implicated in triggering teratoma *in vivo*[71, 72,73,74,75,76,77,78].
Nonetheless, teratoma formation was also described in human Mesenchymal Stem Cells (MSCs) derived products[79]. And MSCs are considered Multipotent Stem Cells, mostly being assumed they are not prone to the creation of teratomas.
Several brain tumors, composed of oligodendrocytes or oligodendrocyte-like cells, were originated on a transplant treatment for Telangiectasia Ataxia based on neuro Stem Cells[80].
Those are relevant problems to be solved simultaneously and with the same level of urgency as compared with other also no lower challenging issues.

I.2.5. – Clinical Trials and hPSCs.

Two different but apparently equivalent opportunities are raised in choosing the human Stem Cell basis to create Cell Therapy products: hESCs and hiPSCs[81].

Presently (January/2021), and comparing clinical trials based on products derived from hESCs and hiPSCs:

1. hESCs - 34 Clinical trials registered, mostly for Macular Degeneration/RPE (19-01-2021_ ClinicalTrials.gov)

2. hiPSCs - 5 clinical trials registered (19-01-2021_ClinicalTrials.gov)

hESCs are thus found as the cell basis for most of those Clinical Trials. It seems that they are the safest cells to work with in preparation for transplants, otherwise, manufacturers' choice would not reflect such a remarkable difference in the number of referred Clinical Trials.

hiPSCs are mostly viewed as potential allogeneic transplantable products. Since their major advantage, (auto-HLA-match), is annihilated in the allogeneic context and as they seem to have reduced safety as compared to hESCs, the option in search for Cell Therapies inclines toward hESCs.

Since the first isolation steps by Thomson J.A. & Odorico J.S.(2000)[82], at WiCellInstitute. Near 2000 hESC lines are believed to be isolated with 1000 registered worldwide. The NIH Human Embryonic Stem Cell Registry, includes 486 hESCs lines,

both healthy and disease-specific Cell Lines, since 2009 to 21-February-2022, https://grants.nih.gov/stem_cells/registry/current.htm.

It could seem quite easy to find the ideal ones to be the foundation of all our work. Within such a plethora of hESC lines, search for the creation of new hESC lines and consequent human embryos sacrifice, would be unnecessary[83].

I.3. Manufacturing of hPSCs derivatives for Regenerative Medicine

I.3.1. – Cell Culture in Serum-, Feeder-, Xeno-Free conditions.

Developments in complete serum-free and feeder-free Stem Cell culture, open the doors to chromosome 6-replaced Stem Cells to be expanded and differentiated within Regulatory Authorities' requirements for clinical grade cells, produced under "strict"/current Good Manufacture Product (cGMP) conditions, to be used in Cell Therapy and Regenerative Medicine[84,85].

I.3.2. – Cell Culture in Human- or CHO-Feeder conditions.

Other hypothesis is to grow hPSCs over a feeder layer of human primary cells like foreskin human fibroblasts[86], human amniotic cells[87], or human Cord Blood MSCs[88]. They do not have to comply with xeno-related viral and other xeno-related concerns. However, must be human viral-free and other human pathogens free. Another major possible concern from Regulatory Authorities is related to every cell-based feeder layer that never have constant and reproducible composition. Those concerns are very well presented in Regulatory Authorities' specifications. (EudraLEX, EMA Guidelines for Cell Therapies, https://ec.europa.eu/health/documents/eudralex_pt)

Chinese Hamster Ovarian Cells (CHO) are used for long time as cells approved by Regulatory Authorities for several human recombinant protein therapeutics[89,90]. And this may ease Regulatory Authorities' approval as feeder layer in human Pluripotent Stem Cell expansion, and differentiation protocols, or for only brief culture periods like for the first steps in single-cell clone expansion that are hard to succeed in feeder-free cell culture of human Stem Cells.

Mainly for clone creation and expansion, feeder layers may be helpful[91]. Again, Regulatory Authorities will raise concerns and high standard safety proceedings must be placed for their approval. In Cell Therapy webinars unanimous recommendations are

issued for Regulatory Authorities advice and if possible, protocol approval(s) prior to any investments be made in any step of Cell Therapies.

Other authors were successfully using **human amniotic fluid** as media for hPSCs culture[92]. However, again, compliance with Regulatory Authorities recommendations is mandatory, otherwise it will be only a waste of time, money, and human lives.

I.3.3. – DNA transfer and deletion techniques in Human Cells.

I.3.3.1. - Cell transfection/transduction (aims and techniques).

Introduction of "construct" inside a cell is designated as "transfection" if DNA is from plasmid origin or "transduction" when "construct" is included in a viral vector. For precise insertions, transfection methods are preferred. That is because in transduction, mainly if provided using Retro/lentiviruses, even if apparently much more efficient, usually introduces "insert" in many places in the genome other than in the correct intended place(s) for precision knock-ins.

For transfection there are available several main techniques:

Lipofection[93], **Electroporation**[94], **Ultrasound-based sonoporation**[94], **Magnetic field-based magnetoporation**[94], **Optoporation - Laser-based transfection**[94], **Microfluidic squeezing**[95], **Single-cell microinjection by micropipette**[96,97], **Multi-cell robotic microinjection**[97].

Usually for research objectives, lipofection and electroporation are the most used. However, for hard-to-transfect cell lines, namely human Pluripotent Stem Cell Lines, alternative solutions might be preferable because both electroporation and lipofection present low efficiency and even lower efficacy[98].

In summary **Lipofection** is a transfection technique that uses the double capability of some lipidic compounds to:

1- form vesicles inside of which, nucleic acids, proteins, and other molecules can be harvested, and

2- the ability to bind lipidic components in cell membrane, allowing to the transfer of vesicles' content inside the cells, mostly by endocytosis, hopefully without killing the cell.

In summary **Electroporation** is a transfection technique based on the creation of small pores in the cell membrane by application of sudden appropriate electric currents in cell membrane. Usually for human pluripotent Stem Cells voltages are within 1200mV to

1400mV and for a short period of time of 1 to 2 milliseconds, one or two pulses. This leads to the momentaneous creation of membrane pores that enable molecules to travel through. Molecules can enter and get out of the cell by traveling through the pores and cell membrane will soon recover normality if cell survival threshold is not exceeded.

When comparing transfection by electroporation and by lipofection, authors consider that electroporation is more efficient but more aggressive to the cells, and more prone for cell death. Depending on researcher's experience, cell line, cell culture passage, confluence and other parameters, results may be equivalent. Because the higher transfection efficiency may be lost by the also higher cell death in electroporation, and the lower transfection efficiency in lipofection may be compensated by procedure-related higher cell survival. Those are important parameters researchers must empirically evaluate *vis a vis* the specific objectives to be fulfilled.

I.3.3.2.- Large DNA content transfer.

I.3.3.2.1. - Microcell-Mediated Chromosome Transfer (MMCT).

Since 1977, starting by Fournier *et al.* [99,100], and continued until today by many other authors, many contributions have been made to solve a significant issue, by creating a protocol that enables entire chromosomes to be transferred by Microcell Mediated Chromosome Transfer (MMCT)[99,100,101,102,103,104,105,106]. (**Figure 7**).

In MMCT, cells are arrested in metaphase by exposing them to colcemide or nocodazole. After the arrest, cells are submitted to cytochalasin B to disrupt the microtubules in the cell nucleus followed by ultracentrifugation. Ultracentrifugation allows for the disruption of cells and at the same time for the formation of the so called "microcells". Microcells are small vesicles composed by a membrane similar in composition to the cell nucleus membrane and might contain one or a few chromosomes inside. By Ficoll density gradient centrifugation, those microcells are isolated and can be stored in N2 for future use. Chromosome transfer from inside the microcells to cells is obtained by polyethyleneglycol (PEG) cell fusion protocol and other protocols.

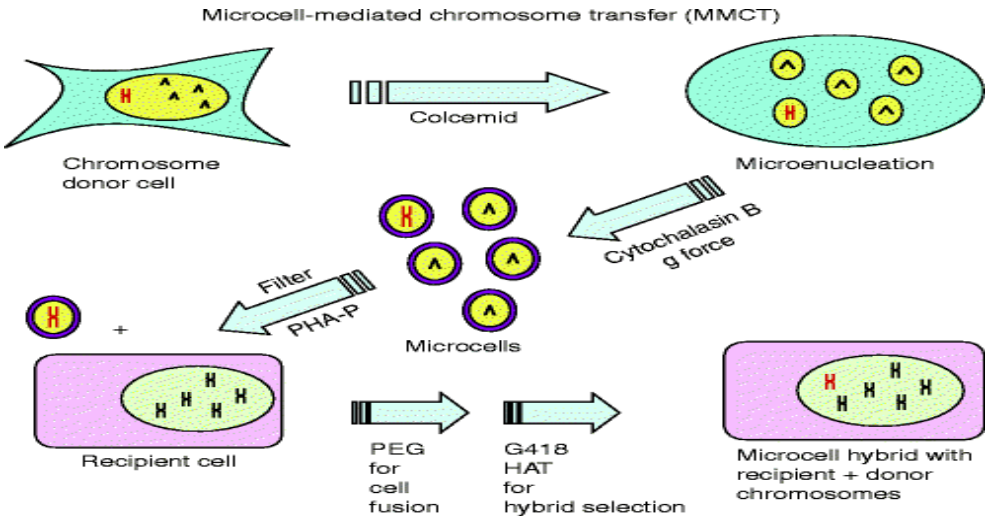


Figure 7 – Microcell-Mediated Chromosome Transfer (MMCT). Lung M.L. (2011)[171]

In: Schwab M. (eds) Encyclopedia of Cancer. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978- 3-642-16483-5_3716.

If entire chromosomes have been successfully transferred by MMCT, could it be possible to replace MHC by entire chromosome 6 pair transfer in humans?

I.3.4. - Tailored Large-DNA deletion.

I.3.4.1. - CRISPR/Cas Nuclease System.

Clustered Regularly Interspace Short Palindromic Repeats (CRISPR) is a nuclease system present in bacteria and archaea, enabling them to fight against phages.

Emmanuelle Charpentier and Jennifer A. Doudna received 2020 Nobel Prize in Chemistry for discovery and development of this important tool for Molecular Biology. However, many others contributed for the birth and are contributing all along the time for the development of this technology. It is fair to mention, Feng Zhang, Broad Institute of MIT and Harvard in Cambridge, Massachusetts; George Church, Harvard Medical School in Boston, Massachusetts; Virginijus Siksnys at Vilnius University in Lithuania; Francisco Mojica at University of Alicante, Spain. They also had and still have relevant contributions in the discovery and development of the technology[107].

CRISPR system is composed of many nuclease types and subtypes. Actual classification (January 2021) includes 2 classes, 6 types and 33 subtypes, compared with 5 types and 16 subtypes in 2015. Of the utmost relevance for development of CRISPR technologies is the ongoing discovery of multiple, novel Class 2 CRISPR–Cas9 systems, which in January 2021 include 3 types and 17 subtypes. Another novelty is the discovery of numerous derived CRISPR–Cas9 variants, often associated with mobile genetic elements

that lack the nucleases required for interference. Some of these variants are involved in RNA-guided transposition, whereas others are predicted to perform functions distinct from adaptive immunity that remain to be characterized experimentally. Third, was the discovery of numerous families of ancillary CRISPR-linked genes, often implicated in signal transduction. In a simplistic way, any CRISPR nuclease relies on three main elements. An enzyme with two active domains, one to cleave target DNA strand (HNH domain), and the other to cleave non-target DNA strand (RuV-C domain)[108]. The enzyme is "conducted" to the cleavage site by an RNA sequence, called single guide RNA (sgRNA). This sgRNA has a "complementary" sequence to the target DNA strand. However, for the CRISPR enzyme to be able to recognize the DNA sequences where to produce the DSBs, a designated Protospacer Associated Motif (PAM) must be present in the non-target DNA strand[108]. Depending on the CRISPR nuclease involved, PAM may have to be located immediately downstream the DNA complementary sequence to the sgRNA (CRISPR/Cas9), or immediately upstream of the referred sequence (CRISPR/Cpf1)[110]. But with such important new discoveries happening, it would not be a surprise if sometime soon a discovery is made of a CRISPR nuclease that could be dependent on a PAM that must be included inside the protospacer.

Moreover, several CRISPR new nucleases have been synthesized improving the natural qualities that wild type CRISPR nucleases present. One example is the creation of a High Fidelity CRISPR/Cas9[109].

With only 8 years in development (2013-2020), CRISPR technology, mainly CRISPR/Cas9, is already the most used nuclease technology for gene editing in academic research[154].

For large deletions of DNA CRISPR-Cas9 is an available tool[112,113,114]. As CRISPR/Cas9 is a friendly-to-use nuclease system among the several available, it was our choice to use it in our experiments.

I.3.4.2. – Other Editing Nucleases

Before CRISPR discovery and development, Molecular Biology already had important and precise tools to perform DSBs in genome DNA. Examples are Zinc-Finger nucleases (ZFN), Transcription Activator-Like Effector Nucleases (TALEN)[115], and Meganucleases[116].

ZFNs are obtained by fusion of several proteins (Zinc-Fingers), each one able to recognize a triplet of DNA bases, then binding the set to the nuclease domain of FokI nuclease. Must use as much Zinc-Finger proteins as multiples of 3 bases we have in the

DNA sequence for the ZFN system to bind and cleave. The many the Zinc-Finger proteins, the larger the DNA it will bind and the more precise will be the DSB produced by FokI. Exceptions are the very highly repetitive sequences mainly in telomeres and in centromeres.

TALENs are obtained by the fusion of appropriate 33-34 repetitions of TAL proteins with great affinity for specific nucleotides, associated to FokI nuclease dimeric domain.

With ZFNs and TALENs is possible to obtain very precise DSBs in DNA.

However, ZFNs and TALENs are large molecular structures, expensive, difficult, and time-consuming to synthesize[115].

Other approaches, based on the use of Transposases, promise to represent a real improvement in precise genome editing in comparison to CRISPR/Cas single systems[118,119,120].

More recently a Meganuclease-based system was published, it is based on tailoring of a algae Meganuclease ICreI and commercialized by Precision Biosciences®, named as ARCUS. "*The ARCUS gene-editing platform, developed by scientists at Precision BioSciences, is based on the homing endonuclease I-CreI, which comes from the Chlamydomonas reinhardtii chloroplast genome, and is part of the LAGLIDADG motif meganuclease family*"[121]. A remarkable feature of this artificially modified/tailored system is its capability to home to a (23bp) wider DNA site as compared to CRISPR-Cas9, but even better, can discriminate DNA sites that are only one base different, as well as promotes hangover cleavages in dsDNA instead of blunt as is the case with CRISPR-Cas9. Those two characteristics can improve specificity of DNA DSBs and HDR up to 49,6%, (https://precisionbiosciences.com as reassessed on 27th March 2022).

Transcription Activator-Like Effector Nucleases (TALENs) also are available to obtain large DNA deletions[122] as well as Zinc-finger nucleases (ZNFs)[123].

I.3.5. – Entire Chromosome deletion in Human Cells.

I.3.5.1. - Chromosome Loss by Cre-LoxP inverted editing.

In 1997, Lewandoski M. & Martin G.R.[124] described a protocol based on a Cre-LoxP inverted system to induce entire chromosome deletions. In 2007, Matsumura H. *et al.*[125] published the targeted chromosome elimination from ES-Somatic hybrid cells based on

the Lewandoski H. *et al.*[124] protocol, and same approach was used to trigger chromosome deletion in mice embryonic stem cells, published by Tada M. *et al*. in 2009[126].

Another proposal was filled for a patent in 2006 in Japan by T. Tada *et al*., to induce Chromosome 6 loss in human cells by an inverted Cre-LoxP-based strategy and simultaneous replacement by the patient's chromosome 6 pair in a patented protocol.

Patent: **US 2009/0264312 A1**. T. Tada *et al.* (43) Pub. Date: Oct. 22, 2009

Patent: **METHOD FOR REMOVING DESIRED CHROMOSOME AND TAILOR-MADE MEDICAL TREATMENT UTILIZING THE SAME**, Japanese applicant, Publication Info: JPWO2006075671 (A) 2008-06-12

This inverted Cre-LoxP-based chromosome loss method induces chromosome deletion by the formation of dicentric chromosomes. Those dicentric chromosomes do not have a normal mitosis behavior and in many mitotic cells will trigger cell death, or chromotripsis, or kataegis[127,128,129]. Chromotripsis, a chaotic event in mitosis that results in tens or hundreds of genomic rearrangements[130,131], would be the origin for mutations and transpositions in cells that could survive and is a pervasive condition in 2-3% of cancers but may grow up to 50% in several specific cancers[132]. Kataegis, is a hypermutation genomic state in a localized region of the genome[128,129]. Being not so widely spread all over the genome, the consequences for Cell Therapy are exactly the same, as increase on genomic mutations is not compatible with the requirements for safety in transplantation.

This condition, (chromosome loss based on the creation of dicentric chromosomes), may prevent Regulatory Authorities' approval based on the risk that cells in transplants may have serious mutations, both from the very beginning of transplant creation, as well as during transplant's lifetime.

The justification is based on the arguments and experiments of many authors revealing that the creation of dicentric chromosomes induces high levels of chromotripsis and kataegis[127,128,129], with "*enhanced in vivo tumorigenic potential*"[129]. (**Figures 8 and 9**).

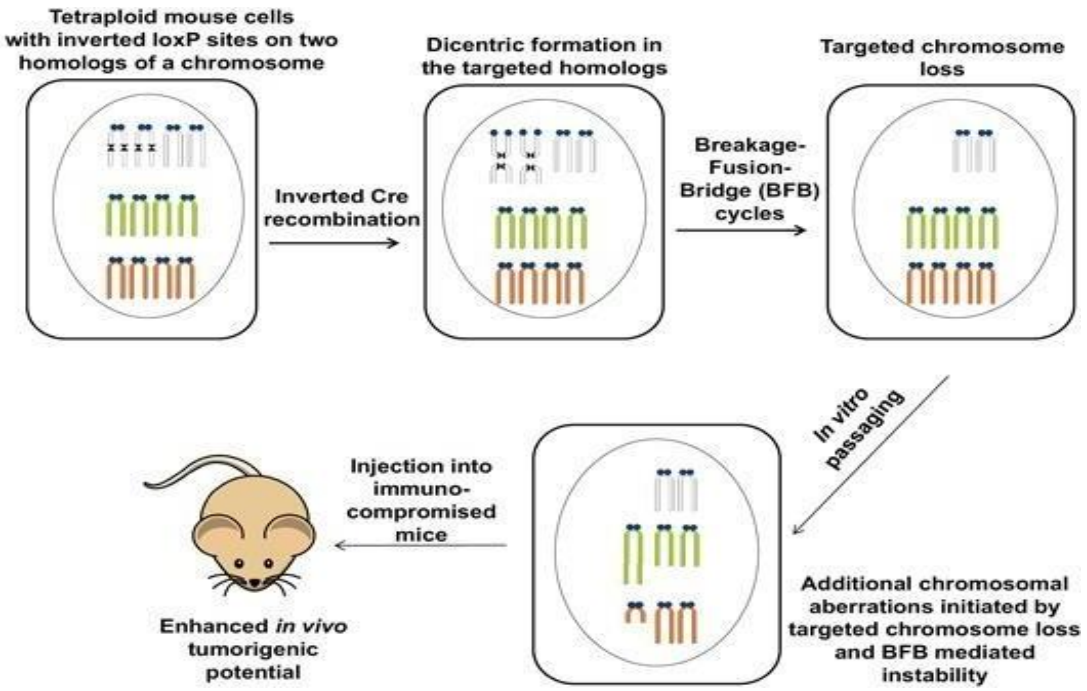


Figure 8 – Chromosome loss by Cre-LoxP dicentric chromosome induction protocol. Notice the Breakage-Fusion Bridge (BFB) cycles promoted by dicentric chromosomes triggering chromosomal aberrations and genomic instability in surviving cells, and "*enhancing in vivo tumorigenic potential*" Thomas R. *et al*. (2018)[129]. Creative Commons-BY-NC- article.

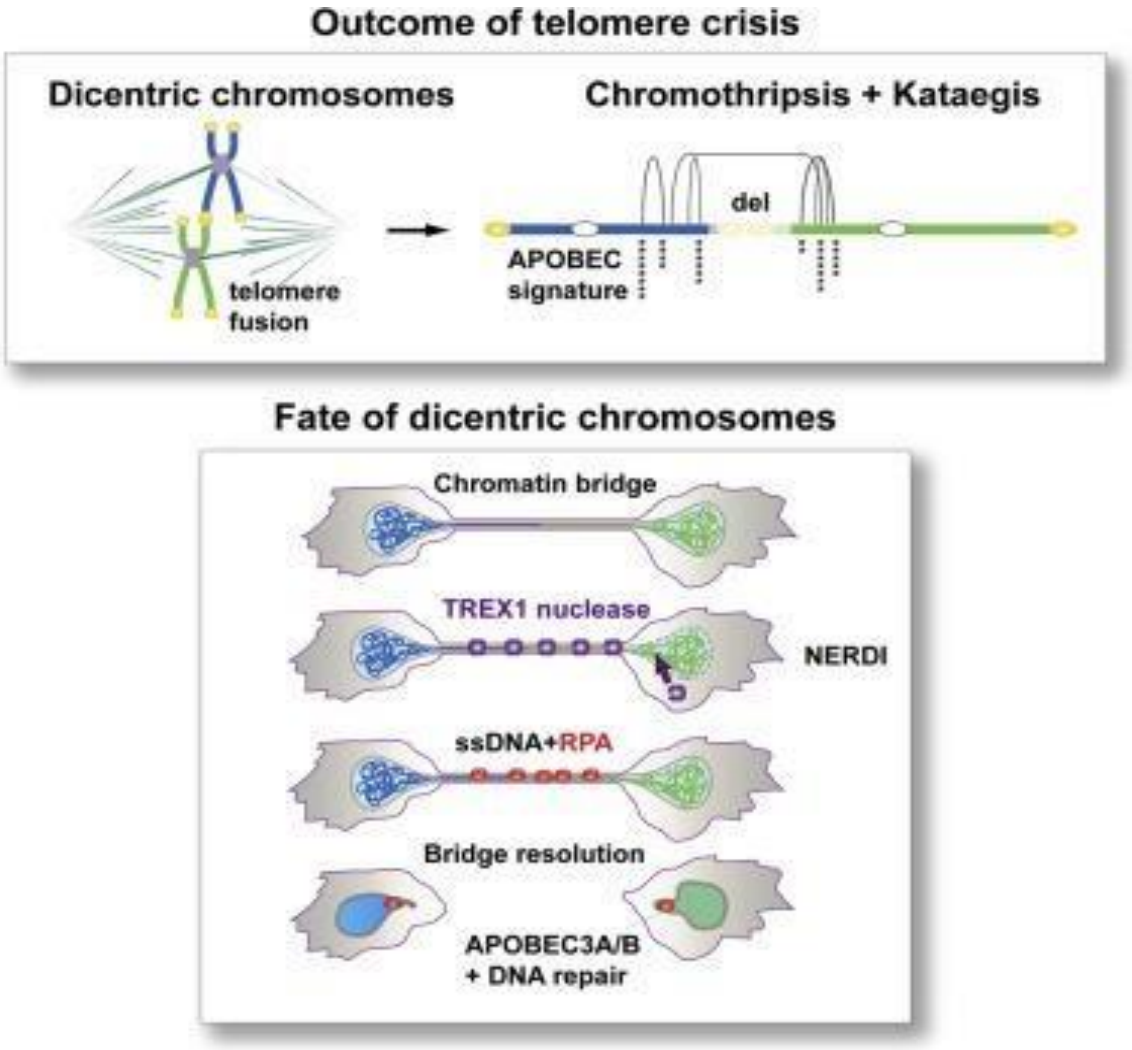


Figure 9 - Dicentric chromosomes are prone for Chromothripsis and Kataegis. Chromothripsis and Kataegis Induced by Telomere Crisis, Maciejowski J. *et al.* (2015)[127]. (Written permission was obtained from the authors and publishers: RightsLink ® License nr: 5033160339918, date: Mar 20, 2021).

I.3.5.2. – Chromosome deletion by CRISPR/Cas9 nuclease System.

In 2017, two different teams, Adikusuma F. *et al.* (2017)[133] in Australia (**Figure 10**) and Zuo E. *et al*. (2017)[134] in China, published a method in which CRISPR/Cas9 is used to promote an entire human Y chromosome deletion, or human 21 chromosome deletion.

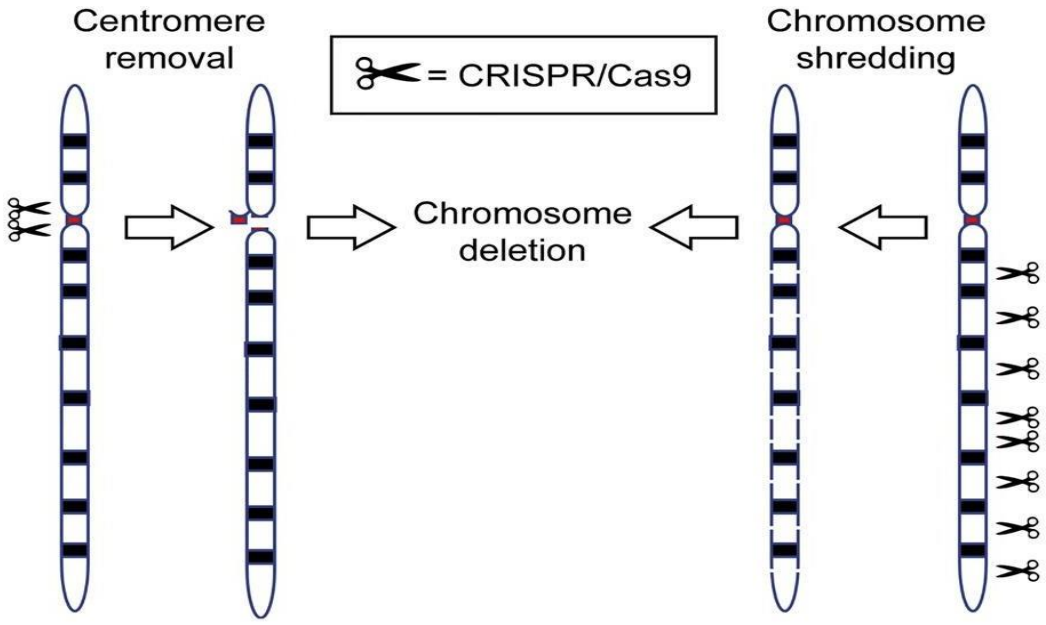


Figure 10 – Different strategies to induce specific chromosome deletion by CRISPR/Cas9. Centromere removal by DSBs flanking the centromere (left in the figure), and by shredding the chromosome by CRISPR/Cas9 cleavage of several repeated DNA clusters inside the chromosome (right in the figure), also possible by centromere-DNA shredding (not represented). Adikusuma F. *et al.* (2017)[133]. Authors' written permission to reproduce this figure was obtained.

Both authors describe several methods enabling to the deletion of entire chromosomes in human cells. In short, chromosome deletion can be achieved by CRISPR/Cas9 if one of the following protocols is used:

1- the sgRNAs are complementary to DNA sequences positioned flanking chromosome centromere. Centromere excision will disable chromosome segregation and induces Chr. loss.

2- the sgRNAs are complementary to repeated DNA sequences inside the centromere. Centromere destruction will disable chromosome segregation and induces Chr. deletion.

3- the sgRNAs are complementary to repeated DNA sequences all over the chromosome. CRISPR/Cas9 will shred the chromosome. A Chr. turned into pieces by several DSBs will be lost.

I.4. - Presently available strategies to improve allo-transplantation.

Authors are trying hard to put into place several strategies that could at least lower or at best overcome rejection in allo-transplantation. Some examples are:

I.4.1. - Allo-transplantation between Identical Twins and in 25% of Siblings with similar HLA.

As identical twins, and 25% of siblings, have remarkably similar HLA expression on their cells, rejection syndromes usually do not have significant expression. In transplants performed between identical twins or in 25% of siblings only in rare occasions immunosuppression is required, for kidney transplantation[208] or in Bone Marrow transplantation[135,136,137].

I.4.2. - Immunosuppression in allo-transplantation.

Immunosuppression is world-wide the main strategy used to "control"/reduce the impact of transplant rejections. Several protocols, based on the transplanted cell type or organ, age of the host, his or her immunological state and severity of the rejection syndromes, are approved all over the world to try to avoid the side-effects of rejections. However, immunosuppression on itself is accompanied by serious complications that in many cases is the main factor for patient's death[138,139].

I.4.3. - By Knock-out or deletion of HLA Class I.

I.4.3.1. - Knock-out of β2-microglobulin.

Some authors proposed to abolish the expression of HLA Class I by **knock-out of β2-microglobulin**, a gene encoding a protein essential for HLA Class I assembly and stabilization, that is expressed from human chromosome 15[140].

Cells without **β2-microglobulin** will not be able, in principle, to have a proper HLA at their surface and will therefore be non-immunogenic. However, knock-out of β2-microglobulin still allows for the synthesis and transport toward the cell membrane of incomplete HLA receptors Class I. These incomplete receptors include mutations/mismatch compared with the ones present in the host, and they still will trigger rejection. In addition, any cell that does not express HLA is killed by NK cells[140] and this will create an additional problem for transplantation efficacy.

Examples of Patents filled based on this approach:

TARGETED DISRUPTION OF MHC CELL RECEPTOR, SANGAMO THERAPEUTICS INC [US], Publication info:KR20180088911 (A)

CELLS LACKING B2M SURFACE EXPRESSION AND METHODS FOR ALLOGENEIC ADMINISTRATION OF SUCH CELLS, HARVARD COLLEGE [US], Publication info: US2018141992 (A1)

PREPARATION METHOD FOR IPS CELL HAVING LOW IMMUNOGENICITY AND CAPABLE OF REALIZING APOPTOSIS UNDER INDUCTION, BEIJING ALLIFE MEDICINE TECH CO LTD, Publication Info: CN108998419 (A)

I.4.3.2. - HLA Class I deletion by CRISPR/Cas9.

Other authors proposed HLA Class I deletion by CRISPR/Cas9, to avoid any possibility of the host immune system to recognize and reject those cells that are expressing new-to-the-host (non-Self) allogeneic antigens when transplanted[112].

In these cells, β2-microglobulin is still present at the cell membrane but it will not be recognized as a new antigen by the host's immunological system. However, the absence

of the other components of HLA Class I receptors on the cell membrane[140] will trigger rejections at least by Natural Killer (NK) cells.

An example of a Patent filled based on this concept:

CRISPR/CAS-RELATED METHODS AND COMPOSITIONS FOR IMPROVING TRANSPLANTATION, EDITAS MEDICINE INC [US] PUBLICATION INFO: KR20180031671 (A)

Those hypotheses, based on β2-microglobulin knock-out or on CRISPR-based HLA deletion, would be hardly compliant with the Regulatory Authorities' requirements, because those cells will be devoid of any capability to alert host's immunological system of any viral, bacterial, fungal, or parasite infection or infestation or/and cancer transformation[141]. Conversely, any HPSC transplant based on these proposals will be completely useless because HLA expression is the very basis of immunological system functioning.

I.4.4. Overexpression of HLA G in Transplanted Cells.

As the overexpression of HLA G in the placenta is one of the mechanisms by which fetal tolerance by the maternal immunological system is achieved, some authors proposed the overexpression of HLA G in allo-transplanted cells to trigger host's tolerance, by inhibiting NK cells. Mainly, in addition to the proposal for specific HLA Class I Knock-out, that in it-self fails because triggers NK cell killing. By the artificial overexpression of HLA G in transplants, supposedly it will be possible to diminish rejection.

However, in many tumors, this is also one of the mechanisms they use to evade patient's immunological system[142,143,144,145,146,147,148]. (**Figure 11**). Transplanted cells with HLA G overexpression that could get cancer transformation would be hardly recognized and killed by host's NK cells. This would have tragic consequences. Those proposals will not comply with the safety recommendations from Regulatory Authorities.

HLA G-based Patent: HLA G-MODIFIED CELLS AND METHODS, ESCAPE THERAPEUTICS INC [US], Publication info: KR20180128096 (A)

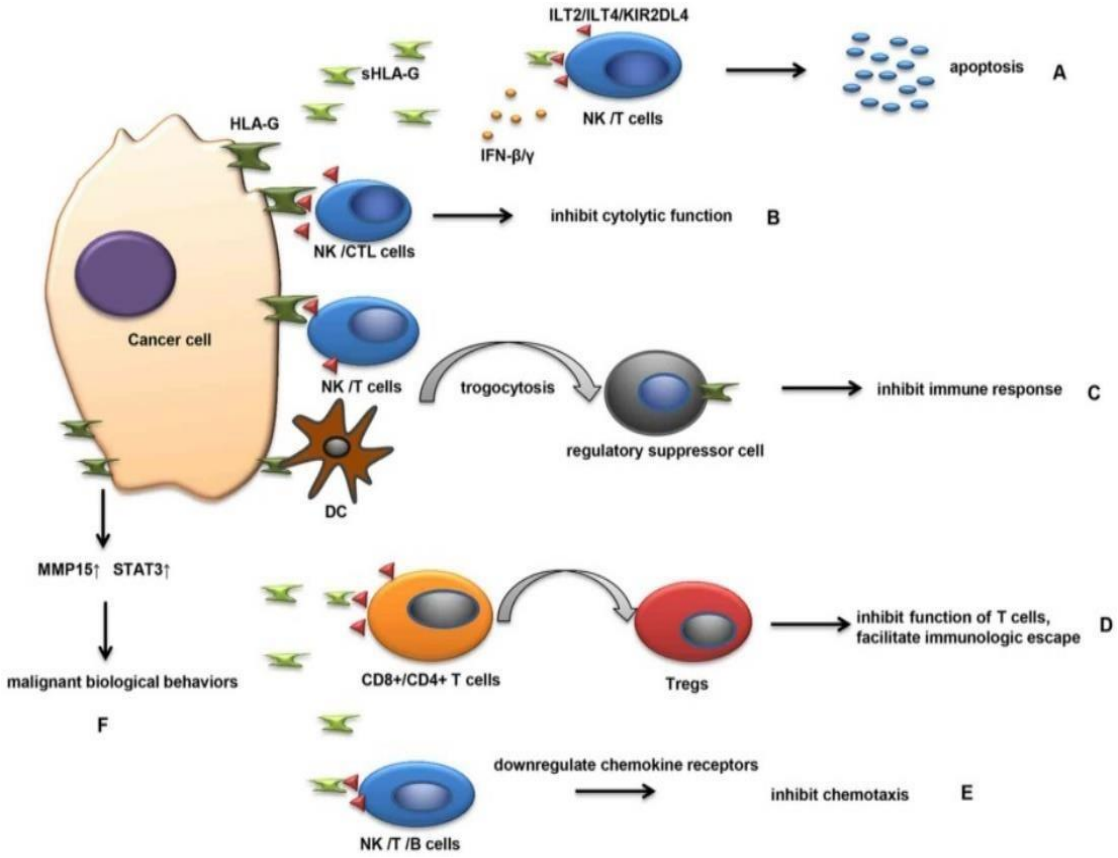


Figure 11 –HLA G overexpression on tumor cells may lead to evasion of NK cells-based elimination, as well as by triggering suppression of the immunological activity on B and T cells and tumor tolerance. Zhang Y. *et al.* (2018)[147]. Creative Commons.

I.4.5. - HLA Class II Knockout.

The solutions to evade allo-transplant rejection that included HLA Class I knockout, and overexpression of HLA G, failed, because NK cell surveillance is not 100% eliminated and still exists lethal surveillance based on T Cells, B Cells, Dendritic Cells and Macrophages. That is one of the reasons some authors performed in hESCs in addition to the HLA Class I knock-out and HLA G overexpression editing, knock-out of the HLA Class II Transactivator (ClassIITA). They try to remediate the referred issues related to the lack of tolerance, that hampers the safe use of those cells in transplantation[149]. At least, this approach annihilates the hypothesis for HPSC transplants to be manufactured because the leukocytes and lymphocytes will be deprived of proper immunological capability by not expressing any HLA receptors. Conversely, for tissue and solid organ transplantation, the absence of HLA receptors devoid the cells from any capability to inform the host's immunological system if a viral infection or a cancer transformation ofthe transplanted cells happen. This is very dangerous for host's survival. And at least

macrophages will be able to kill the transplanted cells, in addition for the NK cells and others. For that, some authors added the overexpression of CD47. Please see **I.4.6.**

I.4.6. - HLA Class I Knockout + Overexpression of HLA G + HLA Class II Knockout + Overexpression of CD47.

As argued in points **II.4.3.**, **II.4.4.**, and **II.4.5.**, such an immunological evasive system, as it was described above, is not only HPSC useless, but also solid organ dangerous. However, as a transplant in which the cells with no HLA Class I, no HLA Class II and overexpression of HLA-G will still be killed by the host macrophages, authors proposed an additional overexpression of CD47 receptor (non-eat-me receptor) in the transplanted cells, to diminish their phagocytosis by macrophages[149]. Such a proposal will not comply with the safety recommendations Regulatory Authorities require in transplantation.

I.4.7. - Expression of Chimeric Artificial Receptors (CAR) in T regulatory Cells (CAR Treg) for transplant protection.

This specific proposal for the manufacturing of special CAR Treg cells tries to create an approach to treat Diabetes with allo- or auto-pancreatic β-cells. The creation of those specific CAR Treg cells will hopefully induce tolerance in the diabetic patient toward the antigens (insulin, β-Cells, and others), he or she created antibodies against. Those antibodies not being synthesized based on the inhibitory effect of the CAR Treg cells, will hopefully allow for diabetes (mainly Type 1 Diabetes) to be overcome.

This patented approach claims that the inhibition of specific HLA allele-dependent effector T cell clones can be achieved by Chimeric Antigen Receptor(s) being expressed in autologous T regulatory cells (CD4+ Foxp3+). The same reasoning is proposed toward the interference with the activity of T effector cell clones engaged in transplant rejection, (allo-immune rejection). Once the alleles engaged in a specific case of transplant rejection are identified, the authors claim that it would be possible to construct a T regulatory cell-based annihilation of the implicated T effector cell clones. As in those cases of allo-transplant rejection, multiple HLA alleles will be implicated, several CAR T reg clones must be prepared. The consequence could be a broad immunosuppression.

This strategy has the equivalent dangers as those in the strategies based on HLA elimination. The reduction in the number of expressed HLA receptors would reduce the availability of HLA receptor(s) to present antigens, and ultimately would also trigger

transplant rejection not by the HLA present on the cell membrane but by the exact absence of some of them. Additionally, the absence of Self-antigen presentation will trigger a new kind of "auto-immune rejections".

Patent: METHODS TO PROTECT TRANSPLANTED TISSUE FROM REJECTION, UNIV PENNSYLVANIA [US], Publication Info: TW201840845 (A).

"Methods to protect transplanted tissue from rejection

Abstract

The present invention includes compositions and methods for an HLA-A2 specific chimeric antigen receptor (CAR). In certain embodiments the HLA-A2 specific CAR is expressed on a T regulatory cell. In certain embodiments, the HLA-AR specific CAR protects transplanted tissue from rejection."

I.4.8. - Other Treg Cell-based approaches to overcome rejection in allo-transplantation.

Other authors are proposing solutions based on Treg cell development as a method to overcome rejections in allo-transplantation by the induction of a tolerance state in the host.

Riley J.S. *et al.* (2020)[150] (**Figure 12**) tried the induction of tolerance by an *in-utero* approach, setting up the host's immune system for a tolerance state when the immune response is still not matured and new antigens can still be included in the "Self" set of antigens during thymus-dependent T Cell clonal immune development and selection. Depending on the animal involved, the period available before any new antigen will be recognized as "non-Self" will vary. However, it must be in the first weeks of embryo/fetal life, in humans[151]. For any time after early fetal life, this approach is not useful. In today's state-of-the-art, only a few applications may be foreseen. In the future, it may be possible for humans to become tolerant to antigens that could be beneficial or otherwise dangerous to Humanity. Nevertheless, this knowledge is relevant for proposals that rely in *in-uterus* allo-transplantation for rare diseases, that can be diagnosed in the fetus and only will trigger a disease phenotype after birth. Examples may be allo-transplants for Angelman's disease, and urease deficiency diseases.

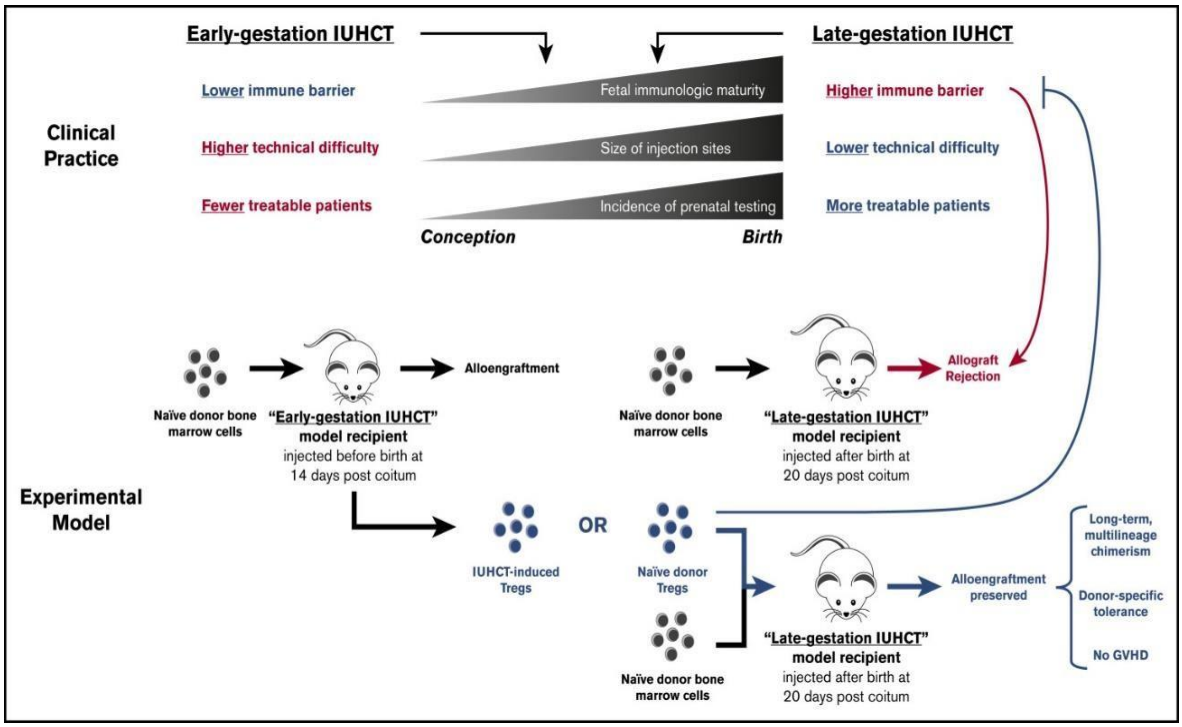


Figure 12 – *In uterus* allogeneic Hematopoietic Cell Transplant (IUHCT). If transplant is performed early, *in-uterus* tolerance toward donor cells may be achieved. After birth, only with the help of donor regulatory T-Cells (T-regs) will specific and low-level rejection possibly be waved. Riley J.S. *et al.* (2020)[150]. Written permission from the authors was obtained to reproduce this Figure.

Odak I. *et al*. (2019)[152] (**Figure 13**) focus was on studying main differences between the populations of $CD4^{+}Foxp3^{+}$ regulatory T cells (Tregs) in High and Low GVHD responding patients 30 days after HPSC allo-transplantation. Authors concluded that in patients with low GVHD presentation (more tolerant) there was a wide diverse set of the T Cell Receptor (TCR) in the isolated bulk $CD4^{+}CD25^{+}CD127^{-}$ Tregs when compared to high GVHD responders. The knowledge how Tregs behave in HPSC transplantation and how to regulate their expansion could be useful to achieve immunological tolerance and avoid Graft-Versus-Host Disease (GVHD) in humans in need for an allogeneic bone marrow transplantation.

This study underlines the relevance of a wide repertoire of Treg cells in lowering GVHD.

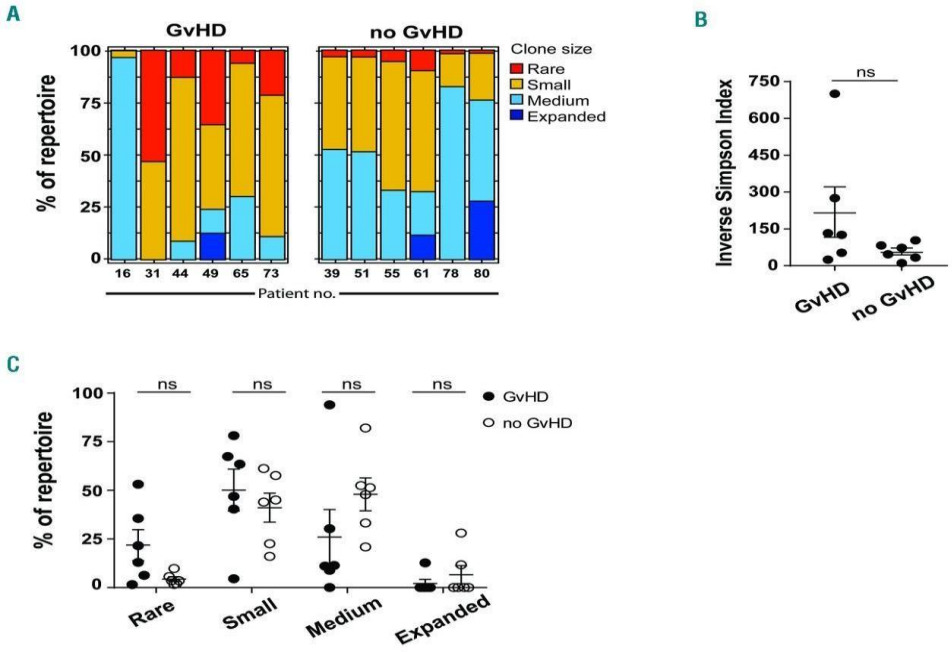


Figure 13– Regulatory T-cell repertoire after Allo - Hematopoietic Stem Progenitor Cell Transplantation and Graft Versus Host Disease (GVHD). The larger the repertoire the lower the GVHD. Odak I. *et al*. (2019)[152]. Written permission from the authors was obtained to reproduce this Figure.

I.4.9. - Transplant Encapsulation.

Transplant encapsulation inside a porous polymeric membrane, made of alginate or any other polymer, aims to separate the allo-transplanted cells from the host immunological system[64]. However, nutrients should still enter the transplant and therapeutical cell products access the blood stream. (**Figure 19**).

These are the two main issues with this technology, because in the short-term fibrosis encapsulation (foreign body reaction) of the encapsulated implant will be the rule, inducing transplanted cell death by lack of nutrients and oxygen, as well as lack in the circulation of the essential therapeutic molecules[153,154].

I.4.10. - Other Tolerance Approaches (TOLEROGENIxX).

A recent (2019) proposal, available for live Kidney transplantation, was proposed by a Start-up: TOLEROGENIxX.com. The proposal claims gains in tolerance in live Kidney transplantation, based on a protocol that requires a double donation from a live donor. **First**, 8 days before the surgery for kidney transplant, monocytes are collected from the donor by leukapheresis, and then are treated with mitomycin C, before being infused in the patient to be submitted to a kidney transplant. These cells, named Modified Immune

Cells (MIC) will not proliferate after transplantation and will develop features of immature dendritic cells (DC) resulting in profound suppression of T Cell response. **Second**, 8 days after the infusion of the donors' Mitomycin C treated monocytes, the transplant is performed. Eight days is the time lapse that the protocol requires for the patient to become tolerant toward the HLA mismatched donor's kidney, by the infusion of Mitomycin C treated donor's monocytes, (**Figure 14 and Figure 15**). However, in the days or weeks near the transplantation day, patient is still submitted to immunosuppression[155,156].

Low response from host to transplanted kidney donor cells is grounded in the demonstration of "*a substantial increase in CD19+ CD24hiCD38hi transitional B lymphocyte regulatory cells (B regs) after transplantation*"[155] also corroborated in [236]. FACS analysis of MICs revealed low expression of stimulatory molecules such as CD80, CD83, CD86, and HLA class II histocompatibility antigen and chain HLA-DR. When injected into the "*prospective experimental graft recipient*", donor-derived MICs preferentially accumulates in peripheral lymphoid organs and induces regulatory lymphocytes[155,156]. A week after MICs infusion into prospective kidney graft recipient, both donor and patient are submitted to surgery for the collection of the healthy donor kidney to be transplanted to the patient.

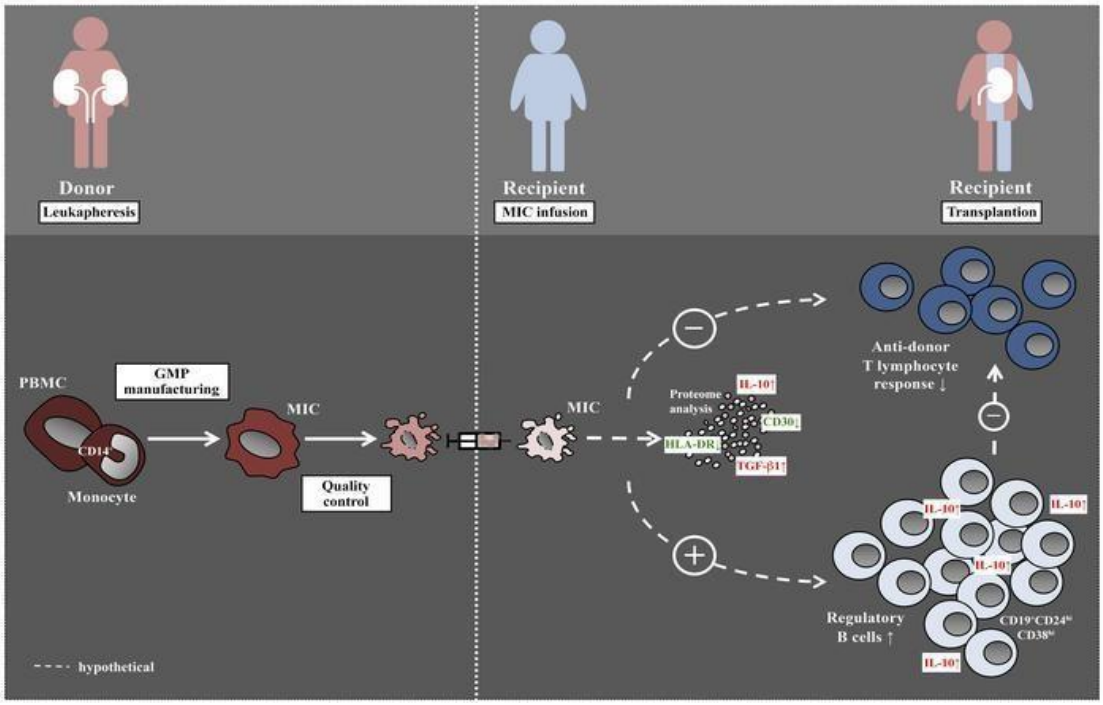


Figure 14 – Protocol basis for immune tolerance in kidney allo-transplantation (live donation). First, donor monocytes are collected by apheresis and treated by mitomycin (MIC). Second, MICs are infused

in the patient to be transplanted, triggering the expansion of regulatory B cells that will inhibit the patient's T cells to trigger rejection. Third, a week after the MIC infusion, donor and patient are submitted to surgery for the kidney donation. Morath C. *et al*. (2020)[155].

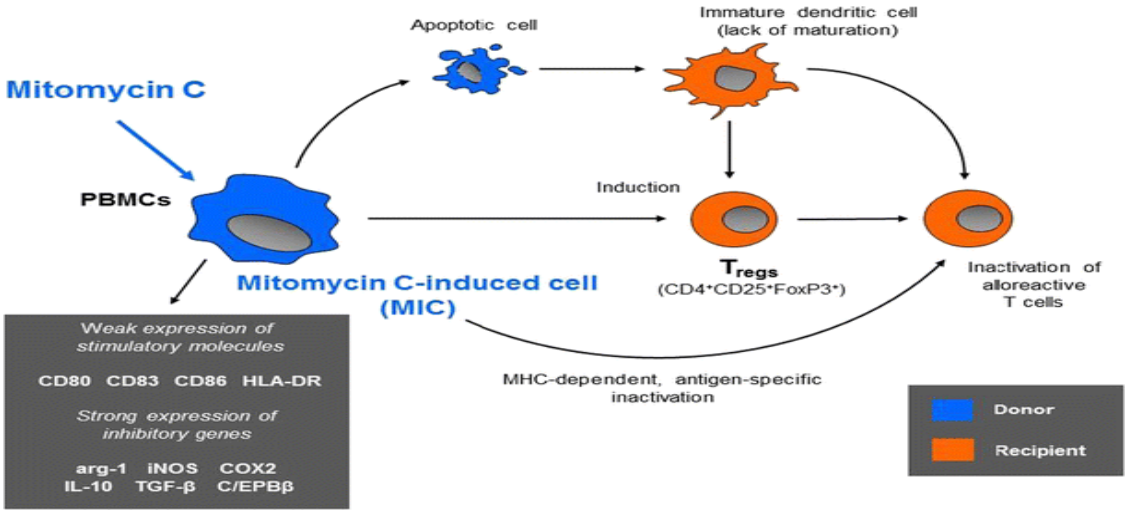


Figure 15 – Cellular mechanisms in immunological tolerance protocol for Allo-Kidney live donation transplant. Authors identify CD4+ CD25+FoxP3 (T-regs) as the providers for immune-tolerance toward the transplanted kidney, after infusion of mitomycin treated donor monocytes eight days before transplantation surgery, Morath C. *et al.*[156] (2018).

I.4.11. - In summary.

A review of many possibilities, both theoretical or already attempted, aiming to solve the long-lasting medical problem of rejections in human allo-transplantation were exposed. Until now, no solution is efficiently solving the issue *de per si*. However, relevant science-grounded approaches were made.

CHAPITER II – OUR PROPOSAL

CHAPITER II - OUR PROPOSAL

II.1. Project Aims and Experimental Strategy.

II.1.1. - Project Aims.

The final goal of the project was to create a patentable Method to obtain the replacement of human chromosome 6 or any other human single chromosome or chromosome pair in Stem Cells (SCs). More specifically, the aim is to create "Off-The-Shelf" hPSC lines, in which the endogenous chromosome 6 can be replaced by the chromosome 6 from a patient to be transplanted. By differentiation of these healthy hPSCs that are in full HLA-match with the host, any cell, micro-tissue, tissue, or organ can be created for any given patient. In (**Figure 16)** a synopsis is presented of the several steps to accomplish our final goal, that comprehends the creation of transplantable materials in full HLA-match with the patient to which it is intended to be transplanted, based on the replacement of human chromosome 6 pair.

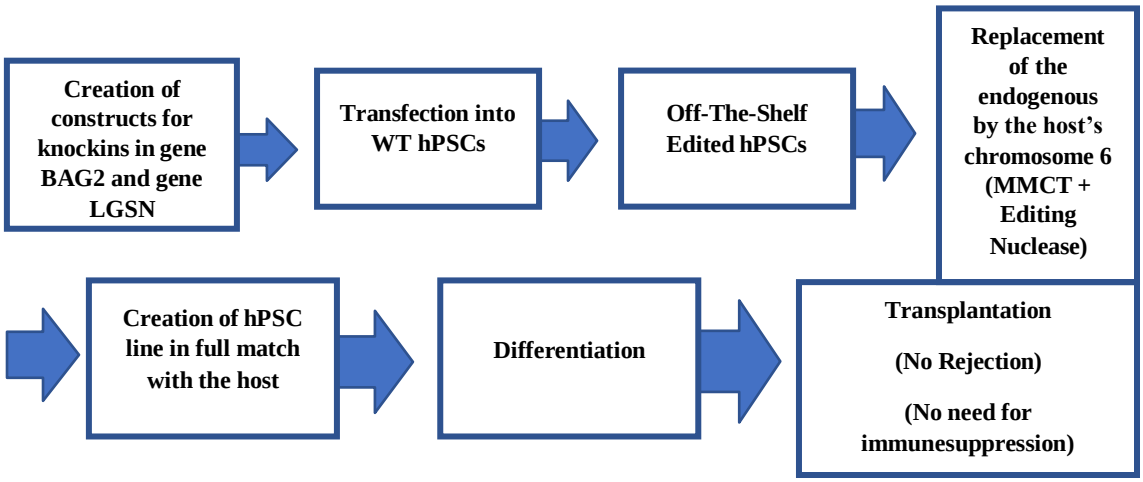


Figure 16. Schematic representation of the main steps along the project. Starting from the creation of the constructs to achieve the needed gene knock-ins in BAG2 and LGSN in chromosome 6 pair of WT hPSCs. After selection of the correctly edited clones, Off-The-Shelf product is created. By simultaneous Microcell-Mediated Chromosome Transfer (MMCT) of host's chromosome 6 pair and deletion of edited endogenous chromosome 6 pair by Editing/DSB Nucleases, new hPSC lines are created. Those cells by differentiation will provide for the creation of all cells, micro-tissues, tissues, and organs the host may be in need. As those cells are in full HLA match with the host, the usual rejection syndromes are not to be expected.

Here, we are proposing a strategy to generate a human "Off-the-Self" pluripotent stem cell line that can be used to generate all types of cells and tissues for human transplantation, without the recurrent problem of immunological rejection.

To achieve this, we will construct a hPSC cell line with a genetically modified Chr 6, ready to be swapped by Chr 6 of the patient to be transplanted, thereby generating a patient-specific PSC carrying its own pair of Chrs 6. This PSC can then be used to generate any tissue that expresses the patient MHC from these Chrs 6 and will therefore not be rejected.

Our strategy involves two main steps:

1- Genetic modification of Chr 6 in a chosen human PSC line, by insertion of 2 specifically designed DNA Cassettes into regions flanking Chr 6 centromere. This will constitute a universal "Off-the-Shelf" PSC line.

2- Isolation and transfer of patient Chr 6 to the "Off-the-Shelf" PSC line, in parallel with elimination of modified endogenous Chr 6.

II.1.2. – Strategy.

Ideally, it would be relevant if we could replace the entire endogenous MHC in donor Stem Cells by the MHC of the patient or, at least, the entire HLA set of genes directly into the nuclei of the cells to be transplanted.

However, MHC contains a large complex of genes[7] and, there is no available vector to achieve safe transfer of such a large piece of DNA. The best available vectors are Human Artificial Chromosomes (HACs), which are independent DNA structures derived from a human small chromosome, like human chromosome 15, 20 or 21. Usually they are introduced in human cells by electroporation and may remain in dividing cells in culture for (only) up to 6 months[157]. As they maintain a centromere-like structure, they will replicate during mitosis. In this case, Regulatory Authorities will have concerns about using HAC's at least because of two major concerns. **First**, it does not solve the problem of endogenous donor HLA expression that must be simultaneously deleted, (only the entire and unique HLA set from the host shall be inside the cells), and **second**, no warranty exists for long-term permanence inside transplanted cells *in vivo*.

However, solutions have been created to delete the entire HLA Class I (by CRISPR deletion of all HLA receptors), or indirectly by knock-out of β2-microglobulin expression from human chromosome 15 (expected to interfere with the normal HLA Class I expression on human cells).

Anyway, if such cells with complete deletion of the endogenous HLA are engineered with HACs to provide the patient's HLA, the lack of stable expression is likely to result in rapid loss of HAC-dependent expression of patient's HLA, and eventual rejection by

Natural Killer (NK) cells[44]. Thus, this proposal is not a solution neither in veterinary (*vis a vis* respective MHC) nor in human transplantation.

Another approach, already mentioned above, is grounded on the fact that HLA Class I receptors also include Chromosome 15-dependent expression of β2-microglobulin as a component to be added to the three protein chains, α1, α2, and α3 to stabilize each HLA class I receptor on the cell membrane. Several authors prepared Stem Cells where β2-microglobulin expression is knocked-out. Without complete HLA Class I expression and/or assemblage on the cell membrane, HLA Class I mismatch between transplanted cells and host will be possibly eradicated in humans. However, mismatched α1, α2, and α3 molecules from HLA-Class I receptors will continuously be produced and transported to the cell membrane, because respective gene expression is not knocked-out. At least, soon as they appear outside of cell membrane, they can still evoke immune rejection, by NK cells, T-Cells, B-Cells, and Macrophages, because they have α1, α2, and α3 chains, expressing at least some donor's different-from-the-host Class I HLA genes that those cells are able to recognize as non-Self. Even if the entire HLA receptors are notcompletely assembled and stabilized in the cell membrane by the lack of β2- microglobulin.

Moreover, any cell that does not expresses HLA Class I molecules, (as in the methods based on complete HLA deletion), will be targeted at least by NK cells[149].

Theoretically, one can reason that if it is not possible to replace the entire MHC, at least it would be possible to replace HLA genes one by one or, as they are represented in gene clusters, by simultaneous deletion of Class I and Class II donor's cluster of genes, followed by replacement with the equivalent copies from the host. But this would represent a huge and difficult genetic manipulation for today's state-of-the-art, in a multistep process in human chromosome 6 pair, given that HLA relies on codominant gene expression, which means that all mismatched HLA receptors would have to be replaced. Genetic "scars" from these multi-manipulations have high probability of compromising all or part of HLA genes or other nearby or distant genes by off-targeting, as well as by other mechanisms.

Human cloning for human reproduction, (another way to produce cells in full HLA-match with a patient), is not allowed by ethical standards, regulations, and laws in many countries. However, human cloning with therapeutic intent or research is in a different drawer in many research leading countries[158]. A consensus has been difficult to achieve worldwide since UNESCO produced the 2005 declaration prohibiting *"all forms of*

human cloning inasmuch as they are incompatible with human dignity and the protection of human life". Because a Convention was not possible to be signed by any country, in 2005 or thereafter, what countries have done is reserving for their own jurisdictions the creation of their own legal instruments to deal with the question[158,159].

Another approach toward solving rejection in human allo-transplantation was proposed by the work of Takahashi K. *et al.* (2007) in Shinya Yamanaka's team[28]. As it will be better explained in this thesis, (see **I.1.3.**) this team was able to reprogram somatic adult cells to a stage of pluripotency. This was a major achievement and by many viewed as a solution for rejection(s) in human transplantation, as all reprogrammed cells assumedly express the exact same HLA set of receptors as it is present in all cells in a patient's body. Pluripotency in those reprogrammed cells designated as human induced Pluripotent Stem Cells (hiPSCs), embodied the opportunity to create whichever cell, tissue, or organ a patient may be in need with simultaneous full HLA-match, avoiding rejection. However, Shinya Yamanaka, Nobel Prize 2012 with John Gurdon for the creation of hiPSCs, publicly declared on several occasions that hiPSC technology was too expensive to be used in Personalized Medicine.

In 2017, the creation of a cGMP (current Good Manufacturing Practices) hiPSC line had a cost over 1M US dollars. However, the figures in 2020 were reduced to near 500K US dollars and as technology improves, better prices will be available without any compromise in cGMP safety[159]. By the way, in recent webinar (2022), it was advanced a 200K US dollars cost for the creation of cGMP hiPSC lines, even though the price is about 600K to 800K US dollars, (www.Catalent.com).

Moreover, hiPSCs are not directly suitable to treat patients with genetic diseases, as hiPSCs reprogramed from their own somatic cells as expected, will reproduce the exact same mutations, perpetuating those diseases in the manufactured transplants. Furthermore, since on their reprogramming process, Klf4 and c-Myc factors must be overexpressed and they are known oncogenic factors, some authors are concerned about the clinical use of hiPSCs[36,37,38].

We should constrain from trying to solve such a difficult problem, like human rejection in allo-transplantation, by proposing the creation of so many new tools or too expensive solutions that only a minority of people may access. We should also refrain from creating proposals that would give rise to new or worse category of problems. Creation of new tools is time and money consuming, and waste of time means many human lives to be

lost, as well as too expensive solutions hampers the creation of universally available products that are needed to save humans independently from economic or/and financial constraints. Despite conscious of the difficulties ahead, we must try to propose the safest, faster, lower cost possible solution among available tools already tested and validated or certified in other environments.

There are a very wide range of genetic diseases that can be cured by transplantation of a relatively small number of suitable cells. Those cells will produce the needed life-saving factors (proteins/hormones), without the need for vascular and other solid organ structural components, to provide for the survival of transplanted cells. Proposals for subcutaneous or intraperitoneal in-sheet or encapsulated implants as a way to provide for rejection waiving have been addressed for example by Tatsumi K. *et al*. (2013)1[60] (**Figure 17**) and Liu Q. (2019) (**Figure 18**)1[61].

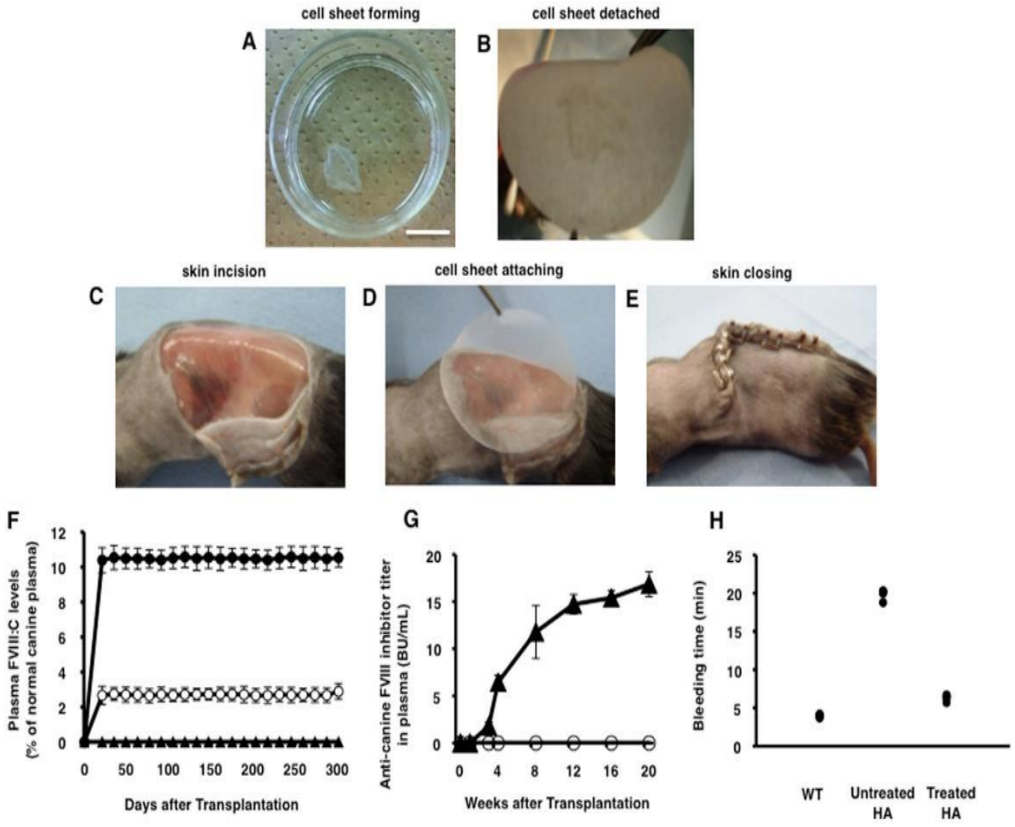


Figure 17 - Transduced FVIII (Hemophilia A) Endothelial Cells subcutaneous mouse sheet, Tatsumi K. *et al.* (2013)[160]. Transplanted cells achieved a good level of bleeding control in a Hemophiliac mouse model by the enhancement of the blood levels of Factor VIII. Creative Commons Attribution License.

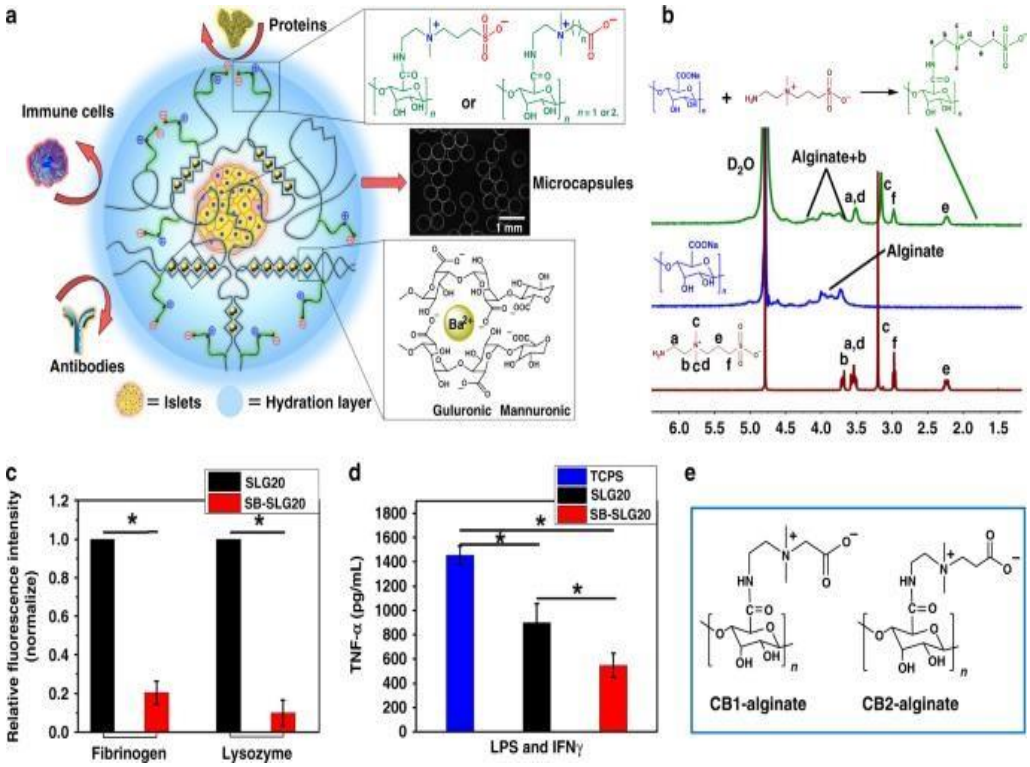


Figure 18 – Alginate encapsulation. Modifications in alginate molecules to overcome Foreign Body Reaction (FBR) and implant rejection, Liu Q. (2019)[161]. Creative Commons.

It may be the case of endothelial cells, hepatocytes, and others, able to produce Factor VIII and Factor IX to cure hemophilia A and hemophilia B respectively, or pancreatic β-cells to produce insulin for diabetes. However, there are multiple immunological challenges, in addition to HLA-related, that are implicated in long-term encapsulated cell transplants' failures as exposed by Ashimova A. *et al.* (2019)[162] (**Figure 19**).

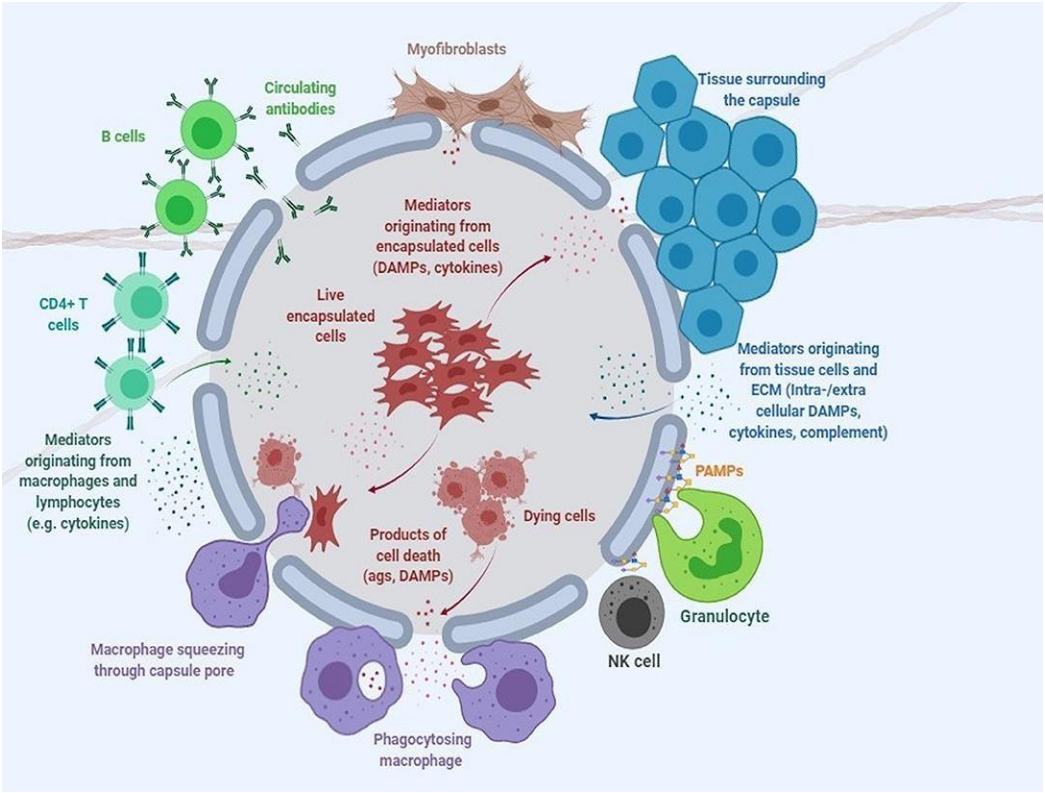


Figure 19 – Cell encapsulation in alginate for allo-transplantation. Host's immunological challenges for encapsulated cells to survive. Ashimova A. *et al.* (2019)[162]. Creative Commons.

Also, for many other life-saving enzymatic and hormonal factors, maintained in correct physiological levels daily by a unique low-dose cell agglomerate or by multi-intra-one-organ or multi-organ low-dose-cell agglomerates, that do not need vascular, excretory, or other complex structures. As may also be the case for insulin-producing pancreatic β-cells as described by Bochenek M.A. *et al.* (2018)[163], (**Figure 20**).

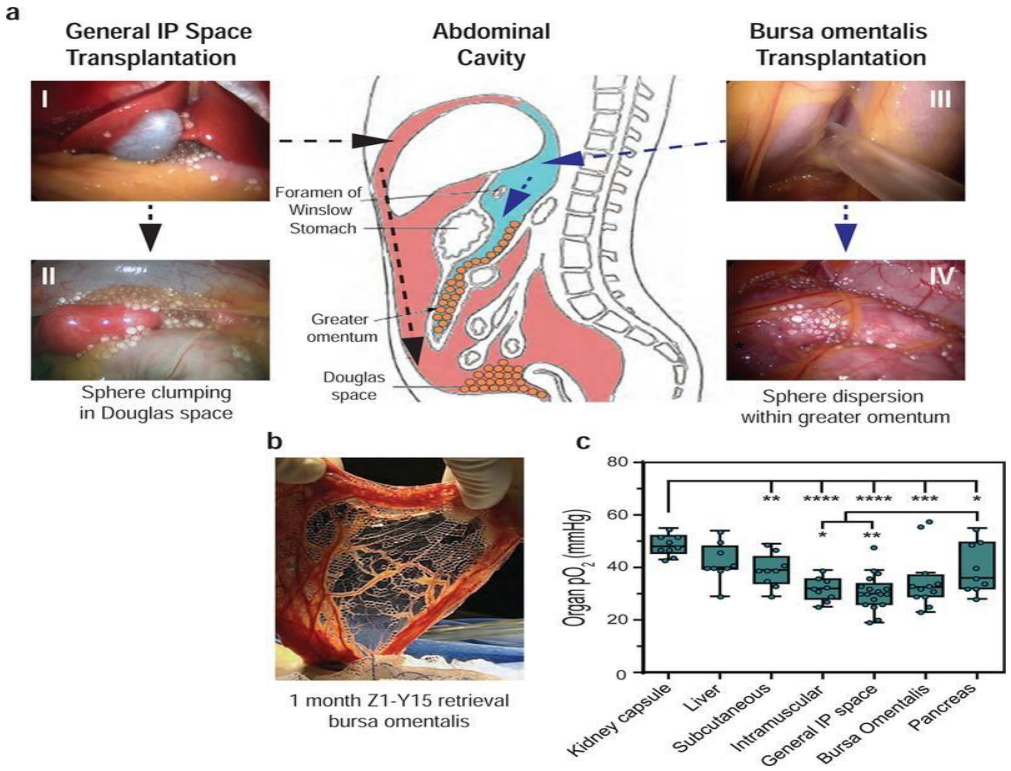


Figure 20 – Intra abdominal encapsulated Allo-pancreatic islets implants inside peritoneal spaces, Bochenek M.A. *et al.* (2018)[163]. Users may view, print, copy, and download text and data-mine the content in such documents, for the purposes of academic research, subject always to the full Conditions of use: http://www.nature.com/authors/editorial_policies/license.html#terms.

II.2. MY PATH TO THE PROJECT

In 2013, after realizing that the entire MHC is inside maternal and paternal human chromosomes 6 and since HLA is the expression of some of the genes inside MHC, I started searching for a solution for human Allo-transplantation Rejection(s), with available state of the art tools.

First, I found a very interesting work from Fournier R.E. & Ruddle F.H. (1977)[99], where authors revealed that transfer of entire human chromosomes was possible from inside microcells where they were isolated, to other human cell nuclei in a protocol named Microcell-Mediated Chromosome Transfer (MMCT). **Second**, in at least two publications from T. Tada's team[125,126], a protocol for deletion of an entire human chromosome by the creation of a dicentric chromosome was published.

Thus, scientists were already in the possession of the two main tools needed to accomplish the objective of human allo-transplantation without rejection.

This led me to the idea of promoting, in healthy human Stem Cells, the simultaneous deletion of the endogenous Chromosome 6 pair, using T. Tada's protocol, and replacing it by the exogenous chromosome 6 pair of a patient, using MMCT. The result would be the creation of healthy human Stem Cells in full HLA-match with a patient, enabling three main simultaneous achievements:

1- avoidance of allo-transplantation immunological rejection/s,

2- maintenance of the complete immunological capabilities/functions in the transplanted individuals, linked to the advantages of no need for reduction of immunological defenses by immunesuppression,

3- as donor cells are healthy, diseases expressed from the other chromosomes but Chr. 6, are expected to be treated and even cured, because the transplanted cells will not be sick, as are the autogenous cells in the patient.

At that time, I did not realize that T. Tada's team already filled a patent application, (JPWO2006075671), aiming the exact same objective and following the exact same strategy. In T. Tada's team protocol the entire human chromosome 6 deletion was based on the creation of dicentric chromosomes, that the cell reject at mitosis as edited chromosome segregation is compromised. Dicentric chromosomes are cause for chromotripsis and kataegis[127]. Based on that knowledge, I concluded that a safety issue restrained T. Tada's team to pursue the development of the technology.

As an alternative, I considered the use of Clustered Regularly Inter-Spaced Palindromic Repeats (CRISPR)/Cas9[107] to drive simultaneous Double Strand Breaks (DSBs) in regions of DNA flanking chromosome 6 centromere, to promote specific excision from the cell.

It also seemed to be a safer hypothesis when compared with the chromosome deletion method proposed by the creation of a dicentric chromosome in T. Tada´s team protocol, patent (JPWO2006075671).

Our strategy includes the creation of human Pluripotent Stem Cell (hPSC) lines prepared with specific insertions in genes flanking Chromosome 6 centromere, hopefully not crucial for Stem Cell survival, allowing for its deletion from the cell. Those edited hPSC lines will be the starting off-the-shelf product, or the raw material to the manufacturing of human allo-transplants in our Method.

By driving endogenous chromosome 6 deletion from off-the-shelf hPSCs (edited), and simultaneously transferring the patient's chromosome 6 by microcells, a second raw

product will be available to produce any cell, micro-tissue, tissue, or organ a host/patient may be in need.

In short, the complete strategy in our experimental work for the creation of off-the-shelf hPSC lines includes:

1- Editing of gene BAG2 in long arm of both maternal and paternal chromosome 6 in healthy hPSC lines.

2- Isolation of correctly edited clones.

3- Editing of gene LGSN in short arm of both maternal and paternal chromosomes 6 in the correctly edited clones in point 2- or *vice versa*.

4- Isolation of correctly edited clones in both BAG2 and LGSN genes.

Step 4-, represents the creation of the off-the-shelf hPSC lines.

5- Ultimately, in the off-the-shelf hPSCs and by replacement of the endogenous chromosome 6 pair by the patient's one, new healthy hPSC lines in full HLA-match with the patient will be created. By differentiation of these last new hPSC lines, (healthy and patient full-HLA compatible), the way is paved for all transplants to be created for the patient in full HLA-match. It represents the creation of a new transplant type: Allo-Auto-transplant.

CHAPTER III - MATERIALS and METHODS

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III.1. - HUMAN CELL CULTURE and ANALYSIS

III.1.1. - Cell culture

All procedures requiring direct exposure to the atmosphere of cells in culture, passaging, thawing, freezing, transfection, or the like, was performed inside a previously UV/20 minutes treated, and 70% ethanol disinfected laminar flow hood to avoid contamination of the samples. The same is true for the sterility of any material or reagent intended to be into contact with the cells. Before placing these materials or reagents inside the hood containers were disinfected using 70% ethanol and even outside surfaces of cell culture plates were disinfected as soon as they arrive inside the hood and before the covers on the plates were lift.

III.1.2. - Cell Lines

Four hiPSC Lines and one hESC Line (Wicell H9) were prepared or/and used all along the experimental tasks:

1- F002.1A.13 (TCLab, Portugal), **TCLab hiPSC line**, a commercialized hiPSC line, is in use in the Lab and regularly tested for pluripotency and correct karyotype. Mycoplasma-free by testing with a commercial kit.

2- **hiPSC E line**, a kind gift from Simão Rocha, Senior Staff Scientist, Maria Carmo-Fonseca Lab, IMM/João Lobo Antunes. This hiPSC Line was developed by Simão Rocha/ CarmoFonseca Lab/IMM and submitted to periodic pluripotency and karyotype evaluations. Mycoplasma-free.

3- **hiPSC F line**, gift from Cláudia Gaspar, prepared in Ricardo Fodde's Lab, Erasmus MC University, Holland. This cell line characteristically, undergo spontaneous neuronal differentiation. Also periodically evaluated for pluripotency and normal karyotype.

4- **GEpi hiPSC line (GIBCO®),** Human Episomal iPSC line, Gibco®, is a commercialized hiPSC line in use in the Pluripotent Stem Cell Lab at SCERG and regularly evaluated for pluripotency and correct karyotype.

5- **H9 hESC line (WA09) (WiCell Research Institute, Inc.)**, a kind gift from Inês Milagre, Instituto Gulbenkian de Ciências, IGC, Oeiras, Portugal. Cells were evaluated for pluripotency and normal karyotype.

6- A transformed cell line was also used: **HEK 293 T cell line**, a commercialized cell line acquired from ATCC: human embryonic kidney, ATCC® CRL-1573™. Regularly evaluated for mycoplasma contamination.

III.1.3. - Cell Thawing.

Cryovials containing **hPSCs** frozen cells were taken out of the - 80°C freezer or of the liquid nitrogen storage (N2) in Master or Working Cell Banks and briefly warmed up in a 37°C water bath. When the cells were almost completely thawed, they were carefully resuspended in 5 mL/37°C Washing Medium (see point **V.1.4.** for Washing Medium composition), transferred to a 15 mL Falcon® tube and centrifuged at 1000 rpm for 3 – 4 minutes in a benchtop centrifuge (Eppendorf™ 5810 R). After, the supernatant was carefully discarded, the cell pellet was resuspended in a volume of about 200uL/well to be seeded in a 6-well plate, 37°C mTeSR™1 or mTeSR™Plus, supplemented with 1% Penicillin/Streptomycin. The solution was homogenized, by very slowly pipetting up and down only a few times for conservation of cell aggregates that are known to improve cell survival and colony formation in hPSCs. The content, (~200uL/well), was distributed among wells pre-coated with Matrigel® and containing 1,5mL cell culture medium. The plates were gently shaken in a cross-like motion, both in the hood and in the incubator after microscope observation, allowing for uniform distribution of the cells across each well and placed in a humidified, 5%CO2 incubator at 37°C, undisturbed for an overnight period to allow the cells to adhere.

For **HEK 293 T** cells, all procedures go as in hiPSCs and hESCs but the cell culture medium was Dulbecco's Modified Eagle Medium (DMEM, Gibco®) + 10%Fetal Bovine Serum (FBS) + glutamine + 1% Penicillin/Streptomycin.

III.1.4. - Cell Maintenance.

hiPSCs F, E, TCLab, GEpi, and hESCs/H9 were cultured on Matrigel® (Corning®)-coated plates (Corning®). hiPSCs F, E and TCLab with mTeSR™1 medium (StemCell Technologies) supplemented with 1% Penicillin/Streptomycin, and hiPSC GEpi and hESCs H9 were culture with mTeSR™Plus medium (StemCell Technologies) + 1% Penicillin/Streptomycin.

III.1.4.1. - Coating of Plate Wells

Before seeding **hPSCs**, bottom of plates or T-flasks for adherent cells (Corning®), were covered with Matrigel® Corning® Matrix following manufacturers recommendations.

Matrigel® Corning® is extracted from Engelbrath-Holm-Swarm laboratory-induced mouse sarcoma. Prior to the coating of the wells, Matrigel® was thawed on ice and diluted 1:100 by chilled Dulbecco's Modified Eagle Medium (DMEM, Gibco®), then stored at -20°C in microtubes for future use. Matrigel®-coated plates were stored at 4°C for at least an overnight period before being used. Cells were grown in a humidified, 5%CO2 incubator at 37°C. Medium was changed daily in mTeSR™1 cell cultures and every other day in mTeSR™Plus cell cultures. The cells in culture were routinely monitored by microscopic observation using Leica DM2500 (Leica Microsystems) microscopes.

III.1.4.2. - Cell Culture Media for hPSCs:

(mTeSR™ 1; mTeSR™ Plus)

In the first years of experimental work with hPSCs, cell culture medium was mTeSR™1, STEMCELL Technologies™, with a daily cell culture medium change. Starting in 2019, STEMCELL Technologies™ produced a new cell culture medium for hPSCs, mTeSR™Plus, that was used routinely in our experiments. This is a culture medium based on mTeSR™1, but including three new important characteristics:

1- manufacturing is under cGMP conditions, allowing transition from Lab Research to (GMP) Cell Therapy applications.

2- Media contains FGF2 that is not thermolabile at 37°C as in mTeSR™1, allowing for cell culture medium change, every other day. This allows for a reduction in cost and better stemness conservation of human Pluripotent Stem Cells in Culture.

3- "*offers enhanced buffering to reduce medium acidification so that cell quality is preserved during skipped media changes.*" (https://www.stemcell.com/why-mtesrplus).

III.1.4.3. - Y-27632 RHO/ROCK pathway inhibitor, STEMCELL Technologies™ (Rock inhibitor/ Rocki)

Y-27632 RHO/ROCK pathway inhibitor, is a selective inhibitor of RHO-associated, coiled-coil containing protein Kinase (ROCK). "*Rho-associated kinases ROCK1 and ROCK2 are serine/threonine kinases that are downstream targets of the small GTPases RhoA, RhoB, and RhoC. ROCKs are involved in diverse cellular activities including actin cytoskeleton organization, cell adhesion and motility, proliferation and apoptosis, remodeling of the extracellular matrix and smooth muscle cell contraction.*"[164].

Y-27632 inhibits ROCK1 and ROCK2 by competition with adenosine triphosphate (ATP) to the catalytic site. It was used in our experiments to enhance Stem Cell survival in anoikis, (a form of programed cell death after they detached from the Extra-Cellular Matrix (ECM) - dissociation-induced apoptosis), and other cell stressful situations like transfections and thawing.

Although some authors reported that rock inhibitors may originate nefarious modifications in hESCs in culture, mainly related to the survival of cells with mutations that otherwise would not be able to survive in culture, (cell culture mosaicism contamination)[165].

For **HEK 293T cells**, there was no need for the use of ROCKi. **HEK 293T** cells are resistant enough to be submitted to thawing, passaging, and transfections with no need for ROCKi.

III.1.5. - Cell Freezing

For **HEK 293T** freezing, first step included 70%-80% confluent wells, cautious washing twice with phosphate-buffered saline (PBS, 1x), followed by an incubation of the cells in trypsin at 37°C for 5 minutes, to detach the cells from the wells in freeing the anchorages between the cells and the matrix at the bottom of the wells.

As enough cell detachment happens, cells were flushed with DMEM and transferred to a 15mL Falcon® tube with 5mL DMEM to neutralize trypsin. After a centrifugation at 1000rpm for 5 minutes benchtop centrifuge (Eppendorf™ 5810 R), supernatant was discarded, and cells resuspended in 250uL of DMEM pure + 10%DMSO (DMSO, Sigma-Aldrich®) solution per million cells. Then, resuspended cells were transferred for cryovials (250uL/Cryovial) and immediately stored at -80°C if to be used in the next 1-2 weeks or after 24 to 48 hours to a nitrogen container for long time storage.

For **hPSCs**, first step included cautiously washing twice with phosphate-buffered saline (PBS, 1x) of culture wells, followed by an incubation of the cells in 0.5 mM Ethylenediamine tetraacetic acid (EDTA) dissociation buffer (Life Technologies), 1 mL/6-well at room temperature (RT) for 4 – 5 minutes. This allowed for the EDTA, (a Ca2+ chelator), to be effective in freeing the anchorages between the cells and the matrix at the bottom of the wells. Thereafter, the EDTA was neutralized by transferring the detached cells into 5 ml 37°C, washing medium in a 15mL Falcon™ tube. For cell detachment the following procedures were performed: the cells were mechanically detached by dispensing 37°C Washing Medium (DMEM/F12 + 1%

Penicillin/Streptomycin, please see below for composition), 3 times on the top of each well with the plate tilted and repeating it from the other side of the wells, by inverting the plate horizontally. It was important not to overdo this step because hiPSCs and hESCs only hardly survive as single cells and up and down pipetting could cause the cell clumps to be too much dispersed.

Transferred cells into 15 mL Falcon® tubes were centrifuged for 3 – 4 minutes at 1000 rpm in a benchtop centrifuge (Eppendorf™ 5810 R), after which the supernatant was discarded, and the pellets resuspended in 1mL DMEM/F12 + 1% Penicillin/Streptomycin, Washing Medium for manual cell counting in a Neubauer hemacytometer. A new centrifugation was performed for 3 – 4 minutes at 1000 rpm in a benchtop centrifuge (Eppendorf™ 5810 R), after which the supernatant was discarded, and the pellets resuspended in a freezing solution of 10 % Dimethyl sulfoxide (DMSO, Sigma-Aldrich®) in Cell Culture medium, (250uL/for 10^6 to 2x10^6 cells/cryovial). Finally, the cell suspension was transferred to pre-labeled 1.5 mL cryopreservation vials (Nunc Millipore), which were placed in a freezing container, named Mr. Frosty™ (Thermo Scientific™) and stored at – 80°C in an ultra-low temperature freezer. From RT to – 80°C, Mr. Frosty™ has a cooling rate of 1 °C/minute, which is believed to be optimum for the successful cryopreservation of cells. The 10% DMSO cryopreservation solution is toxic to the cells at RT, thus the resuspension and storage steps were performed without unnecessary delays. 24 to 48 hours after the cells were frozen at –80°C they were transferred to a liquid nitrogen container, where their preservation is assured for longer periods.

Freezing solution composition (1.5 mL cryopreservation vial):

- 25 µL of DMSO (Sigma-Aldrich®)
- 225 µL of Culture Medium.

Total – 250uL/cryovial for 10^6 cells to 2x10^6 cells.

Washing Medium composition (for a final volume of 1 L):

- 12 g of DMEM/F-12 powder (Thermo Scientific™).
- 2.4 g of NaHCO3.
- 10 mL of Penicillin/Streptomycin (Pen/Strep, Sigma-Aldrich®).
- 10 mL of MEM Amino Acid solution (Thermo Scientific™).

- 100 mL of KSR.

- 900 mL of Milli-Q H2O.

III.1.6. - Cell Passaging.

hPSCs cells were passaged whenever they reached a confluence of approximately 60%-70%. For HEK 293T cells passage was performed when they reach 80-90% confluence. Usually it took 3 – 4 days from the day of thawing or from the previous passage. Passaging of cells consists in transferring them from one culture recipient to another aiming to renovate and/or increase the surface area available for the cells to grow.

First, cultured cells in each well of a 6-well plate were washed twice with 1mL PBS, followed by incubation of the **hPSCs** in 1mL of 0.5 mM EDTA dissociation buffer at RT for 4 – 5 minutes until cell detachment starts to be observed or in the case of HEK 293T cells, a trypsin solution for 5 minutes incubation at 37°C. EDTA (hPSCs) and Trypsin (HEK 293 T cells), was neutralized by transferring mechanically detached cells, (by flushing 3-4 times the well to resuspend the clusters of cells), to 15mL Falcon™ tubes in 5mL Washing Medium (please see **IV.1.5.** for composition). After centrifugation for 3- 4 minutes/1000 rpm, Washing Medium was discarded. The pellet was resuspended in a volume of 1mL for manually cell count in a Neubauer hemacytometer. Depending on the cell seeding number, cells were resuspended in the adequate volume of medium to be distributed to 1,5 mL to 2 mL/ well in 6-well plates (37°C mTeSR™1 + 1% Pen/Strep or mTeSR™Plus + 1% Pen/Strep for hPSCs) and same volume of DMEM + 10% FBS + Glutamine + 1% Pen/Strep of culture medium per well in 6-well plates for **HEK 293T** cells. Wells were pre-coated with Matrigel® only for hPSCs culture. The plates were gently shaken in the hood in a cross-like motion to uniformly distribute the cells across each well, observed under microscopy and placed in a humidified 5%CO2 incubator at 37 °C, were a new cross-like gentle shake was performed and maintained undisturbed for an overnight period to allow the cells to adhere. The most used passage rate was 1:6, meaning that at passage, the cell content of one well is distributed for 6 new wells of the same size. However, other passage rates were used if required by experimental work.

III.1.7. – hPSCs Single-Cell Dissociation.

Before detaching the cells, the medium of the hiPSCs was changed to mTeSR™1 supplemented with 10 μM ROCK inhibitor (ROCKi, Y-27632, STEMCELL Technologies™) or as in the case of hiPSCs GEpi, and hESCs the medium was changed

to mTeSR™ Plus supplemented with 10uM ROCKi and incubated at 37°C/5%CO2 for at least two hours in a humidified incubator. After that, the wells were washed twice with RT PBS and the cells were incubated in 500uL/ 6-well Accutase® cell detachment solution (STEMCELL Technologies™) at 37°C in the incubator for around 7 minutes to accelerate dissociation process. Following this, the Accutase® was gently pipetted up and down the wells to detach all the cells and transferred to Falcon® tubes containing 4mL of Washing Medium (see point **III.1.5.** for composition) to neutralize it. The tubes were centrifuged at 1000 rpm for 4 minutes in a benchtop centrifuge and the supernatant was discarded. The cell pellets were resuspended in 1mL 37°C mTeSR™1 supplemented with 10 μM ROCKi, ahead of manual Neubauer hemacytometer/Trypan blue cell counting. However, some authors consider that Accutase® may produce relevant nefarious alterations in cells, mainly genomic alterations[166].

III.1.8. - Manual Cell Counting.

Manual cell counting was done using a Neubauer-type hemacytometer (LW Scientific), which is a glass device with engraved grids that facilitate cell-counting on a microscope. In a round bottom well of a 96-wells plate, 10 μL of a 1mL Accutase® treated single-cell suspension (see **III.1.7.**) was well mixed with 10 μL of Trypan Blue (Thermo Scientific™), which is a stain for dead cells. Then, 10 μL of this mixture were pipetted onto the hemacytometer and the unstained live cells and stained dead cells were counted on 4 quadrants. An estimate of live and dead cell density per milliliter of suspension was calculated by multiplying the average number of live and dead cells per quadrant by the dilution factor (2, in our experiments) in Trypan Blue and by 10^4 to account for the dimensions of the hemacytometer.

III.1.9. - Electroporation of hiPSCs.

Electroporation is a physical, electric current-based transfection method that consists in the generation of an electrical field on the surface of the cells which increases the permeability of the cell membranes by opening pores. Those pores allow for outside molecules to enter the cell cytoplasm as well as inside molecules to come outside. Nucleic acids that may be present in the cell culture medium where cells are suspended or even in adherent cells, may have access to the cytoplasm of the cells in a first step and thereafter to the cell nucleus by this technology[167].

The Neon® transfection system (Invitrogen™) uses a technique where the tip of a plug-in pipette serves as the electroporation chamber. For this work, the Neon® transfection system 10 μL kit was used, which included specially prepared 10 μL pipette tips,

electroporation tubes, a resuspension buffer (Buffer R) and an electrolytic conductive buffer (Buffer E2).

Medium was changed to mTeSR™1 supplemented with 10 μM ROCKi and the cells were incubated at 37°C/5% CO2 humidified incubator for at least two hours prior to their collection for electroporation. After, cells were detached with Accutase® and dispersed as single cells in solution and counted. The estimated volumes containing around 10^5 cells were transferred to separate 1.5 mL Eppendorf™ tubes (one per each condition). The tubes were centrifuged at 1000 rpm for 5 minutes in a microcentrifuge (Eppendorf™ 5418 R) and the supernatants carefully removed with a pipette. Each cell pellet was then resuspended in 10 μL of Buffer R and the DNA constructs (500ng CRISPR/Cas9/sgRNA-BAG2 + 500ng Cassette1/BAG2 construct) were added to each designated tube. The tubes were placed on ice, along with an electroporation tube with 3 mL of Buffer E2. In all steps, manufacturer's recommendations were followed. Additionally, a 12-well plate (Corning®), pre-coated with Matrigel®, and with 1 mL of 37°C mTeSR™1 supplemented with 10 μM ROCKi for seeding of the electroporated cells was provided. The Neon® tube was plugged into the equipment and a sample was carefully aspirated using the Neon® pipette with a 10 μL tip, which was in turn plugged into the electroporation tube until the tip was submersed in Buffer E2. No bubbles could be present inside the tip as this would cause surface warping and uneven voltage distribution during the electroporation, leading to high cell death. After a sample was loaded, the selected electroporation settings were performed in the device. For selection of the best settings to be used, we tried several conditions, based on the literature, and our experience. Evaluation of the best conditions for electroporation was based on the observation under fluorescence microscopy (GFP+) of the number of GFP + cells after 72-hour period. Conditions used were: 1050V/ 30ms / 2 pulses; 1100V/30 ms/1 pulse; 1200V/30ms/1 pulse; 1400V/ 20ms/2pulses, and the electroporated cells were seeded into the designated well in the 12-well plate. The plate was placed in a humidified 5%CO2 incubator at 37°C and was kept undisturbed for 24 hours. In all experiments, very high cell death happened at 24 and 48 hours after procedure, and despite the fact that always some GFP+ cells were detected in culture by direct fluorescence microscopy, no GFP+ colonies were ever isolated, or at least colonies with a majority of GFP+ cells to be picked and expanded for puromycin selection. After these negative results we changed our

hPSCs transfection methodology for Lipofection, (Lipofectamine®3000 reagent (Invitrogen™) in hPSCs). (Please see next point **III.1.10**.).

III.1.10. - Lipofection

Lipofection is a biochemical transfection method that consists in the creation of DNA, or RNA, or protein-lipid complexes by electrostatic interaction between cationic lipids and the negatively charged DNA molecules. These complexes are then internalized by the cells by endocytosis and the DNA, RNA and proteins released into the cytosol from where DNA can reach the cell nucleus[167].

The method was performed using Lipofectamine®2000 in HEK 293T cells and Lipofectamine®3000 reagent (Invitrogen™) in hPSCs. Manufacturer's recommendations were followed for the preparation of the experimental reagents for transfection protocols. No special equipment is required to be used for Lipofectamine®2000 in HEK 293T cells, or Lipofectamine®3000 reagent (Invitrogen™)in hPSCs.

hPSCs were cultured to ~10% confluence on the day of transfection, usually in 12-well flat bottom plates, Matrigel® coated for hPSCs.

On transfection's day, culture medium supplemented with 10 μM ROCKi was changed at least 2 hours before transfection.

In the meanwhile, the DNA-lipid complexes for transfection were prepared in 1.5 mL Eppendorf™ tubes, following manufacturer's recommendations:

- Tube A: Lipofectamine® 3000 reagent diluted in Opti-MEM medium (Gibco®). Mixed well. (Volumes dependent on the number of wells in 12-well plates).
- Tube B: P3000 reagent and DNA constructs (up to 500ng/well) diluted in Opti-MEM medium. Mixed well. (Volumes dependent on the number of wells in 12-well plates).

After a brief incubation of the well mixed solutions at RT for 5 minutes, the content of tube B was added to tube A, drop by drop. The new tube content was gentle mixed by several inversions and incubated at RT for another 10 – 15 minutes to allow the DNA-lipid complexes to be properly formed. Finally, the complexes were added to the cells drop by drop and distributed across the wells by gently shaking the plates, which were placed in a 5%CO2 humidified incubator at 37°C for 24 hours with no medium change.

At 48 hours first fluorescence microscope observation was performed to evaluate for transient transfection efficiency among the wells.

III.1.10.1. - Iterative Lipofection

In addition to the standard procedure described previously, another strategy was used instead of Lipofection being performed once, where Lipofection protocol was repeated every 8 hours during the first two days. This strategy was performed because Homology Directed Repair (HDR), crucial for knock-in of the large DNA insert, is only available in S and G2 phases of the cell cycle. Performing Lipofections at 8/8hour schedule, enabled to improve the efficiency of knock-in in hPSCs (hiPSCs and hESCs).

III.1.10.2. - Lipofection with CRISPR/Cas9 protein and Synthetic sgRNA.

As alternative to plasmid transfection to introduce sgRNA and Cas9 into cultured cells, we used direct delivery of Cas9 protein in complex with the guide RNA, provided as synthetic gRNA, a method that seems to be better for achieving adequate CRISPR/Cas9 targeting. We used for this procedure a commercial kit: TrueCut Cas9 Protein v2® ThermoFisher® and synthetic sgRNA/BAG2- (TrueGuide Synthetic gRNA® ThermoFisher®). Both the TrueCut Cas9 Protein v2® ThermoFisher® and synthetic sgRNA/BAG2- TrueGuide Synthetic gRNA® ThermoFisher®, must be dispensed to the cells in culture, in suspension or in adherent cell culture, by mean of CRISPRMAX™ (Thermo Fisher®) following manufacturers' instructions. Almost simultaneously, the construct for BAG2, (R52 or p81C), must be dispensed to the cells in a Lipofectamine 3000™ (Thermo Fisher®) preparation, following manufacturers' recommendations.

With this strategy, we diminished half the DNA amount/lipofection/well. Using the Cas9/sgRNA ribonuclease complex might in addition reduce CRISPR/Cas9 off-targeting, since the plasmid derived construct was supposed to be active inside the cells longer than Cas9 protein and synthetic sgRNA/BAG2.

III.1.11. - Flow cytometry.

Flow cytometry uses a laser system to measure or detect certain features of cells and/or particles that are conducted through a nozzle. In this work, flow cytometry was used to obtain by automated fluorescent-cell counting a more accurate result than by direct fluorescent microscopic observation. The fluorescent marker used for positive cell counting was Green Fluorescent Protein (GFP), which was expressed by Co-transfected hPSCs (CRISPR/Cas9-sgRNA-BAC2 + Cassette1). Flow cytometry was only performed

at day 6 or more after transfection. Objective was to avoid as much as possible, contamination of edited cells by cells only expressing GFP transiently.

Samples for flow cytometry, must be in single-cell suspension, so the cells had to be detached using Accutase®. After collecting and centrifuging the cells, pellets were resuspended in 400 μL of PBS for immediate flow cytometry analysis or were fixed in 400uL 2% PFA solution up to 7 days before observation. Results from fresh (non-fixed) cells are more accurate than otherwise and in most of our flow cytometry experiments we were able to program to have freshly collected cells for the experiments.

III.1.12. - Collection of Cultured Cells for Analysis.

The hPSCs were incubated for 5 minutes at RT in 1 mL RT EDTA/ 6-well and mechanically detached with a pipette flush and resuspended in a 15 mL Falcon® tube with 4 mL Washing Medium (see **III.1.5.** for composition). After 4 minutes centrifugation at 1000 rpm, two successive washings with 1 mL PBS were performed. The first washing in the same 15 mL Falcon tube for 5 minutes, the last washing in 1 ml PBS was performed in a 1,5 mL Eppendorf tube at 1000rpm for 10 minutes. After the last centrifugation, supernatant was discarded, and cell pellets were immediately stored at -20°C for future use, mainly for genomic DNA extraction.

For HEK 293 T cells all procedures were the same as for hPSCs, but the incubation was with trypsin for 7 minutes at 37°C to obtain cell detachment from the plate wells.

III.2. - BACTERIA PROCEDURES

III.2.1. - Bacteria culture.

The use of "competent" bacteria, (designating specially prepared *E. Coli* strains that have improved capacity, although fragile, for plasmid transfection), was essential for subcloning works and isolation of plasmid-derived DNAs necessary for transfections in the project. The bacteria strain used was DH5α *Escherichia Coli,* acquired commercially (Thermo Scientific™), or prepared in the lab (Domingos Henrique Lab, Instituto de Medicina Molecular, IMM – João Lobo Antunes). All operations involving the exposure of bacteria, or medium for bacteria culture, and specially prepared petri dishes to the atmosphere were performed under the flame of a Bunsen burner (Campingaz®) to prevent eventual culture contamination by air bacteria.

III.2.2. - Bacteria selection.

Ampicillin (Sigma-Aldrich®) was the only antibiotic needed for bacteria selection in liquid lysogeny broth (LB, Sigma-Aldrich®) or in solid LB Agar prepared petri dishes, as all the cloned DNA plasmids used in this work had a β-lactamase gene for ampicillin

resistance. In medium preparation ampicillin concentration of 100 μg/mL for high-copy plasmids was used. Bacteria cultures in low volumes of liquid medium (5mL) in 15ml Falcon® tubes were grown overnight at 37ºC in a rotating (15 rpm/min), 15 degrees inclined wheel system (shaker) in glass tubes or in 15 mL Falcon® tubes. For higher volumes of liquid medium (LB) up to 100 mL, bacteria were grown in oscillating horizontal shakers with 220 rpm shaking. Bacteria cultures on solid medium (petri dishes) were grown overnight in static incubator at 37ºC.

III.2.3. - Glycerol storage of Bacteria.

For long-term storage of the correctly transformed bacteria, 500 μL of fresh bacterial culture in LB should be mixed in a 1.5 mL Eppendorf™ tube with 500 μL of 30% (v/v) Glycerol (Sigma-Aldrich®), and without delay stored in an ultra-low temperature freezer at -80°C. These samples could be directly used for inoculation in liquid LB or for growth on LB Agar Petri dishes, by simply scraping off a little of the contents of the frozen vial with a sterile plastic loop and inoculating it in liquid LB or spreading it across the surface of an LB Agar Petri dish. The glycerol vial was kept on ice at all collection times and returned to -80°C storage as soon as the collection is over, to avoid as much as possible the thawing/death of the remaining cells in the glycerol. Also, all work was done under the protection of a Bunsen flame (Campingaz®) to avoid any cross contamination with atmospheric bacteria.

III.2.4.* - Transformation of Competent *E. coli.

The DH5α *E. Coli* cells or other in-Lab prepared *E. Coli* competent cells were available in the lab, stored in 7% (v/v) DMSO at - 80 °C. Competent cells are very fragile to handle. To ensure maintenance of the maximum competence capability of bacteria, all manipulations were performed on ice. In sterile 1.5 mL Eppendorf™ tubes, 5 – 10 μL of the plasmid DNA was added to 100 μL of the competent cell suspension, mixed by flicking and incubated on ice for 30 minutes in a round bottom glass tube. Next step, the cells were heat shocked by placing the tubes on a water bath at exactly 42°C for exactly 45 seconds, followed by a recovery period of 2 minutes on ice. The transformed cells were immediately used for the next programmed step in cloning that consisted on cell incubation overnight after inoculation in a 5 mL liquid LB tube in a rotating 15° axle, 15rpm, shaker.

III.2.5. - Plasmid DNA Cloning.

After transformation with the plasmid of interest, 100uL of cells were diluted in 900 μL of LB and incubated at 37°C in a 15 rpm/15° shaker for 60 minutes. Following this, the

tube was briefly centrifuged for 15 – 30 seconds at 13000 rpm in a microcentrifuge, the supernatant was mostly discarded, and the cell pellet resuspended with the remaining volume. The resuspended cells were transferred to a pre-heated 37°C LB Agar Petri dish containing ampicillin 100ug/mL for selection of the transformed bacteria, among the ones that are ampicillin resistant. The cells were spread out across the dish using a sterile plastic loop or a glass spreader recently flamed, (but RT cooled under the flame not to kill bacteria by heat), to cover the whole surface. Finally, the cells were placed overnightin static incubator at 37°C and would be ready for colony picking the next day. Negative controls were also used to evaluate for correct cloning rates.

III.2.6. – Picking Individual Colonies

After overnight incubation at 37 °C, colonies would be present in LB Agar dish. Some may be large enough for picking. At this point, the colonies could be immediately picked or the dish could be sealed with Parafilm® and stored in a fridge at 4 °C for up to 1 month for picking colonies at a later schedule.

Using a pipette with a sterile yellow tip (for pipetting) or a sterile plastic loop, the selected colony was carefully scraped out of the LB Agar surface, avoiding contamination with any other colonies in the vicinity. The yellow tip or plastic loop was then discarded inside a 15 mL Falcon® with a perforate cover, to maintain an aerobic bacteria culture environment, containing a pre-mixed volume of 3 mL of liquid LB with 100 μg/mL of Ampicillin and the tube was swirled to disperse the cells. After covering the tube with the perforate cover, the cells were incubated at 37°C with 15 rpm in a 15° axle shaker overnight. All procedures always under Bunsen flame protection.

III.3. - DNA procedures.

III.3.1. - DNA quantitation.

The quantitation of DNA samples was done using a NanoDrop™ 2000 (Thermo Scientific™) spectrophotometer. This equipment quantifies the DNA by measuring the absorption levels of a single 1 μL drop taken from a sample, against a blank measurement using 1uL drop of milliQ water. DNA molecules usually present a peak of absorption at around 260 nm and by measuring this value the spectrophotometer-based system can calculate the concentration of DNA in a solution. Additionally, the purity of the DNA in the solution was automatically evaluated by the comparison between the absorption level at 260 nm with the absorptions at 280 nm and 230 nm.

III.3.2. - Plasmid DNA preparation.

The extraction and purification of plasmid DNA from transformed *E. coli* was made using the NZYMiniprep kit from NZYTech® following manufacturers' recommendations. All centrifugations were carried out in a bench microcentrifuge. Each colony of transformed competent cells were grown in separate 15 mL Falcon® tubes with 3 mL liquid LB/Ampicilin 100ug/mL medium overnight. After incubation, cell suspension from each Falcon® tube was transferred to 2mL Eppendorf™ tubes and centrifuged at 13000 rpm for 30 seconds.

Then, the supernatant was discarded, and the cells were resuspended in 250 µL of A1 buffer by vigorous vortexing. Subsequently, 250 µL of lysis buffer A2 and 300 µL of neutralization buffer A3 were added to each tube. After the addition of each of these buffers, the tubes were inverted 6 – 8 times to mix the solution, but no vortexing was used as it would shear the chromosomic DNA and prevent it from being correctly retained in the pellet during the next step.

By centrifuging the tubes at 13000 rpm for 10 minutes, the chromosomic DNA and other cell debris were pushed down and formed a pellet, leaving a cleared lysate containing the plasmid DNA in the supernatant. The supernatants were loaded onto separate spin columns placed inside 2 mL collection tubes, both provided in the kit.

The samples were centrifuged at 13000 rpm for one minute, which pushed the remaining cytoplasmic molecules through the collection tubes, while the DNA molecules were selectively bound to the silica membrane of the columns by affinity chromatography. After this step, the flowthroughs were discarded and the spin column membranes were washed by successively loading 500 µL of AY buffer and 600 µL of A4 buffer (with EtOH) onto it, with 1 minute centrifugation steps at 13000 rpm after each addition. The flowthroughs were discarded and the membranes were dried by an additional centrifugation of the spin columns for 2 minutes at 13000 rpm.

Finally, the spin columns were placed in clean 1.5 mL Eppendorf™ tubes and 30 – 50 µL of Elution buffer was carefully added to the center of each column, to detach the DNA from the membrane. The tubes were incubated for one minute at RT and then centrifuged at 13000 rpm for 1 minute to collect the eluted and purified DNA. Repeating the elution step allowed for better final yield. Using a smaller volume of Elution buffer resulted in a higher concentration of DNA in the miniprep. The quality and quantity of DNA minipreps was assessed by 2uL sample 0,8% agarose gel electrophoresis (see **III.3.3**) and by Nanodrop® DNA quantitation (see **III.3.1**). Correct plasmid DNA sequence must be

evaluated by restriction enzyme digestion and/or by Sanger sequencing. The DNA preparations may be stored in a freezer at -20°C for future use or at 4°C for immediate use.

III.3.3. - DNA Isolation by Gel Electrophoresis.

Agarose gel electrophoresis is a technique for analysis and separation of macromolecules based on their charge and size. By the application of an electric current on an agarose gel (immersed in a conductive buffer) that generates an electric potential difference on both ends of the gel, charged macromolecules are forced to migrate according to their mass and charge. In this project, gel electrophoresis was used both for qualitative analysis of DNA preparations and for isolation of DNA fragments for purification (see **III.3.4**).

The first step was to prepare the 0,8% agarose gel. To start with, the measured quantity of agarose was mixed with Tris-Acetate-EDTA (TAE) electrophoresis buffer (1x) in a sterile Erlenmeyer flask, which was then heated in a microwave until the agarose was completely dissolved. The mass of agarose used was 0,8% of the buffer volume mass (w/w). Following this, the solution was cooled until approximately 50 °C in RT water bath, and 5 μL of Xpert Green DNA Stain (GRiSP) was added per 100 mL of the gelified solution and well mixed. Finally, the solution was slowly dispensed, free from air bubbles, onto the casting support already fitted with a comb with the desired number of wells, where it remained for about 20 – 30 minutes until it was completely solidified and ready to use.

The solidified agarose gel was placed in a custom-made acrylic transparent apparatus (Acrílicos Fernando Gil Lda) and completely immersed in TAE (1x) buffer. Then the comb was removed leaving the wells filled with TEA (1x). After loading each sample in each separate well a voltage of 90V (~6 V/cm) was imposed by a PowerPac™ Basic Power Supply (Bio-Rad®). Following the electrophoresis, the gel was analyzed by UV light in a ChemiDoc™ XRS+ Gel Imaging System (Bio-Rad®) or other similar.

III.3.4. - DNA Band Extraction and Purification by Gel Electrophoresis.

The purification of DNA samples embedded in agarose gel was performed using the NZYGelPure kit (NZYTech®) and following the respective protocol with a few adaptations aimed at increasing the yield of the final purified product. All centrifugations were carried out in a microcentrifuge.

3 μL of loading dye (6x) was added to each of the resuspended pellets and each stained sample was loaded into one or more wells of a pre-prepared 0,8% agarose Tris-Acetate-EDTA (TAE) (1x) gel (see **I.V.3.3**). The appropriate DNA ladder was also loaded at this

point. The loaded gel was run for 40 – 60 minutes at a voltage of 90 – 100 V, after which it was carefully transported to a UV table for the excision of the correct DNA bands. The exposure of the gel to the UV radiation was kept to a minimum to minimize DNA damage. Each fragment was excised with a sterile scalpel blade, cutting the gel as close to the fragment as possible, and placed in a clean pre-labeled 1.5 mL Eppendorf™ tube. Each tube was weighed in a scale plate tared to an empty tube to determine the approximate weight of each extracted gel band. The weight in mg was then converted to volume in µL and 3 volumes of Binding Buffer were added to each tube, followed by an incubation period of 10 minutes at 55 – 60 °C in a dry bath. This incubation is to fully dissolve the agarose since the Binding Buffer is a chaotropic agent responsible for breaking its bonds. Once the gel bands were completely dissolved, one volume of isopropanol was added to each tube and well mixed. After a brief incubation at RT, the mixtures were transferred to spin columns, placed on 2 mL collection tubes, and centrifuged for 1 minute at 13000 rpm. The flowthroughs were discarded onto the spin columns and centrifuged again under the same conditions.

After discarding the flowthroughs, two washing steps were performed: the first one with 500 µL of Washing Buffer and 1 minute of maximum speed centrifugation; and the second one with 600 µL of Washing Buffer and 5 minutes of maximum speed centrifugation. Both centrifugations were carried out at 13000 rpm and the flow-through was discarded each time. The spin-column was centrifuged once again with an empty collection tube at 13000 rpm for 2 minutes to properly dry the silicone membrane of any EtOH.

The final step of the purification was to elute the DNA onto clean and pre-labeled 1.5 mL Eppendorf™ tubes in 30 µL of Elution Buffer. After a brief incubation of 1 minute at RT the tubes were centrifuged at 13000 rpm for 1 minute. To increase the yield of DNA in the final solution, the elution step was repeated once again by reloading the eluted 30 µL onto the spin column and re- centrifuging one more time. The purified DNA samples were quantified (see **III.3.1.**) and stored at -20 °C.

III.3.5. - Sanger Sequencing.

The plasmid DNA sequencing was purchased from Sigma-Aldrich®. The only steps required for sequencing were the preparation of samples and shipment to the sequencing facility by Sigma-Aldrich®. Sanger sequencing is done by random incorporation of fluorescently labeled di-deoxynucleotides (chain terminators) into newly synthetized strands of DNA, (usually less than 1000bp), that are consequently separated by denaturing

acrylamide gel electrophoresis. To perform this sequencing, a mixture of the DNA fragment and a specific end-point primer must be prepared in a proportion determined by the sequencing facility. The primers used in different steps of the work are clearly indicated as in the patent application nº 107101 05-03-2021 INPI (Portugal), [00178] annex 1 and below in the text are reproduced. Each sample for sequencing was prepared in 1.5 mL Eppendorf™ tubes using the following quantities:

- 100 – 400 ng of purified PCR product or 400 – 500 ng of purified plasmid DNA
- 2.5 μL of primer (10 pmol/μL)

After mixing, each tube was marked with a specific bar code ID for identification of the respective sequencing results. The data was delivered in a few days after shipment and consisted of a chromatogram (.ab1, ABI file) of the sequencing, a text file (.seq, SEQ file) and a fasta file (.fas, FAS file) both containing the DNA sequence.

Reproduced from the **patent application nº 117101 05-03-2021 INPI** (Portugal), English translation, annex 1:

"[00178] In an embodied form of realization, after clone expansion, selection of the ones correctly edited is performed. For that, PCRs are performed followed by sequencing of gene BAG2 and gene LGSN in genomic DNA of clonal expanded cell cultures, using primers amplifying sequences in gene BAG2 from outside of BAG2 construct (Cassette 1) and in gene LGSN from outside of LGSN construct (Cassette 2), at both 5' and 3' endings. Confirmation of the complete insertion of all inserts is obtained by PCRamplification of sequences inside the inserts. For the confirmation of the correct insertionin gene BAG2 primers with the following sequences may be used: Seq. ID Nº 13, Seq. IDNº 14, Seq. ID Nº 15, Seq. ID Nº 16, Seq. ID Nº 17, Seq. ID Nº 18, Seq. ID Nº 19, Seq. ID Nº 20, and Seq. ID Nº 21. For confirmation of the correct insert in gene LGSN following primers may be used: Seq. ID Nº 45, Seq. ID Nº 46, Seq. ID Nº 47, Seq. ID Nº48, Seq ID Nº 49, seq ID Nº 50, Seq. ID Nº 51, Seq. ID Nº 52, Seq ID nº53, and Seq. ID Nº 54."

Table 1** - Primer sequences for PCR and Sanger sequencing evaluation of correctly edited clones in Gene BAG2 **in:** Table 24: Sequences of primers and [redacted] in **ANNEX 1: Patent Application 1171001 U7 05-03-2021 INPI.

Seq. ID Nº 13	*FWD 5' GGGTTGAATGAGAGATAAAG 3'*
Seq. ID Nº 14	*REV 5' TGGATGTGGAATGTGTGCGA 3'*
Seq. ID Nº 15	*FWD 5' GAACATCCTGCATACAATAACCGT 3'*
Seq. ID Nº 16	*REV 5' TAAAGCGCATGCTCCAGCCT 3'*
Seq. ID Nº 17	*REV 5' GGCCAGCTTTTCTGAGCTTC 3'*
Seq. ID Nº 18	*FWD 5' TCTGGAGCTCTACCCAGCATA 3'*
Seq. ID Nº 19	*FWD 5' GAACGAGATCAGCAGCCTCT 3'*
Seq. ID Nº 20	*REV 5' TTGGAGCTGGCAAAGGAAGT 3'*
Seq. ID Nº 21	*FWD 5' AATCACGCAGTCACCTTGGG 3'*

Table 2 - *Primer sequences for PCR and Sanger sequencing evaluation of correctly edited clones in Gene LGSN* ***in:*** *Table 24: Sequences of primers and* [redacted]*, in* ***ANNEX 1: Patent Application 1171001 U7 05-03-2021 INPI.***

Seq. ID Nº 45	*FWD 5'AGCCAGGTCCCATGATAGGT 3'*
Seq. ID Nº 46	*REV 5'ACTCGAGAGCAAGAACTGTGG 3'*
Seq. ID Nº 47	*FWD 5'GCAGAAAACTGTCTCTCTTCCT 3'*
Seq. ID Nº 48	*REV 5'ATAAACCCGCAGTAGCGTGG 3'*
Seq. ID Nº 49	*FWD 5'GCCAAGATCTGCACACTGGT 3'*
Seq. ID Nº 50	*REV 5'GCAAGGTGAGATGACAGGAGA 3'*
Seq. ID Nº 51	*FWD 5'CCCACACAAAGGAAAAGGGC 3'*
Seq. ID Nº 52	*REV 5'GGTAGCCAACGCTATGTCCT 3'*
Seq. ID Nº 53	*FWD 5'ATACGCTTGATCCGGCTACC 3'*
Seq. ID Nº 54	*REV 5'TCTCGGAGCCTGCTTTTCAA 3'*

III.3.6. - Genomic DNA Extraction.

Cells were lysed by adding 500 µL of "tail lysis buffer", with freshly added 10uL of 20U/uL Proteinase K, to each cell pellet and flicking the tubes until the pellet was dispersed in the solution. The tubes were then incubated overnight in a dry bath at 56°C. Following the overnight cell lysis of the samples, 55 µL of Sodium Acetate (3 M, pH 5.2) was added to each sample, followed by an addition of 500 µL of phenol/chloroform/isoamyl alcohol. The tubes were then mixed by inverting and centrifuged at 13000 rpm in a microcentrifuge for 10 minutes. This resulted in the

separation of the volume in two phases: an organic phase at the bottom containing the phenol/chloroform/isoamyl alcohol, which retains RNA molecules, lipids, proteins (also in the interface), and unwanted cell debris; and an aqueous phase at the top containing the genomic DNA (gDNA) for extraction. The clear top phases were carefully transferred to new 1.5 mL Eppendorf™ tube (each sample a new tube), while avoiding aspirating any of the organic phase or interface.

Successively, 500 µL of chloroform was added to the tubes containing the aqueous phases. These tubes were vigorously mixed by inverting and centrifuged once again at 13000 rpm for 10 minutes. The purpose of this step was to remove any phenol residues that might have been inadvertently transferred in the previous step, and once again two phases were formed after the centrifugation. The top aqueous phases containing the now purer gDNA were carefully transferred to new 1.5 mL Eppendorf™ tube. To precipitate the gDNA, 450 µL of Isopropanol, and 2uL glycogen (for better visual identification) was added to each tube, followed by a couple of gentle inversions of the tubes for mixing, after which the tubes were incubated at -20°C overnight.

The following day, the samples were centrifuged at 13000 rpm for 30 minutes and the supernatant was carefully discarded to avoid disrupting the pellet. Next, 2 washing steps by EtOH were successively performed, each comprising 700 µL of 70% EtOH addition to each tube to wash the pellet of any remaining contaminants and the tubes were centrifuged at 13000 rpm for 5 minutes. The EtOH was removed and discarded using a pipette and the pellets were left to air-dry for a couple of minutes at RT, (not to over dry). After all the EtOH had evaporated, the dried pellets were resuspended in 100 µL of nuclease-free water, until the pellets had been fully dissolved. To avoid shearing of gDNA, is better to allow for gDNA to resuspend at 4°C for a couple of hours. Finally, the extracted and purified gDNA samples were quantified and stored at -20°C, for future use.

[redacted]

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III.5. - Plasmids Used in the Experiments.

Two plasmid DNAs were used in this work: **eSpCas9 plasmid** (eSpCas9, Addgene plasmid #71814)[173], (**Figure 21**) to subclone the sgRNAs and **plasmid 925** (p925) (**Figure 22**) a plasmid expressing a PGK promoter, Puro Resistance gene (PuroR), the gene for Enhanced Green Fluorescent Protein (EGFP) and a polyadenylation signal sequence (pA). p925 was an in-house prepared plasmid, by Domingos Henrique and Evdguenia Bekman, in Domingos Henrique Lab, at Instituto de Medicina Molecular (IMM), Lisbon, Portugal. This plasmid was used to subclone Cassette1 for gene BAG 2 Editing.

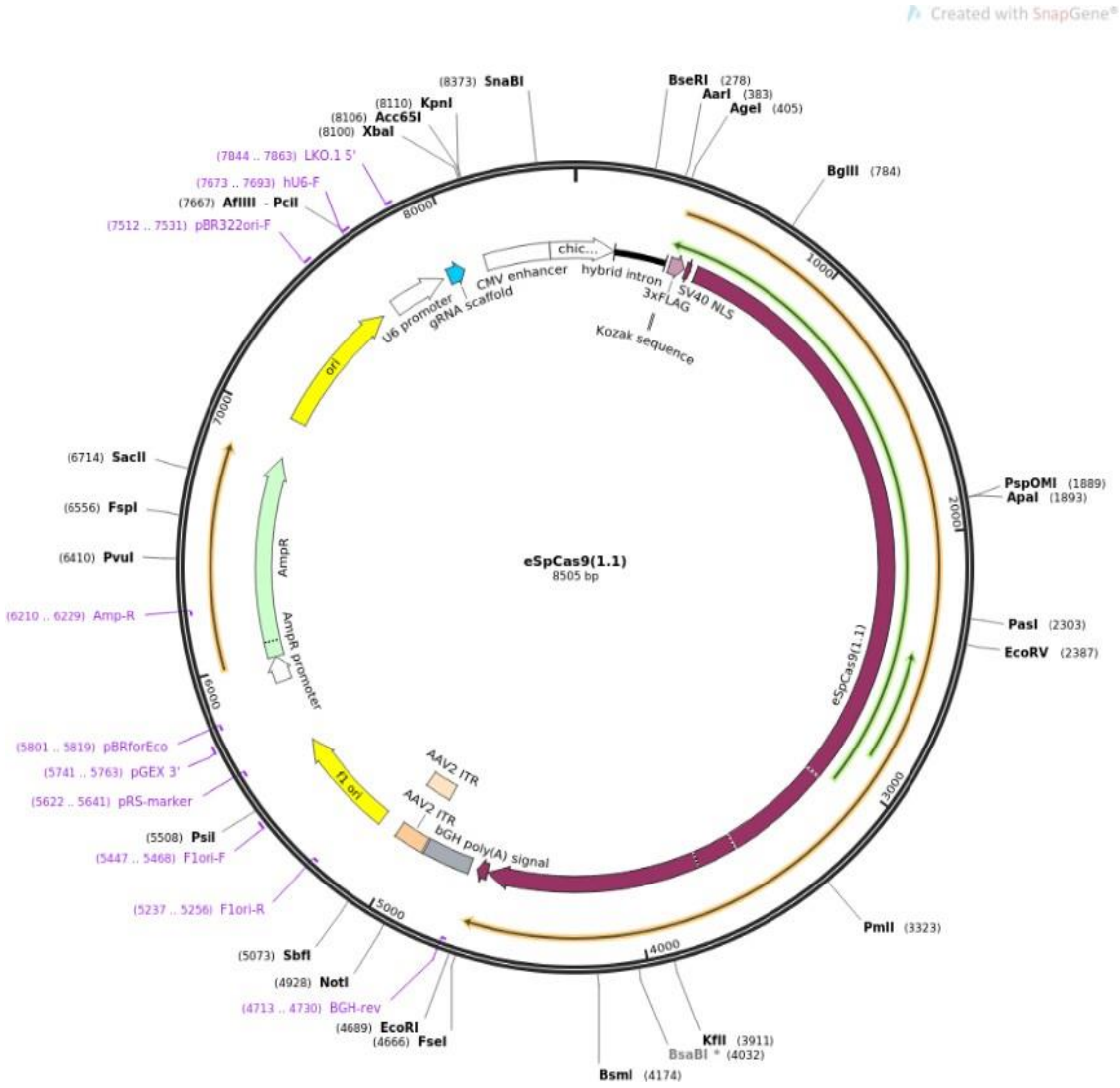


Figure 21 - Map of the eSpCas9 plasmid, Addgene # 71814 a gift from Slaymaker I.M. *et al.* (2015)[173], reproduced from Addgene. Created with SnapGene Viewer.

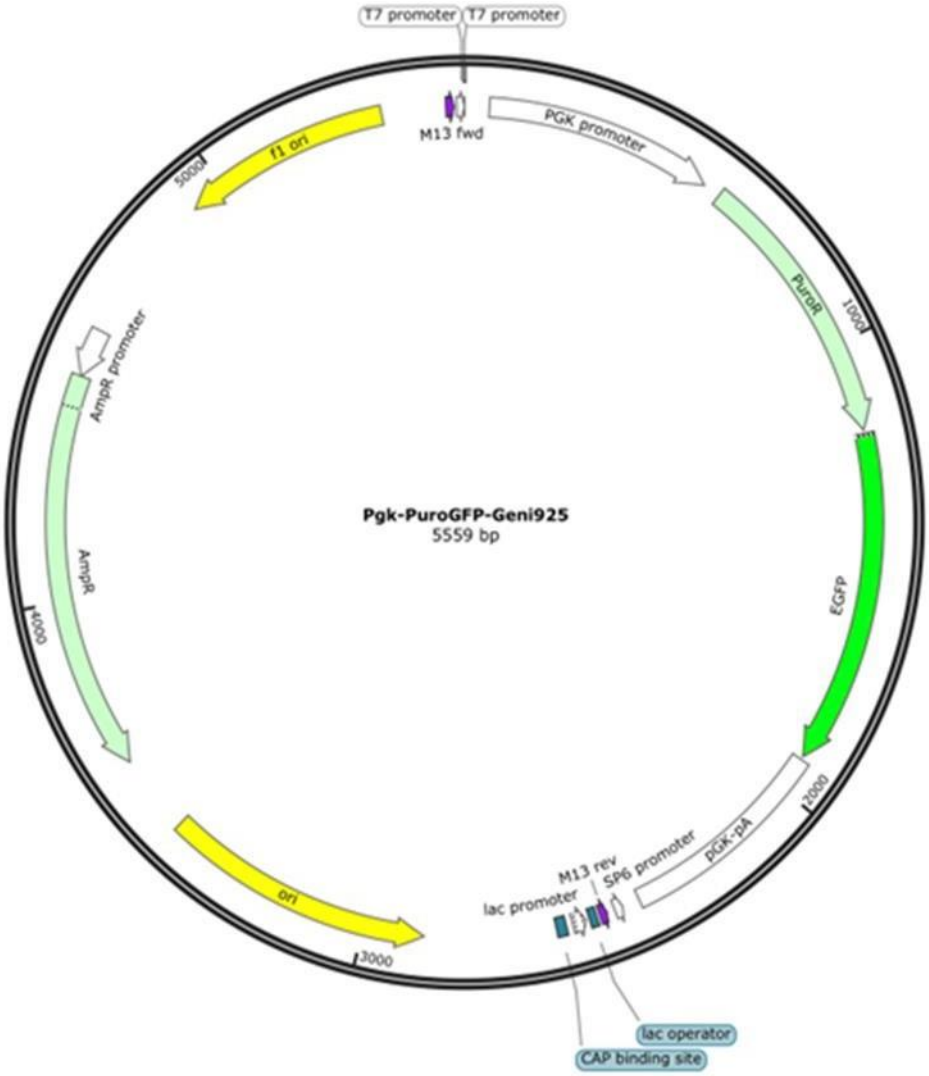


Figure 22 - Map of the pgk-PuroGFP-Geni925 plasmid, used in this work (p295).

III.6. – Subcloning of sgRNAs in eSpCas9 plasmid (eSpCas9, Addgene plasmid #71814), (**Figure 21**).

eCas9 plasmid expresses CRISPR/Cas9 protein and is suitable to express the different sgRNA we may be in need for the Project, by subcloning in a BbsI restriction enzyme site the appropriated oligomeric sequence purchased from the industry. For subcloning ofthe BAG2 sgRNA in eSpCas9, Addgene plasmid #71814 instructions from the authors were followed[372]. In short, For the digestion to take place, the eSpCas9 vector, NEB 2.1 buffer, water to 100uL and *BbsI* enzyme were well mixed in a 1.5 mL Eppendorf™ tubeand incubated at 37°C for 2 hours. To inactivate the enzyme, the tube was placed in a dry

bath at 65°C for 20 minutes and then left in the working bench until the mixture was at RT.

After digestion, an agarose gel was run with 2uL sample digested plasmid vector to evaluate the success of the previous step. In the case of a successful vector digestion, 2 μL of alkaline phosphatase FAST AP (Thermo Scientific™) was then added to the mixture, which was then incubated at 37°C for 30 – 40 minutes to promote the dephosphorylation of the DNA ends, preventing the vector recircularization. To inactivate this enzyme, the tube was placed in a dry bath at 75°C for 5 minutes and then left at RT. After the plasmid eSpCas9 was digested by BbsI and dephosphorylated, a T4 ligase dependent ligation reaction was performed with the different synthetic oligos, already phosphorylated. After an overnight incubation at 15°C, ligation product was used to transform competent cells. After antibiotic selection, (this vector contains a β-lactamase gene, preceded by its promoter, to confer ampicillin resistance, allowing for bacteria selection using this antibiotic, as well as an U6 promoter for mammalian expression and a CMV promoter. Please see **Figure 21**), single colonies were expanded and pDNA(8560bp long) prepared from each, and digested with XbaI/NotI (fragment a- 5334bp long + fragment b- 3172bp long), digested with EcoRV/XbaI (fragment a- 5713bp long + fragment b- 2793bp long) and digested with EcoRV + NotI (fragment a- 5965bp long + fragment b- 2541bp long), to identify the clones containing the desired inserts encoding the adequate sgRNAs. The identity of the clones was confirmed by pDNA sequencing.

Correct clones were expanded and Mini-preps were extracted. The digested plasmid DNA was precipitated and purified using gel electrophoresis. After the purification, the eCas9 vector was ready to be used for promoting DSBs in gene BAG2/sgRNA1, BAG2/sgRNA2, LGSN/sgRNA1 and LGSN/2 ahead of the experiments for selection of the best sgRNAs to perform DSBs in gene BAG2 and LGSN. It was also prepared the vector to promote DSBs in the Nullomer. Samples of correctly transformed cells were store -80°C.

Later, after the selection of the BAG2/sgRNA2 and LGSN/sgRNA1, plasmid Maxi-preps were prepared to be ready for the needed gene BAG2 editing and Nullomer. Several 5uL samples of BAG2/sgRNA2 were digested, precipitated, and thereafter purified by gel electrophoresis extraction and stored at -20°C for the experiments of gene BAG2 editing.

III.7. – Creation by subcloning of Construct for gene BAG2 (Cassette 1) in plasmid p925.

PCR amplifications were performed on a thermal cycler (Applied Systems Thermocycler™), with Taq DNA polymerase (Fermentas®) and using the respective primer dependent protocols, adapted for individual reactions of 50 µL. These PCR amplifications were used to create the Left and Right Homology arms that were subcloned in Plasmid 925 (**Figure 22**) (PGKpmt-PuroR-EGFP-pA), ultimately creating BAG2 construct: Left Homology Arm-PGKpmt-PuroR-EGFP-pA-nullomer-Right Homology Arm (Cassette1).

All experimental work is explained in detail in paragraphs [00114] to [00156] English translation of Portuguese Patent Application 117101/05-March-2021, Instituto Nacional da Propriedade Industrial (INPI). Annex 1 to this Thesis, pages 34 to 53. Referred primers are listed on page 63 - 65 (List of Sequences) same Annex 1 to this Thesis.

"In one embodiment, Cassette 1/Gene BAG2 (Seq. ID No 43) comprises a 5' or left homology arm (5'HA or left homology arm or left HA, 1783 base pairs, bp); a PGK promoter (phosphoglycerate kinase promoter – PGK pmt); a puromycin resistance gene; the enhanced green fluorescent protein gene (EGFP); a polyadenylation sequence (Poly(A) or pA[redacted]; and a 3' or right homology arm (3'HA or right homology arm or right HA, 1590 bp).

In the context of the present description, a plasmid expressing ampicillin resistance for the selection of perfectly edited clones and a cassette comprising the PGK promoter, the puromycin resistance gene, the EGFP protein gene and a Poly(A) sequence are referred to as 'plasmid p925', or simply p925.

In one embodiment, different primers were used in the preparation of the various components of Cassette 1/Gene BAG2:

- *• Seq. ID No 7 and Seq. ID No 8 were used as primers for the synthesis of the 5' homology arm sequence, (1783bp), prepared for subcloning (or insertion) into recognition sites for the restriction endonucleases EcoRI and BamHI on plasmid p925, (PGK-PuroR-EGFP-2pA);*
- *• Seq. ID No 9 and Seq. ID No 10 were used as primers for the synthesis ofthe 3' homology arm sequence, (1590bp), prepared for subcloning (or insertion)*

into recognition sites for the restriction endonucleases BamHI and NotI on the plasmid (p925);

- *• Primers Seq. ID No 11 and Seq. ID No 12 were used for the synthesis of polyadenylation and* [redacted] *sequences, prepared for subcloning (or insertion) into recognition sites for the restriction endonucleases XbaI and NotI on the plasmid (p925).*

The BAG2 gene editing vector can be obtained in two distinct ways. One way is to purchase the complete vector from the industry, delivered as a plasmid in frozen competent cells. Culture of these competent cells allows the vector to be obtained after isolation using purification systems such as the Wizard™ Plus SV MINIPREPS DNA Purification System (Promega™) or some other equivalent system. In an alternative embodiment, the vector can be prepared by subcloning its various promoters and genes, either in a virgin plasmid (empty backbone plasmid) or in a more complete and suitable one.

In implementing this example, subcloning was performed on a plasmid (p925) containing the PGK promoter, the puromycin resistance gene (PuroR), the EGFP protein gene, and a Poly(A) sequence, as well as an ampicillin resistance gene for selection of the correctly transformed competent cells.

In one embodiment, subcloning was carried out in three steps.

[The first step comprises the subcloning of the Poly(A) + [redacted] *sequence into XbaI and NotI sites on the p925 plasmid. The Poly(A) and* [redacted] *sequences were subcloned downstream of the EGFP gene sequence to allow both the selection of correctly edited off-the-shelf cell clones as well as, at a later stage than chromosome replacement, the elimination of clones from cells with maintenance of PuroR and EGFP gene expression. (Figure 4**).** The primers Seq. ID No 11 and Seq. ID No 12 were used in the polymerase chain reaction (PCR) for the synthesis of the Poly (A) +* [redacted] *sequences prepared for their subcloning (binding) to the appropriate XbaI and NotI sites on plasmid p925.*

In one embodiment, for the PCR reaction, a solution was prepared with the mixture of the various reagents (MIX), in accordance with Table 1. After preparation, 47.5uL of the obtained MIX was mixed with 2.5uL/40ng p925DNA [20ng/uL]. The PCR reaction

proceeded in a thermal cycler (Applied Systems Thermocycler™), under the conditions indicated in Table 2.

***Table 1:** Concentration of reagents used in the PCR reaction.*

Reagents (MIX)	***1 reaction***	***2 reactions***
10x Dream Taq Buffer (Fermentas™)	*2.5uL*	*5uL*
dNTPs [10mM]	*2.0uL*	*4uL*
Primer FWD [redacted] + Poly(A) p925 [10uM] (SEQ ID No 11)	*2.0uL*	*4uL*
Primer REV [redacted] + Poly A p925 [10uM] (SEQ ID No 12)	*2.0uL*	*4uL*
PCR H_2O up to 50uL	*41uL*	*82uL*
[5units/uL] Dream Taq (Fermentas™)	*2U/ 0.5uL*	*1uL*
Total	*50uL*	*100uL*

***Table 2:** PCR conditions in a thermal cycler.*

PCR		***Temperature***	***Time***
	Initial denaturation	*95ºC*	*3:00 minutes*
	Denaturation	*95ºC*	*30 seconds*
35 cycles	*Hybridisation*	*55ºC*	*30 seconds*
	Extension	*72ºC*	*1:00 minute*
	Final Extension	*72ºC*	*10:00 minutes*
	Wait	*4ºC*	*10:00 minutes*

After the PCR, a 5uL sample of the PCR product was electrophoresed on an 0.8% agarose gel to confirm the presence of a 470bp band, which contains the PCR product composed of the polyadenylation sequence and [redacted]. After checking for the presence of the band, we proceeded with the electrophoresis of all the remaining PCR product in a 0.8% agarose gel. The 470bp band was extracted from the gel, with an appropriate kit, (e.g. Wizard™ SV Gel and PCR clean-up System – PROMEGA™), following the manufacturer's instructions. The obtained product (PCR DNA) was resuspended in 20uL of PCRH_2O, i.e. water free of the RNAase and DNAase enzymes.

The product obtained, and already resuspended, was subjected to double digestion of the restriction endonucleases XbaI + NotIHF, using the conditions mentioned in Table 3, for 2 hours at 37 ºC.

Table 3: *Conditions used for the digestion of DNA obtained in the PCR reaction by the restriction enzymes XbaI and NotIHF.*

PCR DNA	*20.0uL*
10x Cut Smart Buffer (NEBiolabs™)	*5.0uL*
[50U/uL] XbaI (20U) (Fermentas™)	*1.0uL*
[20U/uL] NotIHF (20U) (NEBioLabs™)	*1.0uL*
PCR H_2O up to 50 uL	*23.0uL*
TOTAL	*50.0uL*

After XbaI + NotI double digestion, the obtained product was purified with a phenol/chloroform/isoamyl alcohol mixture, precipitated in ethanol and resuspended in 20uL of PCR H_2O. After purification and precipitation, the obtained insert contains the sequence Poly (A) + Nullomer, ready to bind to the p925 vector.

In one embodiment, the p925 vector is prepared for binding to the obtained insert. For this, a double digest with restriction endonucleases XbaI and NotI, and a dephosphorylation was performed.

Double digestion with XbaI and NotI was performed with a 5ug sample of DNA from plasmid p925 using the conditions described in Table 4. After preparing the solution, the sample was incubated for 2 hours at 37 ^{o}C.

Table 4: *Experimental conditions for double digest by endonucleases XbaI and NotIHF of plasmid p925.*

[259ng/uL] P925 DNA (5ug)	*19.3uL*
10x Cut Smart Buffer (NEBiolabs™)	*10.0uL*
[50U/uL] XbaI (20U) (Fermentas™)	*2.0uL*
[20U/uL] NotIHF (20U) (NEBioLabs™)	*5.0uL*
PCR H_2O up to 100 uL	*63.7uL*
TOTAL	*100.0uL*

After XbaI + NotIHF double digestion, a 5uL sample of the obtained product was subjected to electrophoresis on a 0.8% agarose gel to confirm the presence of the correct band. After confirmation, the DNA product was purified with phenol/chloroform/isoamyl alcohol, precipitated in ethanol, and resuspended in 20uL of PCRH_2O.

After purification, precipitation, and resuspension of the DNA product, it was dephosphorylated using the conditions described in Table 5. The reaction was carried out at 37 ºC for 10 minutes and was terminated by increasing the temperature (5 minutes at 75 ºC). After the reaction, further purification was performed with phenol/chloroform/isoamyl alcohol and precipitation with ethanol. The obtained product (p925/double digest XbaI + NotI/Dephosphorylated vector) is ready for subcloning (binding) with the insert (Poly (A) + [redacted]*) and can be preserved at -20 ºC for future use.*

Table 5: *Experimental conditions for dephosphorylation.*

P925/XbaI + NotI HF digested DNA	*20uL*
10XFast Alkaline Phosphatase (AP) Buffer (Thermo Scientific®)	*5uL*
Fast Alkaline Phosphatase (AP) (Thermo Scientific®)	*5uL*
PCR H_2O up to 50uL	*20uL*
TOTAL	*50uL*

Before proceeding to binding the p925/double digested XbaI + NotI/Dephosphorylated vector with the insert (Poly (A) + nullomer), a 1uL sample of the former and 2uL of the latter were electrophoresed on a 0.8% agarose gel to assess their relative concentrations. A ratio of 1:5 was obtained between the vector and the insert, respectively.

The binding (subcloning) of the p925/double digested XbaI+ NotI/Dephosphorylated vector with the insert (Poly (A) + [redacted]*) was performed according to Table 6.*

Table 6: *Mixture of binding reagents.*

MIX of reagents - trade names	***Volume***
10x T4 DNA ligase Buffer (Promega™)	*6.25uL*
Vector p925 XbaI+NotI +AP	*2.5uL*
[3U/uL] T4 DNA ligase (Promega™)	*6.25U = 2.5uL*
PCR H_2O up to 25uL	*10uL*
ATP [10mM]	*3.75uL*
TOTAL	*25uL*

In one delivery mode, 8uL of the mix mentioned in Table 6 and 2uL of the insert (Poly (A) A + [redacted] *) were pipetted into a round-bottom tube (Tube 1). In another round-bottom tube (Tube 2, negative control), 8uL of the mixture and 2uL of PCR H_2O were pipetted. Both tubes were incubated at 15 °C for at least two 2 hours, preferably overnight. After incubation, E. coli competent cells (purchased commercially) were transfected with 5uL of the contents of both Tube 1 and Tube 2. The presence of correctly inserted colonies was confirmed by sequencing, and samples were stored at -80 °C in glycerol. (Sigma Aldrich® – 66DE65 and 66DE66). From one correctly inserted colony, an inoculation was made in 100mL of LB ampicillin 100 solution in an Erlenmeyer flask, which was incubated overnight in a horizontal shaker. After incubation, an extraction of a MIDI or MAXI-prep (e.g. QIAGEN™ Midi or Maxi Kit) was performed to obtain a sufficient amount of plasmid to be used in the following steps.*

In one embodiment, the method described in the present disclosure comprises a second subcloning step, for binding the 5' or left homology arm, upstream of the PGK promoter on the p925/Poly(A)+ [redacted] *plasmid). The 5' or left homology arm (1873bp), was bound (subcloned) on an EcoRI site immediately upstream of the PGK promoter on the p925/Poly(A)+* [redacted] *plasmid. A BamHI site was provided at the 5' end of the sequence to allow isolation of the complete vector for BAG2 gene editing.*

In one embodiment, the PCR product was obtained from genomic DNA (gDNA) from HEK293T cells using the NZY Tissue gDNA Isolation Kit (NZYtech®), following the manufacturer's instructions. Using the primers Seq. ID No 7 and Seq. ID No 8, a solution was prepared with the mixture of the various reagents (MIX), according to Table7, and then 45uL of the MIX obtained was mixed with 5uL (200ng) of gDNA [41ng/uL] isolated

from HEK293T. The PCR reaction proceeded in a thermal cycler (Applied Systems Thermocycler™), under the conditions indicated in Table 8.

Table 7: *Concentration of reagents used in the PCR reaction.*

Reagents (MIX) - trade names	***Volume***
10x Dream Taq Buffer (Fermentas™)	*6.25uL*
dNTPs [10mM]	*5.0uL*
Primer FWD Hom. Arm Left BAG2 [10uM] (SEQ. ID No 7)	*3.0uL*
Primer REV Hom. Arm Left BAG2 [10uM] (SEQ. ID No 8)	*3.0uL*
PCR H_2O	*28.25uL*
[5units/uL] Dream Taq (Fermentas™)	*2U/ 1uL*
$MgCl_2^+$ [25mM]	*2.5uL*
TOTAL	*50.0uL*

Table 8: *PCR conditions in the thermal cycler.*

PCR		***Temperature***	***Time***
	Initial denaturation	*95ºC*	*3:00 minutes*
	Denaturation	*95ºC*	*30 seconds*
35 cycles	*Hybridisation*	*58ºC*	*30 seconds*
	Extension	*72ºC*	*2:00 minutes*
	Final Extension	*72ºC*	*10:00 minutes*
	Wait	*4ºC*	*3:00 minutes*

After the PCR, a 2uL sample of the PCR product was electrophoresed on an 0.8% agarose gel to confirm the presence of a 1783bp band, corresponding to the DNA fragment containing the sequence of the left homology arm of Cassette 1. Having checked for the presence of the band, the remaining DNA obtained in the PCR reaction was purified by phenol/chloroform/isoamyl alcohol and digested with the restriction enzyme EcoRI, according to Table 9, for 2 hours at 37 ºC.

Table 9: *Experimental conditions for the digestion of the PCR product (left homology arm BAG2) by the EcoRI enzyme.*

Left homology arm BAG2, PCR product	*10ul*
NEBuffer 2.1	*2.5uL*
EcoRI (Promega™, [12U/uL]	*1.0uL*
PCR H_2O up to 20uL	*6.5uL*
TOTAL	*20.0uL*

After digestion, a 2uL sample of the digestion product was electrophoresed on an 0.8% agarose gel to confirm the presence of a 1783bp band, corresponding to the DNA fragment produced by PCR and containing the left homology arm of Cassette 1. Verifying the presence of the band, the remaining digested product was isolated using the extraction and purification of the band from the 0.8% agarose gel using the Wizard™ SV Gel and PCR clean-up System - PROMEGA™, following the manufacturer's instructions. The obtained product (digested BAG2 left homology arm) was then purified by phenol/chloroform/isoamyl alcohol, precipitated in ethanol and resuspended in 20uL PCR H_2O.

Then, the p925/Poly(A)+[redacted] vector was bound to the digested BAG2 left homology arm insert. In one embodiment, the p925/Poly(A)+nullomer plasmid was digested with the restriction enzyme EcoRI (Table10), to prepare the vector for binding (subcloning) with the previously isolated left homology arm PCR product also digested with EcoRI. Digestion occurred for 2 hours at 37 ºC.

Table 10: *Experimental conditions for the digestion of plasmid vector p925/Poly(A)+[redacted] by the EcoRI enzyme.*

P925/Poly(A)+[redacted]	*5ug = 13.5uL*
10x NEBuffer 4 (NEBioLabs)	*10.0uL*
20U/uL EcoRI HF (NEBioLabs)	*5.0uL*
PCR H_2O up to 100uL	*71.5uL*
TOTAL	*100.0uL*

Complete digestion was confirmed by electrophoresis in 0.8% 5ul agarose gel of the digested product. After checking for complete digestion, the digested product was purified by phenol/chloroform/isoamyl alcohol and precipitated in ethanol. Finally, the product was dephosphorylated following the protocol shown in Table 11. After incubation for 10 minutes at 37 ºC in a water bath, the reaction was terminated by incubating for 5 minutes at 75 ºC. The product obtained was purified by phenol/chloroform/isoamyl alcohol and precipitated in ethanol.

Table 11: *Dephosphorylation protocol.*

P925/Poly(A)+[redacted]/EcoRI purified and precipitated	*20.0uL*
10x Fast Alkaline Phosphatase (AP) Buffer (Thermo Scientific®)	*5.0uL*
[1U/uL] Fast Alkaline Phosphatase (AP) (Thermo Scientific®)	*5.0uL*
PCR H_2O up to 50uL	*20.0uL*
TOTAL	*50.0UL*

In one embodiment, the digested and dephosphorylated p925/Poly(A) vector was ligated to the BAG2 left homology arm insert, also already digested by EcoRI, using the conditions described in Table 12.

Table 12: *Preparation of reagent solution (MIX) for binding the digested and dephosphorylated p925/Poly(A) vector to the BAG2 left homology arm insert.*

Reagents mixture (MIX) - trade names	***Volume***
10x T4 DNA ligase Buffer (Promega™)	*6.25uL*
Digested and dephosphorylated p925/Poly(A)+nullomer/EcoRI Vector	*5.0uL*
[3U/uL] T4 DNA ligase (Promega™)	*6.25U = 2.5uL*
PCR H_2O	*7.5uL*
ATP [10mM]	*3.75uL*
TOTAL	*25uL*

In one embodiment, 4uL of the MIX and 6uL of the insert (digested BAG2 left homology arm) were pipetted into a round-bottom tube (Tube 1). In another tube (Tube 2, negative control tube), 4uL of the mixture and 6uL of PCR H_2O were pipetted. Both tubes were incubated at 15 °C for at least two 2 hours, preferably overnight. After incubation, E. coli competent cells (purchased commercially) were transformed with 5uL of the contents of

both Tube 1 and Tube 2. After transformation, plasmids were extracted from several colonies using the Wizard™ Plus SV Minipreps DNA Purification System, 250 preps, PROMEGA™, and the obtained DNA quantified, using Nanodrop™. The correct insertion of the plasmids was confirmed by restriction enzymes and/or sequencing, using methods widely known in the state of the art.

From one correctly inserted colony, an inoculation was made in 100mL of LB ampicillin 100 solution in an Erlenmeyer flask, which was incubated overnight in a horizontal shaker. After incubation, an extraction of a MIDI or MAXI-prep (e.g. QIAGEN™ Midi or Maxi Kit) was performed to obtain a sufficient amount of plasmid to be used in the following steps.

In one embodiment, after isolation of the correctly inserted plasmid p925/Poly(A)+nullomer/left homology arm, a third subcloning step was initiated to effect the binding of the right homology arm downstream of the nullomer to the vector p925/Poly(A)+ [redacted] */left homology arm.*

The 3' or right homology arm, (1590bp), was bound(subcloned), on a NotI site immediately downstream of [redacted] *in plasmid p925/Poly(A)+nullomer/left homology arm. A BamHI site was provided at the 3' end of the sequence to allow future isolation of the complete vector for BAG2 gene editing.*

The PCR product was obtained from genomic DNA from HEK293T cells using the NZY Tissue gDNA Isolation Kit (NZYtech®), following the manufacturer's instructions. Using the primers Seq. ID No 9 and Seq. ID No 10, a solution was prepared with the mixture of the various reagents (MIX), according to Table 13, and then 45uL of the MIX obtained was mixed with 5uL (200ng) of gDNA [41ng/uL] isolated from HEK293T. The PCR reaction proceeded in a thermal cycler (Applied Systems Thermocycler™), under the conditions indicated in Table 14.

Table 13: *Concentration of reagents used in the PCR reaction.*

Reagents (MIX) – trade names	***Volume***
10x Dream Taq Buffer (Fermentas™)	*6.25uL*
dNTPs [10mM]	*5.0uL*
Primer FWD Hom. Arm Right BAG2 [10uM] (Seq. ID No 9)	*3.0uL*
Primer REV Hom. Arm Right BAG2 [10uM] (Seq. ID No 10)	*3.0uL*
PCR H_2O up to 50uL	*29.25uL*
[5units/uL] Dream Taq (Fermentas™)	*1.0uL*
$MgCl_2^+$ [25mM]	*2.5 uL*
TOTAL	*50.0uL*

Table 14: *PCR conditions in the thermal cycler.*

PCR		***Temperature***	***Time***
	Initial denaturation	*95ºC*	*3:00 minutes*
	Denaturation	*95ºC*	*30 seconds*
35 cycles	*Hybridisation*	*50ºC*	*30 seconds*
	Extension	*72ºC*	*2:00 minutes*
	Final Extension	*72ºC*	*10:00 minutes*
	Wait	*4ºC*	*3:00 minutes*

After PCR, a 2uL sample of the PCR product was electrophoresed on 0.8% agarose gel to confirm the presence of a 1590bp band that corresponds to the DNA fragment produced by PCR and that contains the right homology arm. Having checked for the presence of the band, the remaining DNA obtained in the PCR reaction was purified by phenol/chloroform/isoamyl alcohol, precipitated in ethanol, and digested with the restriction enzyme NotI, according to Table 15, for 2 hours at 37 ºC.

Table 15: *Experimental conditions for the digestion of the right homology arm BAG2 by the NotI enzyme.*

PCR product (Right Homology Arm BAG2)	*10ul*

NEBuffer 3.1	*2.5uL*
NotIHF (NEBioLabs, [20U/uL]	*1.0uL*
PCR H_2O up to 20uL	*6.5uL*
TOTAL	*20.0uL*

Complete digestion was confirmed by electrophoresis of the digested product in 0.8% agarose gel. Having checked for complete digestion, the digested product was isolated and purified by gel extraction after electrophoresis in 0.8% agarose gel using the Wizard™ SV Gel and PCR clean-up System - PROMEGA™, following the manufacturer's instructions. Then, purification of the obtained product was performed by phenol/chloroform/isoamyl alcohol, followed by precipitation in ethanol. Finally, the precipitate obtained, which contains the BAG2 right homology arm 2 (Cassette 1) ready to be bound (subcloned), was resuspended in 20uL PCR H_2O.

Before binding of the BAG2 right homology arm insert, it was necessary to prepare the p925/Poly(A)+ [redacted] */left homology arm BAG2 vector by digestion with the NotIHF enzyme. For this, the vector was treated with Plasmid-Safe™ ATP-Dependent DNase (EPICENTRE®) using the reagents described in Table 16. The mixture was incubated at 37 ºC for 30 minutes, and after this period the Plasmid-Safe™ DNAse enzyme was inactivated by incubation at 70 ºC for 30 minutes, stopping the reaction.*

Table 16: *Treatment of the p925/Poly(A)+[redacted]/left homology arm BAG2 vector with Plasmid-Safe™ ATP-Dependent DNase (EPICENTRE®).*

Reagents (MIX) – trade names	***Volume***
p925/Poly(A)+nullomer/left homology arm BAG2 [1214.9ng/ul].	*20ug = 16.5uL*
PCR H_2O up to 50uL	*25.5uL*
10x Plasmid-Safe™ Buffer (Epicentre®)	*5.0uL*
ATP [25mM] (Epicentre®)	*2.0uL*
[10U/uL] DNAse Plasmid-Safe™ (10U) (Epicentre®)	*1.0uL*
TOTAL	*50.0uL*

The reaction product was purified by phenol/chloroform/isoamyl alcohol, precipitated with ethanol and resuspended in 20ul PCR H_2O.

After treatment with Plasmid-Safe™, the plasmid p925/Poly(A)+[redacted]/left homology arm was digested with the restriction enzyme NotIHF for 2 hours at 37 ºC in a water bath according to the protocol shown in Table 17.

Table 17: *Conditions used in the digestion of plasmid p925/Poly(A)+nullomer/left homology arm by the restriction enzyme NotIHF*

P925/Poly(A)+[redacted]r/left homology arm (1ug/uL)	*1.0uL*
10x Cut Smart Buffer (NEBioLabs)	*10.0uL*
20U/uL NotI HF (NEBioLabs)	*3.0uL*
PCR H_2O up to 50uL	*36.0uL*
TOTAL	*50.0uL*

After complete digestion, confirmed by electrophoresis in 0.8% agarose gel, the resulting plasmid was purified by phenol/chloroform/isoamyl alcohol, precipitated with ethanol, and resuspended in 20ul PCR H_2O. Finally, the product was dephosphorylated following the protocol shown in Table 18. After incubation for 10 minutes at 37ºC in a water bath, the reaction was terminated by incubating for 5 minutes at 75 ºC. The product obtained was purified by phenol/chloroform/isoamyl alcohol, precipitated in ethanol, and resuspended in 50uL PCR H_2O.

Table 18: *Dephosphorylation protocol of the vector p925/Poly(A)+nullomer/left homology arm treated and digested.*

P925/Poly(A)+[redacted]r/left homology arm vector treated and digested	*20.0uL*
10x Fast Alkaline Phosphatase (AP) Buffer (Thermo Scientific®)	*5.0uL*
[1U/uL] Fast Alkaline Phosphatase (AP) (Thermo Scientific®)	*5.0uL*
PCR H_2O up to 50uL	*20.0uL*
TOTAL	*50.0UL*

In one embodiment, the digested and dephosphorylated p925/Poly(A)+[redacted]r/left homology arm vector was bound to the right homology arm BAG2 insert, also already digested by NotI, using the conditions described in Table 19.

Table 19: *Preparation of the MIX solution for binding the digested and dephosphorylated Poly(A)+[redacted]/left homology arm vector to the right homology arm BAG2 insert.*

Reagents (MIX) – trade names	***Volume***
10x T4 DNA Ligase Buffer (Fermentas®)	*6.25uL*
Digested and dephosphorylated p925/Poly(A)+[redacted]r/left homology arm vector	*2.5uL*
[5U/uL] T4 DNA Ligase (Fermentas®)	*2.5uL*
PCR H_2O up to 10ul	*12.5uL*
ATP [10mM]	*3.75uL*
TOTAL	*23.0uL*

In one embodiment, 4uL of the MIX and 6.4uL of the insert (digested BAG2 right homology arm) were pipetted into a round-bottom tube (Tube 1). In another tube (Tube 2, negative control tube), 4uL of the mixture and 6uL of PCR H_2O were pipetted. Both tubes were incubated at 15 °C for at least two 2 hours, preferably overnight. After incubation, E. coli competent cells (purchased commercially) were transformed with 5uL of the contents of both Tube 1 and Tube 2. After transformation, plasmids were extracted from several colonies using the Wizard™ Plus SV Minipreps DNA Purification System, 250 preps, PROMEGA™, and the obtained DNA quantified, using Nanodrop™. The

correct insertion of the plasmids was confirmed by restriction enzymes and/or sequencing, using methods widely known in the state of the art.

From one correctly inserted colony, an inoculation was made in 100mL of LB ampicillin 100 solution in an Erlenmeyer flask, which was incubated overnight in a horizontal shaker. After incubation, an extraction of a MIDI or MAXI-prep (e.g. QIAGEN™ Midi or Maxi Kit) was performed to obtain a sufficient amount of plasmid to be used in the following steps.

After isolation of the correctly inserted plasmid p925/poly(A)+ [redacted]r/left homology arm/right homology arm, designated R52, the complete vector construct for editing the BAG2 gene on the long arm of human chromosome 6 was completed (Figure2), (Seq. ID No 43).

In one embodiment, the R52 construct prepared for editing the BAG2 gene is flanked by two recognition sites for the restriction enzyme BamHI. Taking advantage of this intentional feature, the vector for editing the BAG2 gene was prepared by its excision by digestion with BamHI through the reaction described in Table 20.

***Table 20:** Reagent mixture prepared for the digestion of construct R52 by the restriction enzyme BamHI*

Reagents (MIX) – trade names	***Volume***
10x NEBuffer 3	*6.0uL*
10U/uL BamHI (ThermoScientific®)	*3.0ul*
PCR H_2O up to 100uL	*88,0uL*
R52 pDNA [981ng/uL]	*3,0uL*

In one embodiment, 94uL of MIX and 6uL of R52 plasmid DNA were pipetted into a microtube, which was then incubated for 2 hours at 37 ºC in a water bath. After digestion with BamHI, the DNA was purified by phenol/chloroform/isoamyl alcohol, followed by precipitation in ethanol. After precipitation, the R52 construct was ready for cell transfection, where transfection is the procedure where DNA is taken into cells in order to achieve changes in their genome.

After construct preparation, human pluripotent stem cells (hiPSCs) were transfected into the BAG2 gene with the R52 vector containing Cassette 1."

List of Primer sequences:

Table 24: *Primer sequences and* [redacted].

Seq. ID No 3	*FWD 5' CCACTGTCCTGCTTCTATCTGG 3'*
Seq. ID No 4	*REV 5' CTTGCTGCTGGGGGTTTCTA 3'*
Seq. ID No 5	*FWD 5' GCTGTTTGAGAGCTGAGAGTACA 3'*
Seq. ID No 6	*REV 5'ATCCAACTCTAAGCCAGTGACA 3'*
Seq. ID No 7	*FWD 5'cggaattcggatccTATAGGGTTGAAGCTTTGAGAGAAGC 3'*
Seq. ID No 8	*REV 5'gcgaattcAACGGTTTGCAGTCAGATTTAATTC 3'*
Seq. ID No 9	*FWD 5'ataagaatgcggccgcTGATGGGAAGAACTCTCACCGT 3'*
Seq. ID No 10	*REV 5'ataagatagcggccgcggatccAAAATAGTATCCAGGGAAGTTG T 3'*
Seq. ID No 11	*FWD 5'ctgcatcccgctctagaTATTACTTGTACAGC 3'*
Seq. ID No 12	*REV 5'catagaagcggccgcatCCGATAACGTCGGTCCGAGCG TACCCAGCTTCTGATGGAATTAGAACTTG 3'*
Seq. ID No 13	*FWD 5' GGGTTGAATGAGAGATAAAG 3'*
Seq. ID No 14	*REV 5' TGGATGTGGAATGTGTGCGA 3'*
Seq. ID No 15	*FWD 5' GAACATCCTGCATACAATAACCGT 3'*
Seq. ID No 16	*REV 5' TAAAGCGCATGCTCCAGCCT 3'*
Seq. ID No 17	*REV 5' GGCCAGCTTTTCTGAGCTTC 3'*
Seq. ID No 18	*FWD 5' TCTGGAGCTCTACCCAGCATA 3'*
Seq. ID No 19	*FWD 5' GAACGAGATCAGCAGCCTCT 3'*
Seq. ID No 20	*REV 5' TTGGAGCTGGCAAAGGAAGT 3'*
Seq. ID No 21	*FWD 5' AATCACGCAGTCACCTTGGG 3'*

After expansion of correctly transformed cell clones, samples were stored at -80°C and plasmid Maxi-preps were prepared to be ready for the needed gene BAG2 editing. Several 5uL samples of BAG2/sgRNA2 were BamHI digested, precipitated, and thereafter

purified by gel electrophoresis extraction and stored at -20°C for the experiments of gene BAG2 editing.

For Gene LGSN, decision was made to purchase construct (Cassette 2) from the industry, as it was understood that this approach will significantly save money and time.

III.8. - PCR Amplification of gDNA from Transfected hiPSCs (in bulk) and WT hiPSCs.

PCR amplifications were performed on a thermal cycler, (Applied Systems Thermocycler™), with Taq DNA polymerase (Fermentas®) and using the primers dependent protocols, adapted for individual reactions of 50 μL.

All experimental work is explained in detail in paragraphs [00161] to [00164] English version of Portuguese Patent Application 117101/05-March-2021, Instituto Nacional da Propriedade Industrial (INPI). Annex I to this Thesis, pages 54 to 56. Referred primers are listed on page 63 - 65 (List of Sequences) same Annex I to this Thesis and transcribed below.

" *Figure 5 shows the results of PCR reactions obtained on genomic DNA samples from Wild Type hiPSCs (WT) as a negative control, and genomic DNA from hiPSCs of the same line as the WT but co-transfected, using SEQ primers. ID No 15, SEQ. ID No 16, SEQ. ID No 17, SEQ ID No 18, SEQ. ID No 19, SEQ. ID No 20 and SEQ ID No 21, which allowed the presence of correctly edited cells to be assessed. The conditions for the PCRs are listed in Table 21 and were prepared with the tubes of all reagents placed on ice.*

Table 21: *Concentration of reagents used in the PCR reactions.*

Reagents (MIX)	***1 reaction***	***12 reactions***
10x Dream Taq Buffer (Fermentas™)	*2.5uL*	*30uL*
dNTPs [10mM]	*2.0uL*	*24uL*
WT genomic DNA	*5.0uL*	*30uL*
Genomic DNA Co-transfected	*5.0uL*	*30uL*
PCR H_2O up to 50uL	*41uL*	*492uL*
[5units/uL] Dream Taq (Fermentas™)	*2U/ 0.5uL*	*6uL*

In one embodiment, 279uL of MIX was pipetted into each of 2 separate microtubes (microtube 1 and microtube 2).

A- *To microtube 1, 30uL of WT= Wild Type genomic DNA was added;*

B- *To microtube 2, 30uL of the genomic DNA of the co-transfected hiPSCs was added;*

C- *Five microtubes marked with the numbers corresponding to the respective SEQs were prepared. ID No 15 to 21: SEQ. ID No 15 + SEQ. ID No 16 = 5'off construct up to PGK promoter (PGKpmt); SEQ. ID No 15 + SEQ. ID No 17 = 5'off construct up to homology arm 5'(5'HA)(~500bp); SEQ. ID No 20 + SEQ. ID No 21 = 3'out of construct up to homology arm 3'(3'BH)(~900bp); SEQ. ID No 16 + SEQ. ID No 18 = homology arm 5'(5'HA) up to PGK promoter (PGKpmt); SEQ. ID No 19 + SEQ. ID No 20 = 3' off construct to the Nullomere, for the 5 different negative control PCR reactions (WT= Wild Type genomic DNA);*

D- *Another 5 microtubes were prepared marked with the numbers corresponding to the respective Seq. ID No 15 to 21: SEQ. ID No 15 + SEQ. ID No 16 = 5'off construct up to PGK promoter (PGKpmt); SEQ. ID No 15 + SEQ. ID No 17 = 5'off construct up to homology arm 5'(5'BH)(~500bp); SEQ. ID No 20 + SEQ. ID No 21= 3'off construct to homology arm 3'(3'HA)(~900bp); SEQ. ID No 16 + SEQ. ID No 18 = homology arm 5'(5'BH) up to PGK promoter (PGKpmt); SEQ. ID No 19 + SEQ. ID No 20 = 3' off construct to the Nullomer, for the 5 different PCR reactions performed with genomic DNA from co-transfected cells.*

E- *46uL of the reagent mixture from microtube 1 (A-), were pipetted into each of 5 microtubes (C-) of PCR reaction (genomic WT) with the addition of 2uL of each of the labelled FWD and REV primers (SEQ. ID No 15 to 21), and*

F- *46uL of the reagent mixture from microtube 2 (B-), were pipetted into each of 5 microtubes (D-) of PCR reaction (co-transfected) with the addition of 2uL of each of the labelled FWD and REV primers (SEQ. ID No 15 to 21).*

Immediately, PCR reactions were started in a thermal cycler under the conditions mentioned in Table 22.

Table 22: *PCR conditions in the thermal cycler.*

PCR		***Temperature***	***Time***
	Initial denaturation	*95 ºC*	*3:00 minutes*
	Denaturation	*95 ºC*	*30 seconds*
35 cycles	*Hybridisation*	*57 ºC*	*30 seconds*
	Extension	*72 ºC*	*1:00 minute*
	Final Extension	*72 ºC*	*10:00 minutes*
	Wait	*4 ºC*	*10:00 minutes*

After PCR, a 3uL sample from each of the microtubules was electrophoresed in a 0.8% agarose gel, (Figure 5)."

The digested plasmid DNA was precipitated and purified using gel electrophoresis (Please see **III.3.3.**). After the purification, Cassette 1 vector was ready to be used for transfections aiming to Gene BAG2 editing.

CHAPTER IV - RESULTS

CHAPTER IV - RESULTS

IV.1. – *In Silico* Creation of Constructs for gene BAG2 and Gene LGSN.

The rational for the creation of the two constructs to obtain the needed knock-ins in the two centromere flanking genes in human chromosome 6 was based on three assumptions:

1- Knock-in of the DNA constructs would be needed to accomplish the creation of Off-the-Shelf hPSC lines for the safe deletion of endogenous Chromosome 6, as well as a method for clonal selection.

2- All knock-ins would be performed requiring an HDR-dependent insertion of the needed sequences.

3- All knock-ins would rely on DSBs to be performed by CRISPR/Cas9 nuclease system.

After the selection and testing for the locations where CRISPR/Cas9 should cleave the DNA double strand in Gene BAG2 and in Gene LGSN to provide for the DSBs where the HDR cellular mechanism will insert the Construct 1 and 2 sequences, time came to think about the composition of the different constructs in order to generate the adequate Off-the-Shelf hPSC lines.

Any HDR-based knock-in event needs a piece of DNA homologous to the region flanking the DSB, to act as a template for the HDR cellular machinery to insert them in the DSB. In our work, these DNA templates should therefore include the regions of BAG2 and LGSN immediately flanking the predicted DSB and will be named 5´ Homology Arm (5'HA or Left Arm) and 3'Homology Arm (3'HA or Right Arm). Each homology arm should be in the range of 1-1,5Kb, to ensure an efficient rate of homologous recombination.

Apart from these homology arms, Constructs should include also various other DNA sequences, according to the desired functions to be available after insertion in the endogenous Chr. 6.

First, we thought about the sequences we would need in our construct for gene BAG2. As the location for the DSB was known, the construct has long homology arms each sideof the DSB, over 1.5 Kb in length, to promote correct insert integration.

For correct clone selection and identification after transfection into human PSCs, the insert contains a gene cassette encoding a fusion between a protein conferring Puromycin Resistance (puromycin *N*-acetyl-transferase) and the fluorescent protein GFP. This fusion gene is under control of the PGK (Murine Phosphoglycerate Kinase-1) promoter, an ubiquitous housekeeping promoter that drives long-term persistent expression in human cells.

Also, as explained before, the construct contains a [redacted] that, once inserted in endogenous Chr 6, should provide a specific gRNA target for cleavage of the endogenous Chr 6.

For gene BAG2 a construct fulfilling those desiderates was designed (**Figure 23**).

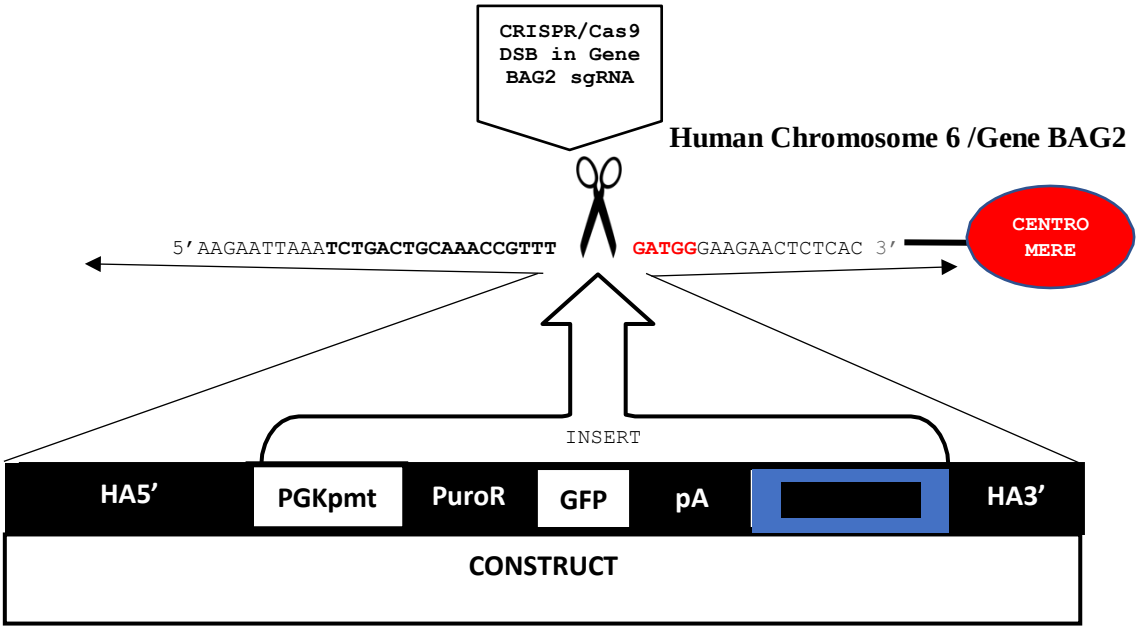


Figure 23 - Representation of Construct for gene BAG2, (patent Cassette 1) and sequence were Cas9/sgRNA-BAG2 will produce DSB (TCTGACTGCAAACCGTTTGATGG). HA5'-Left Homology Arm 1783 bp upstream from CRISPR/Cas9-sgRNA-BAG2 DSB; PGK pmt- Murine Phosphoglycerate Kinase-1 promoter; PuroR- Puromycin Resistance N-acetil-transferase gene; GFP- Green Fluorescent Protein Gene; pA- polyadenylation sequence; [redacted]; HA3'-Right Homology Arm, 1590 bp downstream from CRISPR/Cas9-sgRNA-BAG2 DSB.

The construct for Cassette 2, to be inserted at the LGSN gene located on the other side of Chr. 6's centromere (in relation to Bag2), contains 5' and 3' homology arms complementary to the regions of Chr 6 flanking the chosen sgRNA target, where insertion of the Cassette 2 is aimed. The insert is planned to contain at 5' end a copy of the herpes simplex virus-thymidine kinase (HSV-Δ*tk*) gene (truncated but functional version), under control of the EOS-S(4+) promoter, which contains multimerized *Oct-4* core enhancer element CR4 (conserved region 4) and the *Sox2* core enhancer element SRR2 (Sox2 regulatory region 2)[170]. This promoter has been shown to drive specific expression in pluripotent undifferentiated cells[170] and will ensure high TK expression in hPSCs for negative selection. A SV40 polyadenylation signal is included to ensure proper expression of this HSV-Δ*tk* gene.

The construct contains [redacted] as used in the BAG2 construct (Cassette 1), followed by another selection marker (NeoR) that provides resistance to neomycin/G418 (encoded by the amynoglycoside phosphotransferase gene from Tn5), fusioned in frame with the mCherry fluorescent protein. This selection marker is under control of the human EF-1α promoter, which drives strong expression in human cells. A polyadenylation signal from the bovine Growth Hormone gene follows, before the 3' homology arm at the end of the construct.

The position of the negative selection marker (HSV-Δ*tk*), [redacted], was chosen to ensure that when the endogenous modified Chr 6 is eliminated by cleavage at the two nullomer target sequences flanking the centromere, transfected hPSCs that might retain the "central fragment" containing the centromere of cleaved Chr 6 do express the knocked-in HSV-Δ*tk* gene and can be eliminated by ganciclovir treatment. (Please see **Figure 24**).

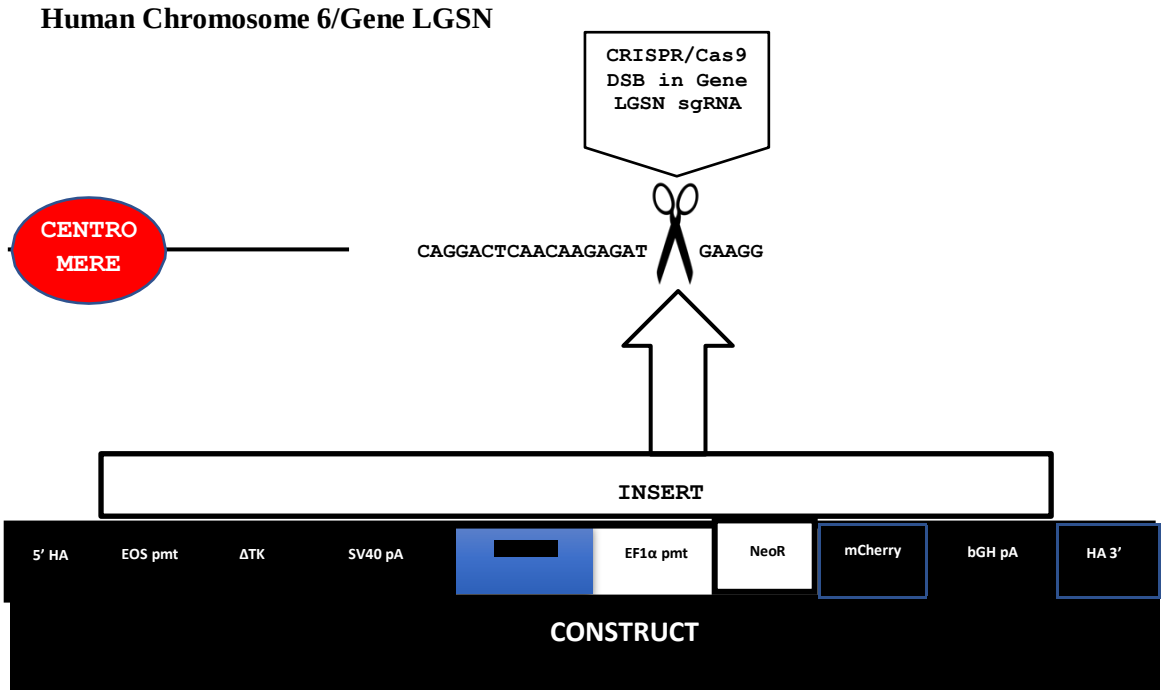


Figure 24 - Representation of Construct for gene LGSN (patent Cassette 2) and sequence were Cas9/sgRNA-LGSN will produce DSB (CAGGACTCAACAAGAGATGAAGG). HA5'=Left Homology Arm 1032bp upstream form CRISPR/Cas9/sgRNA-LGSN DSB; EOS pmt = Gene sox2 promoter; ΔTK = Thymidine Kinase gene; SV40 pA = simian vacuolating virus 40 polyadenylation sequence; [redacted]); EF1αpmt = human elongation factor 1 alpha promoter; NeoR = Amynoglicoside phosphoriltransferase from Tn5; mCherry = Fluorescent Protein Red gene; bGH pA = bovine growth Hormone polyadenylation sequence; HA3' = Right Homology Arm, 1069bp downstream from CRISPR/Cas9/sgRNA-LGSN DSB.

IV.2. - Selection of DNA Homology Arms of BAG2 and LGSN.

To start assembling the DNA constructs (Cassette 1 and 2) required to build the "off-the-shelf" hESC line with a modified Chr. 6, we first selected the DNA regions of BAG2 and LGSN genes to be used as homology arms in the Homologous Recombination (HR) events required for precise insertion of the constructs in Chr.6.

BAG2 and LGSN are two genes in human chromosome 6 located near (flanking) the centromere at the long arm and short arm, respectively.

The sequences to be used as homology arms in the DNA constructs for the two genes, located each side and near Chr. 6 centromere, were identified in Ensembl.org, Genome Assembly GRCh38.p13.

For BAG2 gene the sequence coordinates of the DNA regions to be amplified by PCR and included in Cassette 1 construct are:

Left Homology Arm - 6:57182026:57183813

Right Homology Arm – 6:57183814:57185394

For LGSN gene, the sequence coordinates of the DNA regions to be included in Cassette 1 construct are:
Left Homology Arm - 6:63294000:63295031
Right Homology Arm - 6:63295032:63296100

IV.3. - PCR Amplification of DNA Homology Arms from WT HEK 293 T Cells for Creation of Gene BAG2 Construct (Cassette1).

The DNA fragments containing the regions to be used as homology arms were amplified by PCR from genomic DNA prepared from HEK293T cells. The following primers were used to amplify the DNA fragments, which were then cloned in the p925 plasmid.

5' Homology Arm (1783bp long):

FWD 5' CGGAATTCGGATCCTATAGGGTTGAAGCTTTGAGAGAAGC 3'

REV 5' GCGAATTCAACGGTTTGCAGTCAGATTTAATTC 3'

3' Homology Arm (1530bp long):

FWD 5' ATAAGAATGCGGCCGCTGATGGGAAGAACTCTCACCGT 3'

REV 5' ATAAGATAGCGGCCGCGGATCCAAAATAGTATCCAGGGAAGTTGT 3'

The complementary region to the genomic DNA is underlined, with the other sequences at 5' end containing recognition sequences for Restriction Enzymes to be used in cloning steps. (Please see point **III.7.** for full experimental explanation).
For Gene LGSN, decision was made to purchase the whole construct (Cassette 2) from the industry as synthetic DNA, as this approach will significantly save money and time. The DNA fragments to serve as homology arms will thus be obtained by DNA synthesis.

IV.4 - sgRNA Design to clone into eSpCas9 vector.

To choose the target site, within the BAG2 and LGSN genes, for the guide RNAs to be used with CRISPR/Cas9 to create the desired DSBs, we ~~a~~accessed the website crispr.mit.edu, (from Zhang Lab at Broad Institute in Cambridge, Massachusetts, no more available since 2000),, and CCTop - CRISPR/Cas9 target online predictor - http://crispor.tefor.net/ (from Heidelberg University, Stemmer, M. *et al.* (2015)[312]). But

there are many other CRISPR tools available to perform the same task, free from charge[171].

As a result, from both tools, CRISPR/Cas9 sgRNA selection for the creation of DSBs in Genes BAG2 (Human Chromosome 6 Long Arm near the Centromere) and LGSN (Human Chromosome 6 Short Arm near the Centromere) were:

Table 4. – Sequences of single guide RNA for Gene BAG2 and Gene LGSN. CCTop - CRISPR/Cas9 target online predictor from Heidelberg University, Stemmer, M. *et al.* (2015)[174].

Gene	sgRNA	PAM	QUALITY SCORE
BAG2	(1) GATCAACGCTAAAGCCAACG	AGG	93
BAG2	(2) TCTGACTGCAAACCGTTTGA	TGG	86
LGSN	(1) TCTTAGACCTGGACACGCCG	TGG	92
LGSN	(2) ACTGAGCAGCTCACGATCAC	TGG	86

At the time these sgRNAs were selected, the targets (**Table 4**) were chosen as those with the best score for each gene. However, as technology and knowledge evolved extensively, if our sgRNA choice was to be made today, other sgRNAs would be picked. For instance, in CRISPOR tool[172] the sgRNAs we selected using CCTop tool are not proposed as adequate targets (Please see **Table 5**)

For each selected sgRNA, a pair of oligonucleotides was ordered from Sigma-Aldrich®, one with the sense sequence of the sgRNA and the other with its reverse complementary. Each oligonucleotide had additional nucleotides on their 5' end that were complementary to the overhangs of the BbsI digested eSpCas9 (Addgene # 71814), expression vector. This was done to enable the unidirectional ligation of each sgRNA duplex (formed by the annealing of each pair of oligonucleotides) to the expression vector.

Table 5 – Selected sgRNAs (red) from CCTop in 2015, and new sgRNA (green) for genes BAG2 and LGSN as in CRISPOR (Sept 2021). Haeussler, M. *et al.* (2016)[172].

GENE	GUIDE	OFF-TARGET	MIT SPECIFICITY SCORE	CFD SPECIFICITY SCORE	PREDICTED EFFICIENCY: DOENCH'16	PREDICTED EFFICIENCY: MOR.-MATEOS	OUTCOME: OUT OF FRAME	OUTCOME: LINDEL
BAG 2	Our guide TCTGACTGCAAACCGTTTGA TGG	91	90	90	38	24	67	80
BAG2	New guide GAATCCCGTCCGCGAGGTTA GGG	4	99	100	37	62	70	76
BAG2	New guide GTTCGCGGGCGGTTAGCGGA CGG	15	98	99	52	74	70	77
LGSN	Our guide CAGGACTCAACAAGAGATGA AGG	212	69	81	58	43	65	73
LGSN	New guide GCCATCTGACGAAACGATAG TGG	20	97	98	57	22	64	74
LGSN	New guide TCCACTATCGTTTCGTCAGA TGG	23	97	98	36	23	63	75

IV.5. - Creation of eSpCas9/sgRNA Plasmids

After obtaining a miniprep of the **eSpCas9** Addgene #71814 vector, the plasmid was digested using the restriction enzyme *BbsI* (New England Biolab®). The digested pDNA was then treated with alkaline phosphatase to promote the dephosphorylation of the DNA ends, preventing vector recircularization. The eCas9 vector prepared in this way was used for subcloning of the sgRNAs oligos (BAG2, and LGSN, and Nullomer).

Each subcloned plasmid was prepared to make DSBs in BAG 2.1; BAG2.2; LGSN1 and LGSN2 sgRNAs, and in nullomer sequence (`GTACGCTCGGACCGACGTATCGG`), of inserts Cassette1(BAG 2) and Cassette2 (LGSN). In short, after the plasmid eSpCas9 was digested by BbsI and dephosphorylated, a T4 ligase dependent ligation reaction was performed with the different synthetic oligos, already phosphorylated. After an overnight incubation at 15°C, ligation product was used to transform competent cells. After antibiotic selection, single colonies were expanded and pDNA prepared from each, and digested with XbaI/NotI, EcoRV/XbaI, and EcoRV/NotI to identify the clones containing the desired inserts encoding the adequate sgRNAs. The identity of the clones was confirmed by pDNA sequencing.

Selected clones were stored -80°C, and plasmid *maxipreps* were prepared ahead of cell co-transfection.

IV.6. - sgRNA Functional Validation Assay (T7E1)

To evaluate the DSB efficiency of each gRNA at its target site, decision was made to perform T7E1 assays (Endonuclease1; New England Biolabs (NEB), in HEK 293T cells, transfected with plasmid CRISPR/Cas9 BAG2(1), and BAG2(2), and LGSN(1), and LGSN(2).

[redacted]

HEK 293T cells were transfected at 70%-80% confluence, 1 well in a 6-well plate, with gRNA/Cas9 plasmid: BAG2(1); BAG2(2); LGSN(1), and LGSN(2). Transfection was performed by lipofection with Lipofectamine 2000™ (ThermoFisher Scientific®) following the manufacturer's recommendations. Next day, cells were passaged 1:6 in separate 6-well plates and grew until 70%-80% confluence. At 70%-80% confluence cells were collected for genomic DNA isolation by commercial Kit (NZY tissue isolation DNA Kit, or Wizard™ SV Gel and PCR clean-up System – PROMEGA™), purification, and stored -20°C ahead of T7E1 assay.

For T7E1 assay, (EnGen™Mutation Detection Kit, New England BioLabs™ (NEB), primers were purchased from industry for the four different sgRNAs, BAG2(1); BAG2(2); LGSN(1), and LGSN(2).

Table 6. – Primers for T7E1 Assays in Gene BAG2 and LGSN.

GENE	sgRNA	PRIMER
BAG2	(1)	FWD 5' CTCGCGAACCTCTAACTCCA 3'
BAG2	(1)	REV 5' TTGCTCTCAATGGATTGCTG 3'
BAG2	(2)	FWD 5' GACATCTGATCTCTGGAGCTC 3'
BAG2	(2)	REV 5' CCATGTGGCACCTCAGATGA 3'
LGSN	(1)	FWD 5' ATAGTACCGACCTGATGTCC 3'
LGSN	(1)	REV 5' TCTAAGCCAGTGACATGATG 3'
LGSN	(2)	FWD 5' GCTGACAGAACTGCAAGAGT 3'
LGSN	(2)	REV 5' TCTGTGCTCTCATCTGGACC 3'

T7E1 assay, (EnGen™Mutation Detection Kit, New England BioLabs™ (NEB), rational:

When CRISPR/Cas9 cleaves DNA, NHEJ will produce “scars” in the genome of cells that survive. When PCRs are performed in genomic DNA (gDNA) from “bulk” cells submitted to CRISPR-Cas9, PCR DNA will contain different sequences at the NHEJ “scar” site. When, by submitting those PCR products to denaturation, forward and reverse strands will split from each other. Then, by providing a slow gradient pass from denaturation temperature back to 37°C reannealing will happen. However, in many cases reannealing will give rise to forward strands not annealing with the exact sister reverse strand, giving rise to heteroduplex formation. This creates DNA double strands with short mismatches. T7E1 endonuclease identifies those mismatches and cleaves them. When an electrophoresis gel of this T7E1 digestion is performed, it gives a relative idea about the power CRISPR/Cas9 coupled with a specific sgRNA have in promoting DSBs at the specific site we intended to, by allowing evaluation of the difference between cleaved and non-cleaved amounts of DNA in transfection survivors. Most of the dead-by-transfection cells were discarded by PBS washings before cells were collected for genomic DNA extraction.

Results of T7E1 assay in HEK 293 T transfected with eSpCas9/sgRNAs for gene BAG2 and Gene LGSN are represented in images of 0,8% agarose gel electrophoresis in (**Figure 25** and **Figure 26)**. In **Figure 25** lane **1** marked as BAG2.2, represent 0,8% agarose gel electrophoresis of HEK 293T DNA of cells that were transfected with CRISPR/eSpCas9 expressing the sgRNA (BAG2.2) no T7E1 assay was performed. A thick 600bp expected band is visible however in the 500bp raw. In lane **2**, marked as BAG2.2 T7E1 represents the T7E1 assay in the HEK 293T DNA after CRISPR/eSpCas9/sgRNABAG2.2 transfection. The expected ~400bp band and ~200bp band are present and they present half the intensity of the BAG2.2 column 600 bp band, what is expected in a correct T7E1 assay. In lane **3** marked as LGSN 2 T7, represents the result of the T7E1 assay in HEK 293T DNA after CRISPR/eSpCas9-LGSN2 transfection. The expected bands from the T7E1 assay in lane **3**, would be represented by the splitting of the original 868bp band in two different bands of 552bp and 316bp. In the image the 868bp band is clearly visible, however neither the 552bp band nor the 316bp band are present, meaning that CRISPR/Cas9.sgRNA-LGSN 2, is not a good sgRNA to use in our experiments.

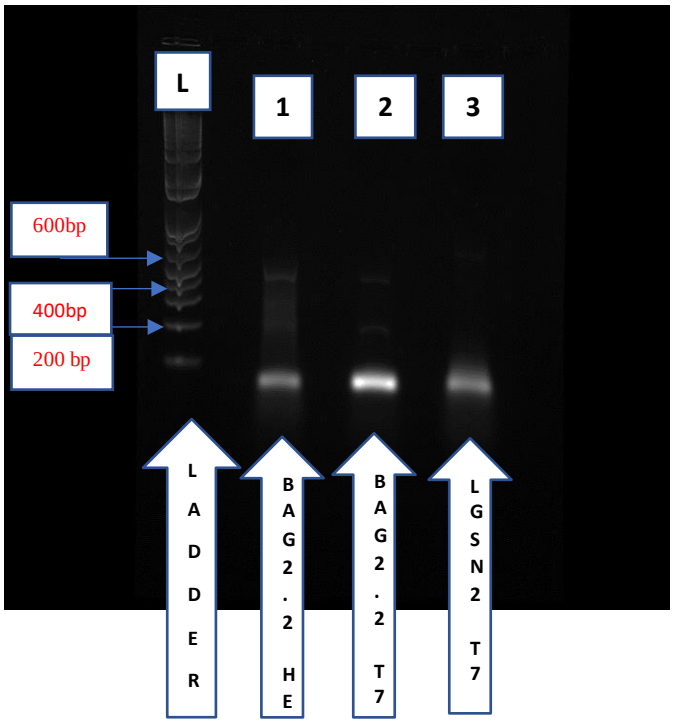


Figure 25 –

0,8% agarose gel electrophoresis of T7E1 assay in HEK293T cells gDNA after transfection with CRISPR/Cas9 sgRNA BAG2.2(sgRNA2). Two correct bands (403bp + 199bp) (Lane **2)**.

and

LGSN 2(sgRNA2) T7. Only the 868bp band is visible. 552bp + 316bp bands of efficient CRISPR/Cas9-sgRNA-LGSN cleavage are not present. (Lane **3).**

L – reference ladder.

In **Figure 26** the lane marked as LGSN 1 (Lane **1**) represent 0,8% agarose gel electrophoresis of HEK 293T DNA of cells that were transfected with CRISPR/eSpCas9 expressing the sgRNA LGSN1. No T7E1 assay was performed in LGSN 1, (Lane **1**). A thicker band 900bp is visible in lane **1**. Lanes **2** and **3**, marked as LGSN T7 represent the T7E1 assay in the HEK 293T DNA after CRISPR/eSpCas9/sgRNA LGSN 1 transfection. The expected ~688bp band and ~316bp band are present, (however better visible in the original one ChemiDoc/ Bio-Rad systems screen) and they present much less than half the intensity of the LGSN 1 what is expected in a correct T7E1 assay. However, it also indicates that LGSN sgRNA 1 may represent a better choice to perform the editing of gene LGSN in hPSCs as compared to LGSN sgRNA2. Lane **4** represents a leftover sample of LGSN 2 T7E1 assay (Lane **3** in **Figure 25**), only for confirmation.

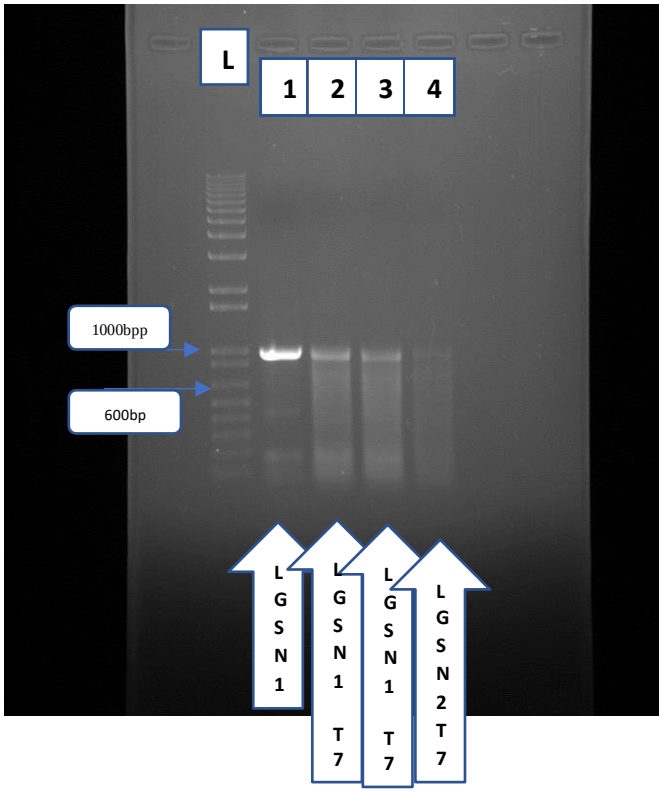


Figure 26 – Better to look at this image in the digital text format and with zoom, as the bands are thin and present low intensity.

0,8% agarose gel electrophoresis of T7E1 assay in HEK293T cells gDNA after transfection with CRISPR/Cas9 sgRNA LGSN 1 and 2(sgRNA1 and 2). In lane **1** a correct band of 1014bp is present. In lanes **2** and **3** two correct bands (688bp + 316bp) are present. They represent the expected correct T7E1 assay after CRISPR/Cas9 sgRNA LGSN 1 cleavage.

and

in lane **4** leftover sample from CRISPR/Cas9-sgRNA LGSN 2(sgRNA2) T7 E1 assay electrophoresis already used in **Figure 33 (**for confirmation). Bands of efficient CRISPR/Cas9-sgRNA-LGSN 2 cleavage are not present.

Conclusion: As from all four sgRNAs assayed, no bands were present in the T7E1 assay with CRISPR/eSpCas9/sgRNA BAG2.1 (image not shown). Only correct bands were obtained for sgBAG2. 2 and better results for LGSN 1, decision was made for their use in the next experiments.

IV.7. [redacted]

[redacted]

[redacted]

IV.8. - Creation of Vector for Gene BAG2 Editing.

[redacted]

DNA sequence containing the 5' Homology Arm (1783bp) was obtained by PCR amplification using genomic DNA from HEK 293 T cells, and ligated in the previous vector p925+[redacted], at the unique EcoRI site. Clones with the correct integration were selected and confirmed by restriction enzyme mapping.

DNA sequence containing the 3' Homology Arm (1503bp) was obtained by PCR amplification using genomic DNA from HEK 293 T cells and ligated in the previous vector p925+[redacted]+5'HA, at the unique NotI site. Clones with the correct integration were selected and confirmed by restriction enzyme mapping.

Maxi-preps from the final vector (named LRArmsPGKpuroGFPnullo) (**R52**) were prepared and stored for future use (**Figure 27**). Linear constructs were isolated by digestion with unique BamH1 sites flanking the DNA insert and purified for future transfection protocols.

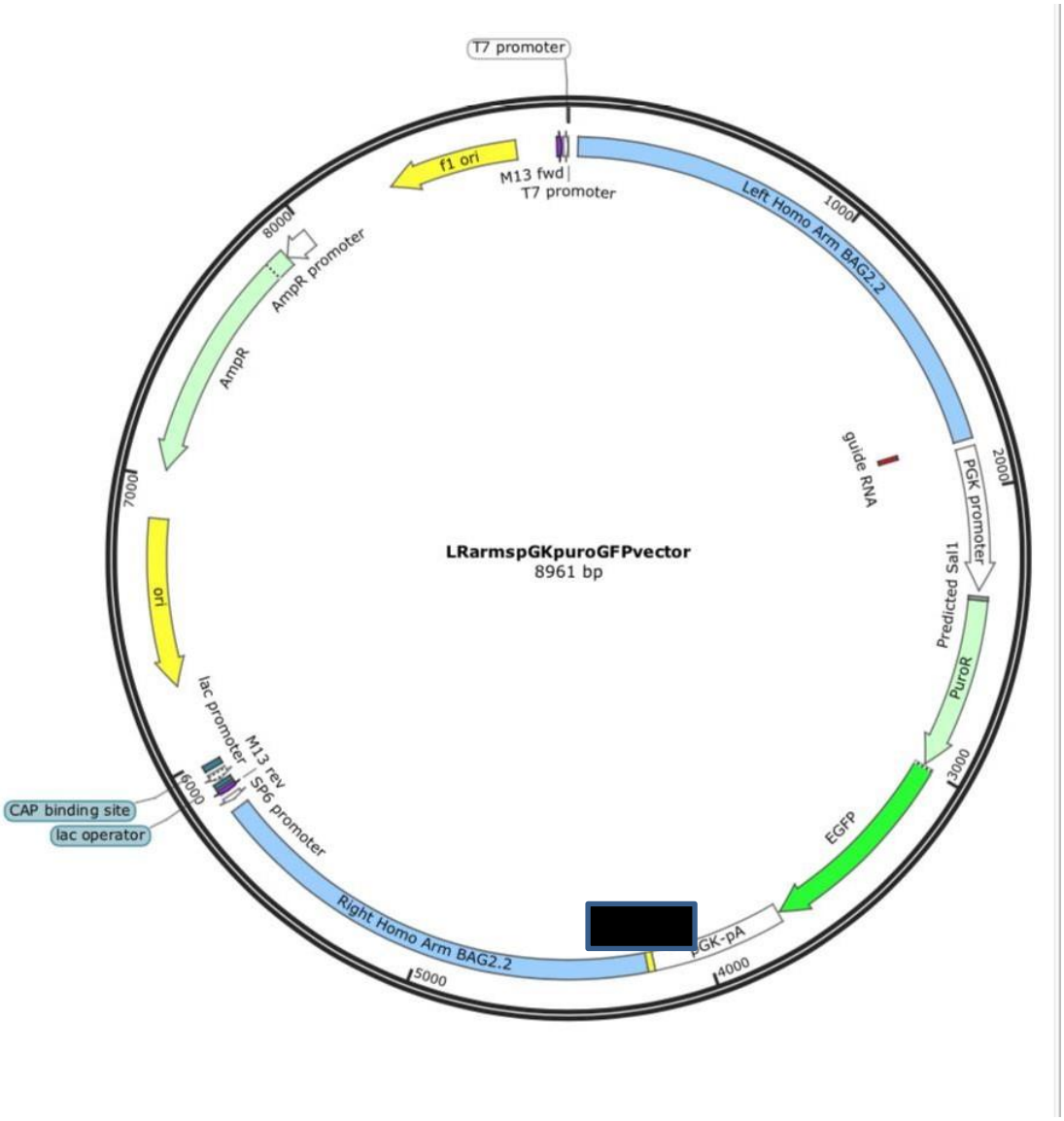


Figure 27 - Map of LRarmsPGKpuroGFPvector (**R52**), prepared for knock-in in gene BAG2. Composed by HA5'-Left Homology Arm 1783 bp upstream from CRISPR/Cas9-sgRNA-BAG2 DSB; PGK pmt-Phosphoglycerate Kinase promoter; PuroR- Puromycin Resistance N-Acetyl-transferase gene; GFP- Green Fluorescent Protein Gene; 2pA- polyadenylation sequence; [redacted]; HA3'- Right Homology Arm, 1590 bp downstream from CRISPR/Cas9-sgRNA-BAG2 DSB. For linear vector purification a BamHI restriction site was provided at vector extremities.

Another version of this Cassette 1 vector was built in which the PGK promoter was replaced by a CAGG promoter, composed of the CMV enhancer and the chicken ß-actin promoter, also known to drive strong expression in human cells. This new Cassette1 - (LHArm-**CAGGpmt**-PuroR-EGFP-2pA-[redacted]-RHArm) – is contained in plasmid **p81C**. The aim was to have two similar vectors for Cassette 1, driven by different promoters, which might have different efficiencies in driving expression in human PSCs.

Maxi-preps from this new final vector (**p81C**) were prepared and stored for future use (**Figure 28**). Linear constructs were isolated by digestion with unique BamH1 sites flanking the DNA insert and purified for future transfection protocols.

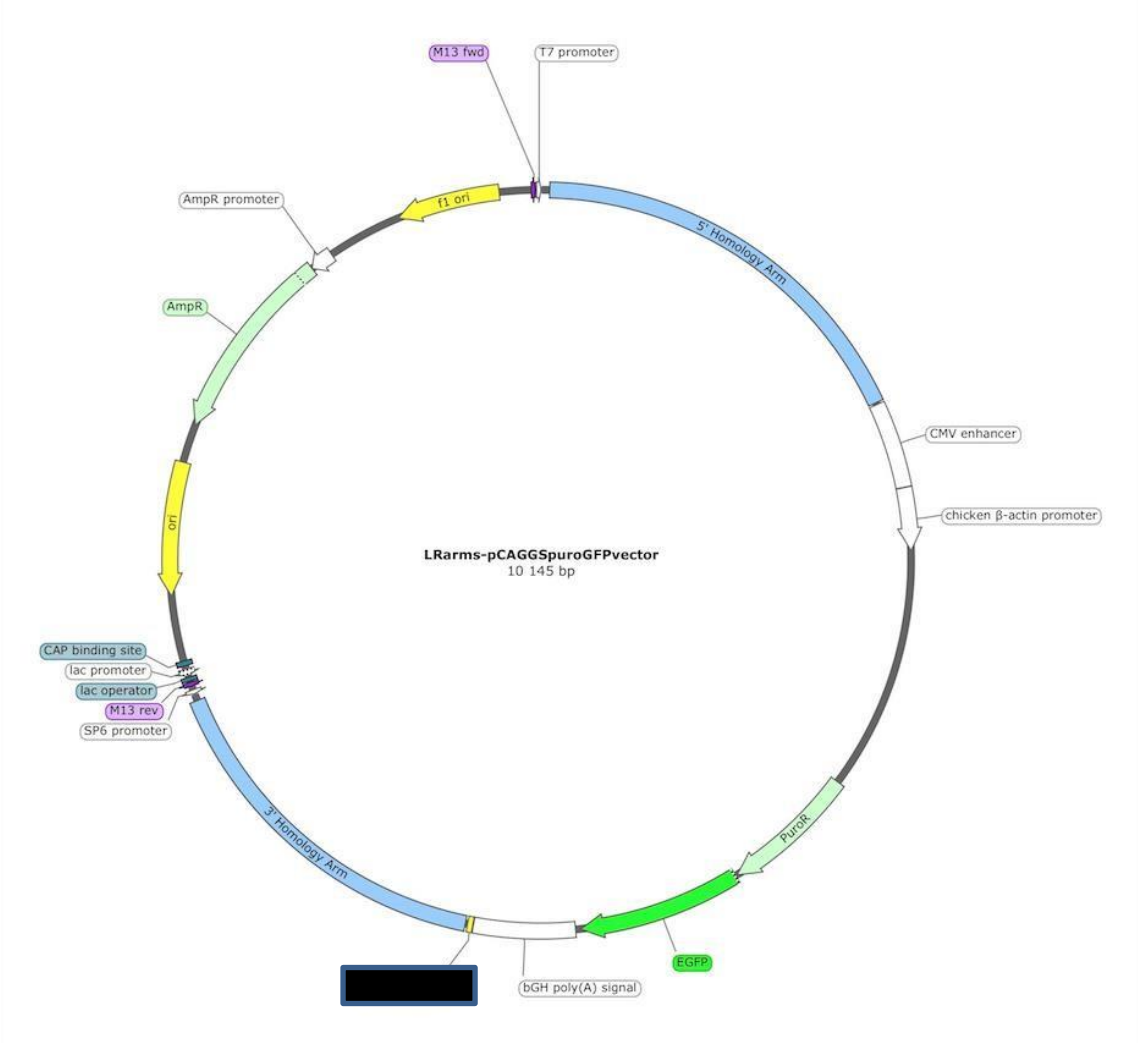


Figure 28 - Map of LRarmspCAGGSpuroGFPvector (**p81C**), prepared for knock-in in gene BAG2. Composed by HA5'-Left Homology Arm 1783 bp upstream from CRISPR/Cas9-sgRNA-BAG2 DSB; CAGG promoter, composed of the CMV enhancer and the chicken ß-actin promoter; PuroR- Puromycin Resistance N-Acetyl-transferase gene; GFP- Green Fluorescent Protein Gene; 2pA- polyadenylation sequence; [redacted]) ; HA3'- Right Homology Arm, 1590 bp downstream from CRISPR/Cas9-sgRNA-BAG2 DSB. For linear vector purification a BamHI restriction site was provided at vector extremities.

IV.9. - Transfections in hiPSCs.

All experimental work on Human Pluripotent Cell Lines were developed at Pluripotent Stem Cell Laboratory, *Stem Cell Engineering Research Group* (*SCERG*),

iBB - Institute for Biosciences and Bioengineering of Instituto Superior Técnico, Universidade de Lisboa.

In our first assays to introduce the BAG2 constructs into hiPSCs, electroporation was used, with the Neon® Thermo Fisher® system. However, there was always huge cell death, even when using Rocki (10mM) 2 hours before electroporation and for 24 hours after electroporation. We then decided to adopt Lipofection as the main method to transfection of hiPSCs (and hESCs), using Lipofectamine 3000™ (Thermo Fisher®), in which much less cell death was observed, even in absence of Rocki.

Analysis of our transfection assays was done by observation of the culture plates under fluorescence microscopy. We could observe various GFP+ cells (**Figure 29**) but most did not survive beyond 72 hours. We could never obtain single colonies of GFP+ cells. All GFP+ cells in culture were consistently disperse, only a few in each cellular aggregate, not dividing, and getting apoptotic (as from observation along several days).

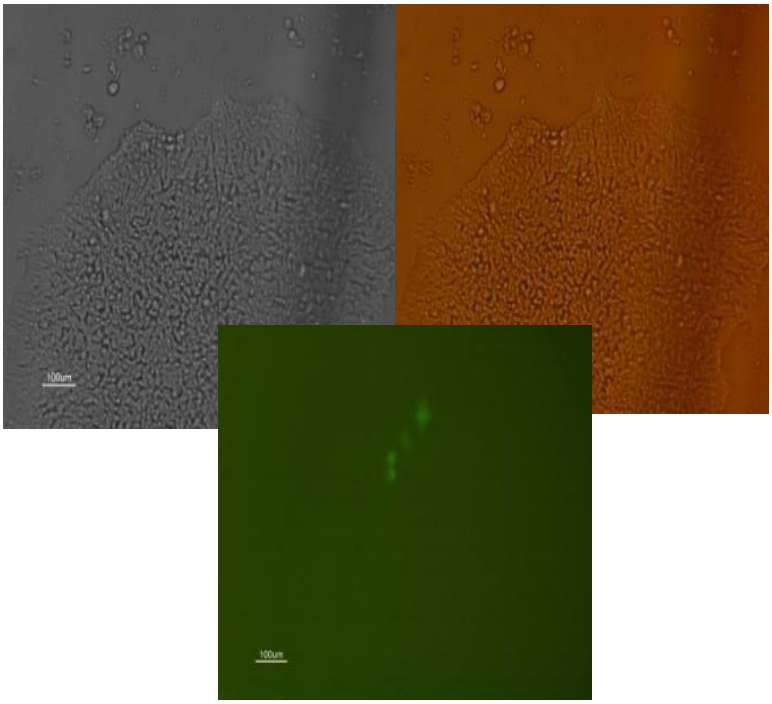


Figure 29. Co-transfection of CRISPR/Cas9-sgRNA/BAG2 + Cassette1 in hiPSCs, Lipofectamine 3000® (ThermoFisher). Co-transfection in hiPSCs line E, day 5 Co-transfection and day 3 puromycin. Usually, there were no more than two to three GFP+ spots per cellular aggregate. Here we present a colony with 4 GFP+ cells. By direct fluorescence microscopy observation.

We then tested the alternative BAG2 construct, (LHArm-CAGGpmt-PuroR-EGFP-2pA-nullomer-RHArm, plasmid **p81C**) to evaluate whether another promoter could be driving better expression of the selection cassette. We noticed an increased number of GFP+ cells using p81C, still, no GFP+ colonies could be obtained even with p81C. We thenwondered whether it could be a problem of puromycin selection and tested various concentrations of puromycin. We found that concentrations above 0,2ug/mL were toxic to hiPSCs and used this concentration in further experiments, sometimes starting with 0,1ug/mL for the first 24h post-transfection.

Next, as HDR is dependent on cells being in S/G2 phases of cell cycle, we decided to perform iterative Lipofection, to increase the probability of cells getting in contact with lipofected DNA in these phases. In our protocol, lipofection was performed with 8 hour intervals, for 48 hours in total. We noticed an increase in the number of GFP+ cells by direct fluorescence microscopy, from the first 24 hours to 48 hours. Still, no single GFP+ colonies were obtained, with most GFP+ cells disappearing by 5 days.

We tested also various hiPSC lines (TCLab, E, F and GEpi, see **III.1.2**) but no improvements in transfection efficiency were evident.

A different transfection method was also used to introduce sgRNA and Cas9 into hPSCs, by direct delivery of Cas9 protein in complex with the guide RNA, provided as a synthetic gRNA, a method that seems to be better for achieving adequate CRISPR/Cas9 targeting. A commercial Kit was used to perform these transfections: TrueCut Cas9 Protein v2® ThermoFisher® with synthetic sgRNA/BAG2- (TrueGuide Synthetic gRNA® ThermoFisher®).

To be more precise in our quantification of transfected GFP+ cells, we used Flow Cytometry analysis. As shown in (**Figure 30**), analysis of hiPSCs transfected with Lipofectamine 3000 revealed that between 0,31 and 0,71% of transfected cells were GFP+, after 4 days of puromycin selection. To further evaluate if GFP+ cells were alive, we used a staining kit that discriminates between live and dead cells (LIVE/DEAD™Fixable Dead Cell Stain Kit, ThermoFisher Scientific™). This revealed that between 0,11 and 0,25% of live cells were GFP+ (**Figure 31**), a value in the range determined for the whole population of cells.

This contrasts with the lack of observable GFP+ cells by direct fluorescence analysis of the transfected plates and raises the question of why we were not successful in obtaining isolated GFP+ colonies in our experiments, as there was a significant number of live GFP+ in the transfected cultures after 4 days of puromycin selection.

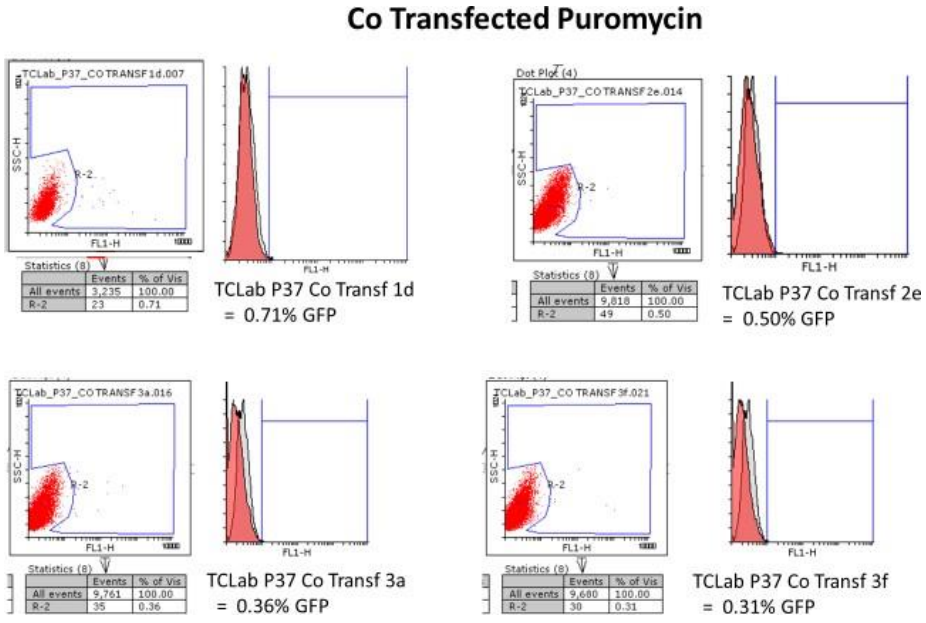


Figure 30 - Flow cytometry analysis of 4 separated samples, each composed of 3 wells content of a 24-well plate. hiPSC TCLab passage 34, co-transfected with Cas9/sgRNA-BAG2 plasmid + Cassete1 (LHArm-PGKpmt-PuroR-EGFP-2pA-nullomer-RHArm), after 4 days puromycin selection (starting at 0,1ug/mL). Acquisition was obtained in a Beckman Coulter Flow Cytometer and analysis of GFP+ cells was performed by Cláudia Miranda and Mariana Branco, using Flowing software®.

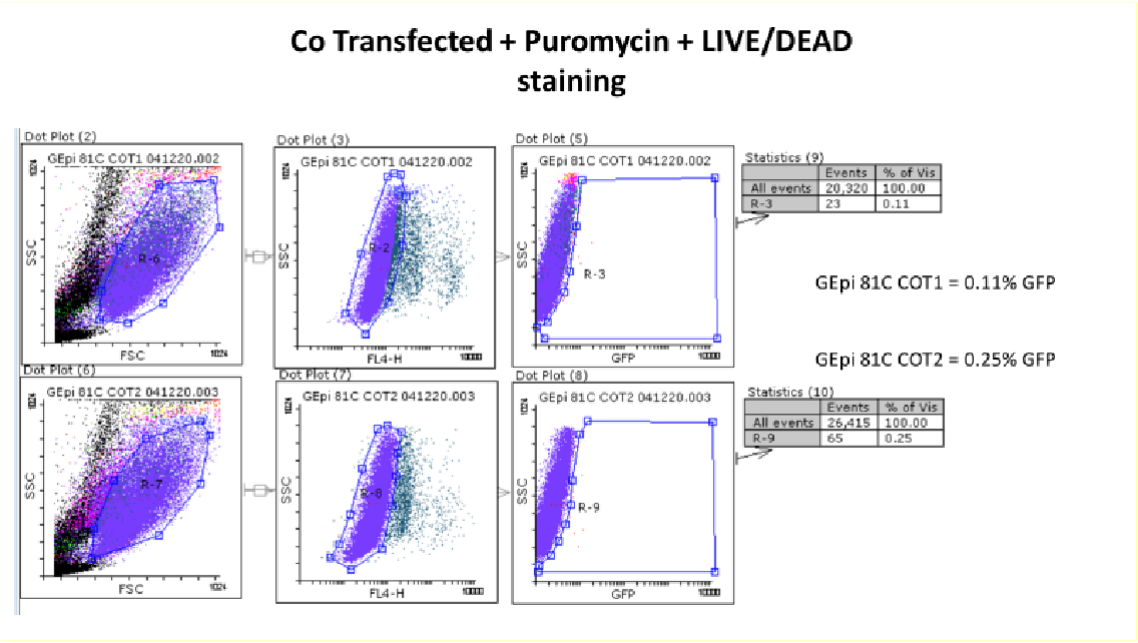
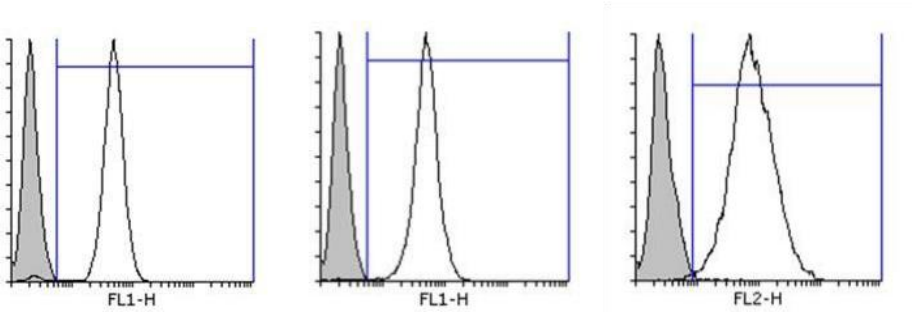


Figure 31 - Flow cytometry analysis of 2 samples, each composed of 10^6 cells. hiPSC Gepi/GIBCO passage 50, co-transfected with Cas9 protein- (TrueCut Cas9 Protein v2® ThermoFisher®) and synthetic sgRNA/BAG2- (TrueGuide Synthetic gRNA® ThermoFisher®), + Cassete 1 (LHArm-CAGGpmt-PuroR-EGFP-2pA-[redacted]-RHArm), after 4 days puromycin selection (starting at 0,1ug/mL). Cells were labeled for 15 minutes at dark using: LIVE/DEAD™Fixable Dead Cell Stain Kit, ThermoFisher Scientific™, following manufacturers' instructions. Acquisition was done in a Beckman Coulter Flow Cytometer and analysis of GFP+ cells was performed by Cláudia Miranda using Flowing software®.

IV.10. - Transfections in hESCs.

In later experiments, we had access to a hESC line (WA09 or H9, a kind gift from Inês Milagre, IGC), a cell line that is commonly used nowadays. We grew these cells (a passage 42) in mTeSR®Plus (Stem Cell Technologies) culture medium. After creating a frozen sample stock, we tested these cells for their pluripotency, using various markers (OCT4, SOX2, SSEA-4), as shown in (**Figure 32**). Results show that these cells maintained high levels of pluripotency in our culture conditions.



OCT4 - 97.6% **SOX2 - 99,0%** **SSEA-4 – 96,3%**

Figure 32 – Flow cytometry evaluation of pluripotency markers in hESCs/H9, passage 42, grown in mTeSR®Plus (Stem Cell Technologies).

We then transfected H9 hESCs with the R52 and p81C plasmids, using TrueCut Cas9 Protein v2® ThermoFisher® with synthetic sgRNA/BAG2- (TrueGuide Synthetic gRNA® ThermoFisher®). Analysis was done by flow cytometry to quantify the amount of transfected GFP+ cells, after 4 days of puromycin selection (up to 0,2ug/mL). This analysis revealed an average transfection efficiency of 2,44% (values varied between 0,2 and 5,42%, in different pooled wells), as shown in (**Figure 33)**. These results show a better transfection efficiency for hESCs when compared with hiPSCs (average 0,47%, (**Figure 30**).

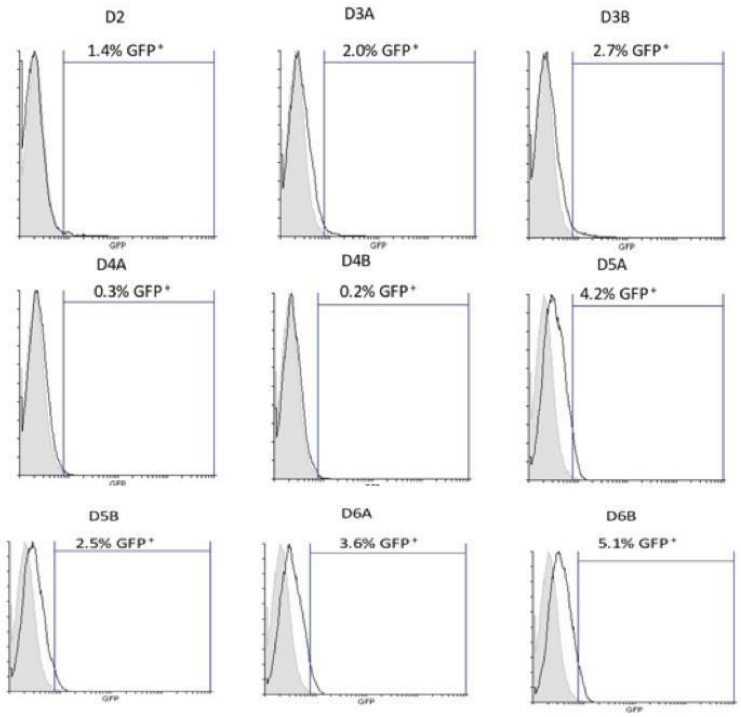


Figure 33 –H9 hESCs transfected with CRISPR/Cas9-BAG2 + Cassette 1- R52, at day 6, with, 4 days of puromycin selection. Flow Cytometry in a Beckman Coulter Flow Cytometer and analysis of 9 different samples by Flowing Software (performed by Cláudia Miranda). Each sample composed by the cell content of 3 successive wells in 24 well-plate for adherent cells.

We next compared transfection efficiencies of H9 hESCs grown from different passages (Passage 44 versus 54), in the same culture conditions. We found that earlier passages produced better transfection efficiencies (2,4% versus 0,6%), after 12 days of puromycin selection. When these cells were further expanded after a new passage, and cultured for 5 days more, the number of detected GFP+ cells increased to 4,5% and 8,1%, for Passage 54 and 44 respectively. This indicates that GFP+ cells continue to proliferate in our culture conditions and opens the possibility that single colonies of correctly edited cells can be obtained after prolonged culture if an adequate selection and cloning procedure is performed.

In (**Figure 34**) flow Cytometry analysis of 3 different samples of younger (P44) and older (P54) 1st passage after H9/hESCs co-transfection and 2 samples of younger (P45) and older (P55) 2nd passage after H9/hESCs co-transfection.

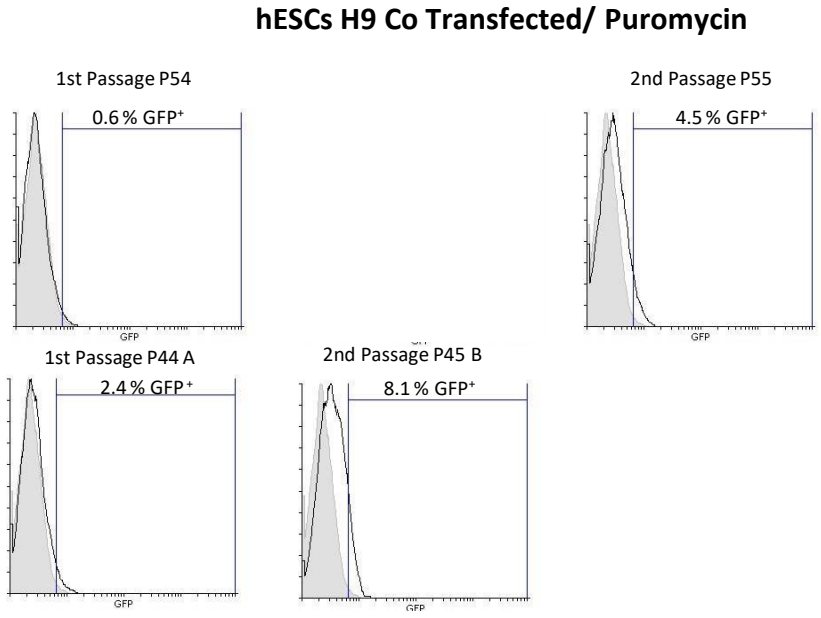


Figure 34 – Flow Cytometry analysis of GFP+ cells after co-transfection [CRISPR/Cas9- sgRNA/BAG2 + Cassete 1 (R52)]. Each sample was a pool of 3 adjacent wells of younger (P44) and older (P54) H9 hESCs. Analysis was done in 1st and 2nd passages after transfection followed by puromycin selection. 1st Passage = 12 days after transfection/ 10 days Puromycin selection. 2nd Passage = 18 days after transfection/

16 days Puromycin selection. Acquisition in a Beckman Coulter Flow Cytometer and Flow Cytometry analysis by Flowing Software (performed by Cláudia Miranda).

IV.11. - Strategy for PCR screening of edited hPSCs

To detect correct integration events of Cassette 1 in the BAG2 gene of transfected hPSCs, we designed a PCR-based strategy to amplify DNA fragments that would only be present as result of Cassette 1 successful knock-in. This strategy (**Figure 35A**) uses a primer that is complementary to a DNA sequence just outside the DNA regions used as Homology Arms (primer "A forw" at the 5' side and and "F rev" at the 3' side), together with a primer that is complementary to a sequence only present in the vector DNA ("B rev" and "E forw", for the 5' and 3' sides, respectively). As internal positive controls, we also designed primers that would amplify DNA fragments from the normal BAG2 gene (Primer "C rev" to be used with "A forw", and "G forw" to be used with "F rev"), to check for genomic DNA quality and PCR conditions, and from the transfected Cassette 1 DNA (Primer "D forw" to be used with "B rev"), to check for integrity of the transfected DNA.

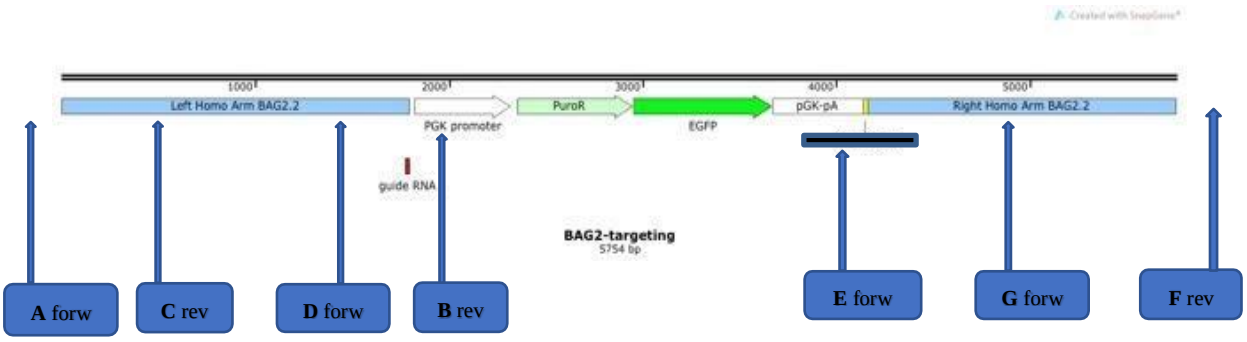


Figure 35A – Scheme with the predicted DNA structure of the BAG2 genomic region after correct integration of the Cassette 1 DNA. Position of the various primers to be used in PCR screening of clones is depicted.

Expected sizes of PCR fragments:

- "A forw" x "B rev": 1999 bp
- "F rev" x "E forw": 1903 bp
- "C rev" x "A forw": 447 bp
- "G forw" x "F rev": 1077 bp

- "D forw" x "B rev": 516 bp

Genomic DNA was extracted from ~6X10^6 hiPSCs WT and co-transfected with CRISPR/Cas9-BAG2 + R52-Cassette1 and used in PCR reactions. All PCRs were performed comparing WT and transfected genomic DNA samples. (**Figure 35B**)

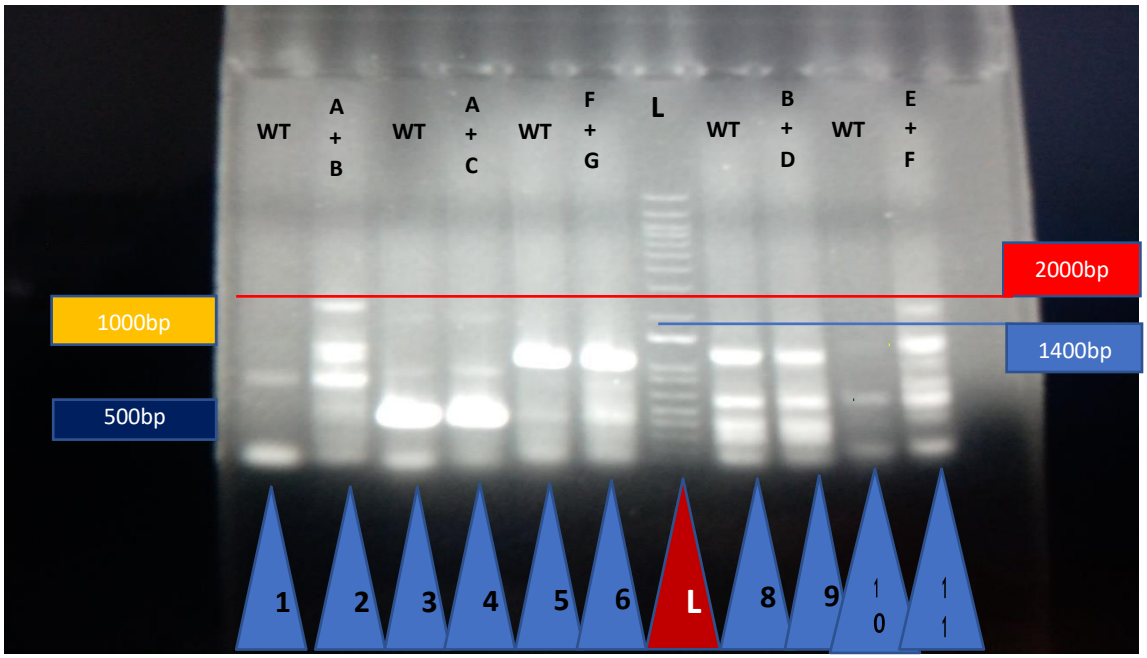


Figure 35B – PCR evaluation for the presence of correctly edited hiPSCs. Each PCR was performed both in genomic DNA (gDNA) extracted from 6x10^6 Wild Type (WT) hiPSCs and 6x10^6 of cells transfected with CRISPR/Cas9-BAG2 + Cassette1-R52. Image represents the results of 0,8% agarose gel electrophoresis of all PCR products. Bands to be compared to the Ladder (L) reference NZY III.

In lanes **1** and **2**, PCRs were performed in gDNA from WT and Transfected cells respectively, using "A forw" and "B rev" primers. A band of the expected size (~2kb) is present in lane **2** but not in lane **1,** and indicates the presence of correctly edited cells.

In lanes **3** and **4**, PCRs were performed in gDNA extracted from WT and Transfected cells respectively, using "C rev" and "A forw" primers. An amplification product with

the expected size (447bp) is present in both lanes, as it arises from a genomic region that is present in both samples.

In lanes **5** and **6**, PCRs were performed in gDNA extracted from WT and Transfected cells respectively, using "G forw" and "F rev" primers. An amplification product with the expected size (1077bp) is present in both lanes, as it arises from a genomic region that is present in both samples.

In lanes **8** and **9**, PCRs were performed in gDNA extracted from WT and Transfected cells respectively, using "D forw" and "B rev" primers. An amplification product with the expected size (516bp) seems to be present in both lanes, but PCR conditions need to be improved to get rid of the background of unspecific bands.

In lanes **10** and **11**, PCRs were performed in gDNA extracted from WT and Transfected cells respectively, using "F rev" and "E forw" primers. A band of the expected size (~1,9kb) is present in lane **11** but not in lane **10,** and indicates the presence of correctly edited cells.

Together, this analysis seems to indicate that there are transfected cells in which the Cassette 1 DNA seems to be correctly integrated at the BAG2 gene, by HDR. This offers good perspectives that our strategy to target the BAG2 gene might be working, and that it will be possible in future work to isolate single colonies of correctly edited hiPSCs.

CHAPTER V – DISCUSSION

CHAPTER V - DISCUSSION

Human allo-transplantation is a relevant and vast field in medicine. For many generations now, different proposals have been made to overcome poor results in human allo-transplantation. Many types of rejection syndromes are the main problem, undermining success and ultimately causing premature death of patients.

Multiple and varied immunosuppressive protocols have been proposed, like HLA-ablative protocols[112,149], allo-transplant encapsulation[153,154], or Tolerogenic approaches[155,156], but all have caveats that will hamper future implementation in the clinic. These protocols attempt to reduce rejection by unspecifically and broadly reducing the patient's overall immunological capabilities to reject an allo-transplant. Notwithstanding, immunosuppression is accompanied of serious complications like infections, infestations, cancers, cardiac, gastrointestinal, and nephrotoxic complications that hampers patients' survival and quality of life (QoL).

Approaches that are based on reducing expression of HLA receptors, so called "transparent" or "immune opaque" transplants, are (exceedingly) dangerous because these HLA-minus immune cells are not anymore able to identify and eliminate viral infected cells or cancer cells, for example. Any of these "transparent" transplants would thus be defenseless and may end in patient's death as the transplant.

Any immunological cell that does not express HLA would also be rejected by the host immunological system, through the activity of Natural Killer cells (NK), Lymphocytes T and B, and macrophages. Those are cells instructed from the fetal lifetime to reject any cell that presents a "different HLA-ID card" or that does not present any "HLA-ID card" at all.

Our proposal of a new method to prepare immuno-compatible transplants is aimed at overcoming many caveats present on the above-mentioned proposals, and most relevantly, will enable the host to not only maintain a healthy non-rejected transplant but also keep a functional and complete immunological system.

Our methodology, which is grounded on human chromosome 6 replacement, was inspired on the one proposed by T. Tada's team[125,126,175], and aims to address the caveats related to high levels of chromotripsis and kataegis. It does not intend to "fight" against, or eliminate HLA expression, but instead aims at maintenance of its functional integrity.

This will provide for full expression of patient's immunological capabilities and avoidance of rejection syndromes.

One of the distinctive features of our method is the insertion of Nullomer sequences into the endogenous Chr. 6 to be replaced in hPSCs. These sequences will be exclusive to endogenous Chr. 6 and provide unique targets to create DSBs by CRISPR/Cas9 at these sites. In this way, endogenous Chr 6 can be eliminated from hPSCs, while sparing the "exogenous" Chr. 6 from the patient. We have designed two different DNA Cassettes to be inserted each side of the centromere, with the main aim of introducing the nullomers sequences into the endogenous Chr. 6. In these Cassettes, most the DNA is composed by promoters and genes intended to assist for the selection, isolation, and ulterior expansion of correctly edited clonal hPSC cells, ahead of the creation of correctly edited Stem Cell lines. As a result, each Cassette is a large construct with more than 5Kbs of DNA, to achieve the integration of a nullomer sequence of around 2.5Kbps. This is a limitation of current methods of gene manipulation in mammalian cells and is not a very efficient process. We are in need of simpler methods to insert DNA in stem cells and to select correctly edited cells. This will be of outstanding relevance in cGMP creation of cell lines, providing for faster, easier, safer, and cheaper results.

Still, our initial PCR analysis of hiPSC cells transfected with Cassette 1 seems to indicate that correct targeting of the DNA construct did occur in some cells (**Figure 35B**). However, we are aware that we need to purify and sequence the diagnostic PCR bands, to confirm Cassette integration at the correct genomic location. Given the observed mean transfection efficiency in hiPSCs of 0,47%, we expect to have 28200 edited cells. However, only a very small part of them will be correctly edited. Authors claim the correctly edited cells will be between 1 cell to 10 cells/ million of transfected cells. In our experiment we transfected 6X10^6 cells. That gives between 6 and 60 correctly edited cells in the $6X10^6$ cells that were analyzed. This illustrates the sensitivity of the PCR analysis method that we designed. On the other side, it stresses the need for very efficient selection procedures to isolate correctly targeted cells, as well as of methods to obtain clonal lines of hPSCs that can be stored and used for the following steps. Conversely, it points to the need for cell selection means, like Flourescence-Assisted Cell Sorting (FACS), as a technique to enhance the selection probabilities of correctly edited clones to be expanded.

Other improvements to our proposed methodology can be anticipated. For instance, more precise in silico methods are now available to design target sequences for sgRNAs, that

will in principle produce less off-targeting damage to DNA. As described before, the gRNAs we used in our protocol do not show the best scores in these new in silico tools, and indeed one of these gRNA did not show good results in the T7E1 assays.

In addition, homology arms should be amplified from genomic DNA of the hPSC lines to be used in genome editing, to ensure a 100% match between the homology arms and the genome of transfected cells. As well as the T7E1 assays would be better also to be performed in genomic DNA isolated from cells belonging to the cell line we will work with.

Together with better methods for single-cell isolation and establishment of clonal lines of hPSCs, we anticipate that a better protocol can now be designed, with more possibilities of success in clinical translation.

Need For Efficient Cell Expansion and Bioreactors.

For some treatments like Hematopoietic Stem and Progenitor Transplants (HSPC), efficient cell bioreactors are needed to grow millions of cells for transplantation in cGMP Cell Therapy BioTechs, to provide for transplantation of millions of patients. Space, volumes, media consumption, staff, strategy, tactics, logistics, cGMP, and many other issues must be improved.

Bioreactors are especially important to obtain the high quantities and quality of cells are needed for Cell Therapies. For now, the main Cell Therapies available are in the field of hematological applications. Apart from erythrocyte concentrates, platelet concentrates, and HSPC transplants, other more recently available are auto- and allogeneic CAR T cells. But the hematological organ, as a “liquid” organ as it is, does not need the usual complex structure solid organs need linked to arterial, venous, and neural networks. It is easier to put a Cell Therapy in place based on HSPCs. However, the usual minimum quantities of cells to provide for a Bone Marrow transplant is 2 to 5x10^6 cells per Kg of the patient weight[177]. This number of cells is not efficiently created/expanded in the usual T-flasks or plates we have in research Laboratories or Contract Development and Manufacturing Organization (CDMO) facilities. For this aim more efficient technology must be provided. This technology is known as bioreactor. The efficiency of bioreactors depends on several parameters one of the main are the cell types to be cultured and the purpose, they are in culture. In Cell Therapies the main goal is not any molecule the cells may be producing (antibodies, growth factors, coagulation factors) as it happens in

relation to recombinant proteins manufacturing to be infused in patients, but the cells themselves sometimes as protein producers *in vivo* in the patients.

There are several types of bioreactors we can consider: The most used in the industry are Stirred Tanks with capacities from 15mL to several tens of Liters. Other types of bioreactors are: Fixed bed, Hollow fiber, Rotary Cell Culture (microgravity cell culture mimicking), Rotating bed, Rocking motion, and Vertical-wheel. For research in SCERG Labs, Stirred bioreactors and Vertical-wheel bioreactors are extensively used as tools to optimize several cell lines expansion and differentiation conditions. An interesting, recent review on this relevant thematic was done by Nogueira, D.E.S. *et al*. (2021)[176].

Our Patent application and future Patent applications.

Our methodology needs the creation of at least two edited hPSC lines. Those will constitute the starting healthy off-the-shelf product for all transplantation protocols. The creation of the off-the-shelf hPSC lines is expensive, time consuming, and technically challenging, mostly because hPSCs are among the most difficult cells for editing. Our experimental results are indicative that this aim is feasible.

From the very beginning I was deeply aware of the importance of not losing the opportunity to patent the Method, by an extemporaneous publication. Losing the patentability could mean the loss of interest from investors to work with us, by lack of protection for fair investment reimbursements. A patent must be looked mainly as a tool. A tool that in a world of relative mounting financial difficulties, turns possible to explain to Investors that guarantees for reimbursements are not only possible but mainly highly rewarding. By this mechanism, patients will start to be saved sooner as it could happen without a patent.

In our patent application, (Annex1, INPI 117101 U/05-03-2021), we make proposals to overcome the issues leveraged in other proposals by providing one HLA full-match solution that avoids rejections, and at least with as much relevance, prevents any need to lower immunological system capabilities, allowing for full maintenance of host's immunological protection. Moreover, a new kind of Allo-Auto-Transplantation may emerge, where not only maybe possible to take advantage of full HLA-match by the Auto-HLA representation in the transplanted cells, but is also true that, as transplanted cells have not pathological mutations, any congenital disease a patient may complain, will potentially be relieved or cured by the presence of the (healthy) Allo-piece of genetic

information present in the transplanted cells. Only monogenic diseases based on mutations on the Auto-Chromosome 6 cannot be directly addressed by this approach.
It also provides the conditions to produce the best Hematopoietic Progenitor and Stem Cells to take advantage of GVL with a lower or absent GVHD, as soon as science understands the *modus faciendis*.
To turn it into reality, high investment for long time will be necessary. Several patents must be filled to protect investors' money and a team of many highly skilled and passionate collaborators shall be gathered, many years before any patient may be cured by our technology. This is a huge amount of permanent work and responsibility, a challenge that only highly committed people will be in condition to deal with. Everyone must know in advance that this is a highly demanding task, and all effort must be directed to find solutions for the highly challenging problems ahead.
After our participation in the program Lab2Market@IST 2020, we obtained the confirmation by experts that we should do better to go for a Contract Development Manufacturing Organization (CDMO) for an Advanced Therapy Medicine Product (ATMP) early in the process. This would allow for the needed experimental work (including in non-cGMP conditions) and will establish a platform to progress toward the several steps needed before and during Clinical Trials.

A potential financial support was assumed by Hovione Capital, (today Bionova Capital) at the end of the Lab2Market@IST 2020 acceleration program.

All business is streamlined if communications are built among and inside companies. This is pushing us to construct a Start-up to ease the process even further in the follow of Capital Venture Companies advice.

One A to Z structured project is what we are constructing in a teamwork of people that knows the project details for up to 6 years now, and that is prepared to take the challenges forward for the next decades ahead.

CHAPTER VI - CONCLUSIONS and FUTURE WORK

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Our work on hiPSCs and hESCs, although not yet fully conclusive, is indicative that replacement of Chromosome 6 in hPSCs is achievable. We got evidence that the necessary cell editing to the creation of off-the-shelf product is feasible. Experimentation may confirm or point out innovative solutions for questions rising by the specific way they will work inside the protocols.

Success in human Stem Cell Chromosome 6 replacement will be a warranty for full or best HLA compatibility in human allo-transplantation. Immunosuppression will no more be required or will be required in much lower doses in a few cases. Furthermore, full expression of HLA gives the patient full immunological defense capability against infections, infestations, and cancer transformations, improving considerably Overall Survival (OS) and Quality of Life (QoL)..

Differentiation of hPSCs to Hematopoietic Progenitor and Stem Cells (HPSC) is the safest in comparison with all other available protocols, and those cells can fix many diseases. This will be one of the first objectives to achieve.

In the meanwhile, protocols to differentiate hPSCs or hMSCs into pancreatic β-cells will be available, to fix the huge Diabetes catastrophe, all over the world. But there are already good protocols to differentiate hPSCs and hMSCs into hepatocytes, endothelial cells, and other interesting cells for Cell Therapies. A small quantity of hepatocytes or endothelial cells will be able to treat any hemophiliac patient.

In Hemato-oncology it would be possible a two stages therapeutic approach:

a) First stage would be a Best-HLA mismatch HPSC allo-transplant, [Lower Graft versus Host Disease (GVHD) and Highest Graft Versus Leukemia (GvL) effect]

b) After tumor eradication, second stage HPSC transplantation with Full-HLA compatibility transplant, could be performed, if indicated by clinically relevant chronic GVHD.

c) HPSC transplantation will not rely on related or unrelated live donations and several HLA full-match transplantation proceedings will be possible for same patient, no matter where, when, and how many times relapses may occur.

d) Another solution could be a double transplant, composed by tailored partial HLA-mismatched HPSCs to fight the tumor by GvT that will be rejected over time, and full HLA-matched HPSCs to completely replace the tailored partial HLA-mismatched cells and cure the patients.

e) A "Human Chromosome 6 Bank" for special GvL effects in Hematooncology, would be easier to manage and wide in donations than actual Bone Marrow Banks, as would be dependent on only 4 mm^2 surgical skin procurement ahead of the manufacturing of off-the-shelf Stem Cell lines.

f) Tissues like skin for burnt patients or pancreatic β-cells for diabetics will survive longer and behave better as compared to the approaches that have been described, because HLA full-match will not trigger the levels of rejections we have today, (2022), and no deleterious complex encapsulation systems will be needed to wave rejection(s).

g) Evidence for the needed knock-in in hiPSCs and hESCs was obtained, with efficiencies within or higher than the ones reported by other authors, paving the way to the creation of off-the-shelf Stem Cell Lines prepared for specific chromosome 6 loss.

h) For compliance with the Regulatory Authorities requirements since pre-clinical steps and future clinical use, off-the-shelf human Stem Cell lines must be created in cGMP conditions by a CDMO ahead of any ATMP.

i) Creation of a startup is crucial to attract investors. Investors' money is the only way patients may be saved.

j) Great attention must be put into place, to protect all investments/Investors by Patents, Trade Secrets, Non-Disclosure Agreements (NDAs), contracts and other legal documents.

k) Several partnerships will contribute to the optimization of the processes.

l) Complementary work would provide for the study of animals surviving extreme drought conditions in the tropics envisioning better new protocols to, at least partially, replace cold-based cell preservation and transportation protocols.

m) Creation of hPSC lines prepared to provide for simultaneous activation or inhibition of many genes in a timely controlled manner, will be also a must, as it will support the creation of cells, tissues and eventually organs in a very controlled/precise way.

n) Creation of new optical instruments, based on RAMAN spectroscopy instead of fluorophores or organic dye markers, for live cell selection and purification of cell cultures.

o) Creation of new systems able to allow for the culture of cell aggregates (organoids) well over 300μm in diameter, with no central necrosis or apoptosis related to lack of oxygen or/and nutrients, is a must for reliable drug screening as well as reliable cell differentiation protocols enhancing the horizons and pushing actual frontiers far away.

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ANNEX 1

PATENT Application PCT PT2022050010PT117101.

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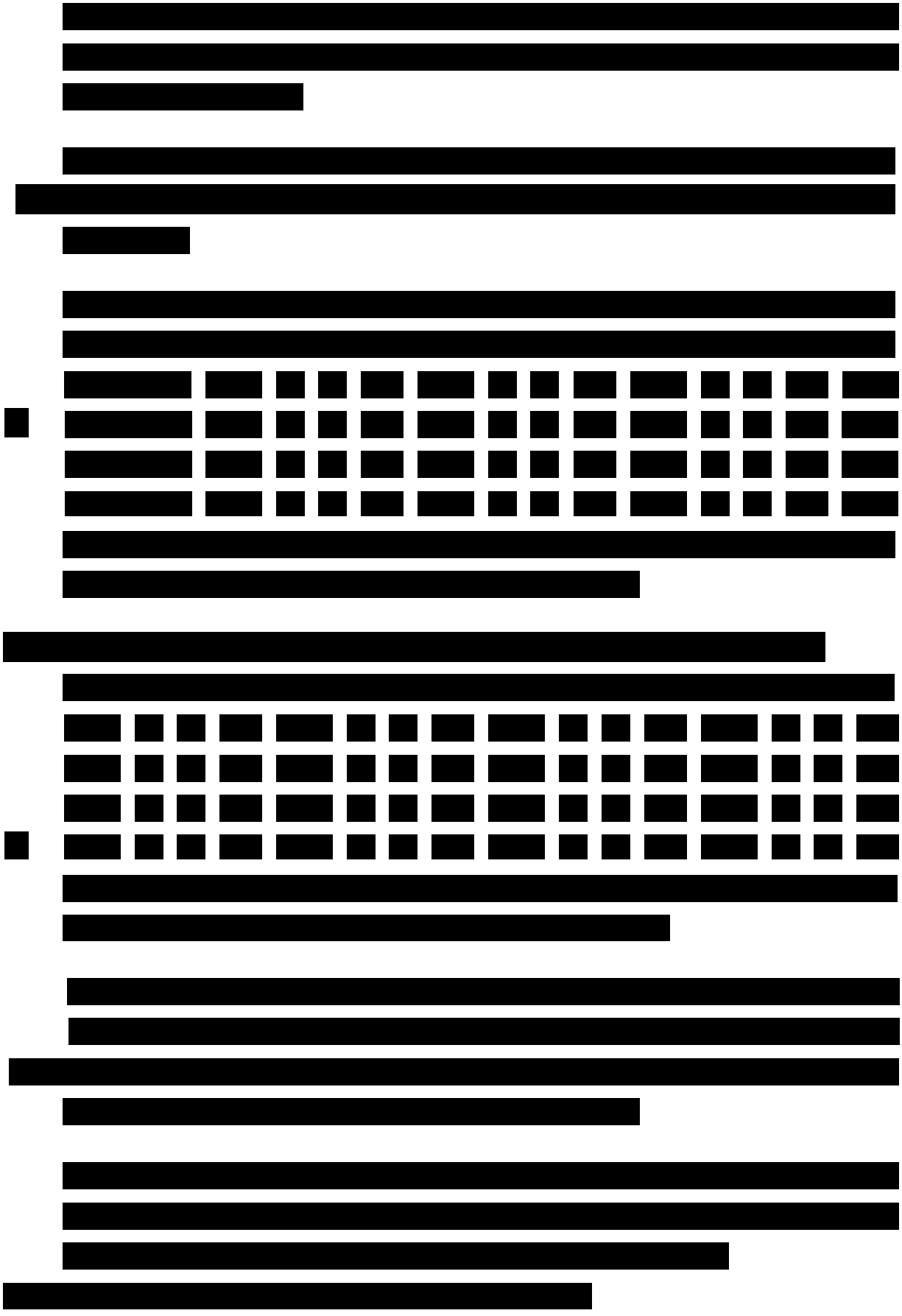

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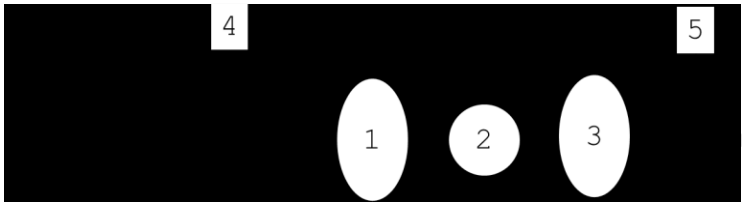
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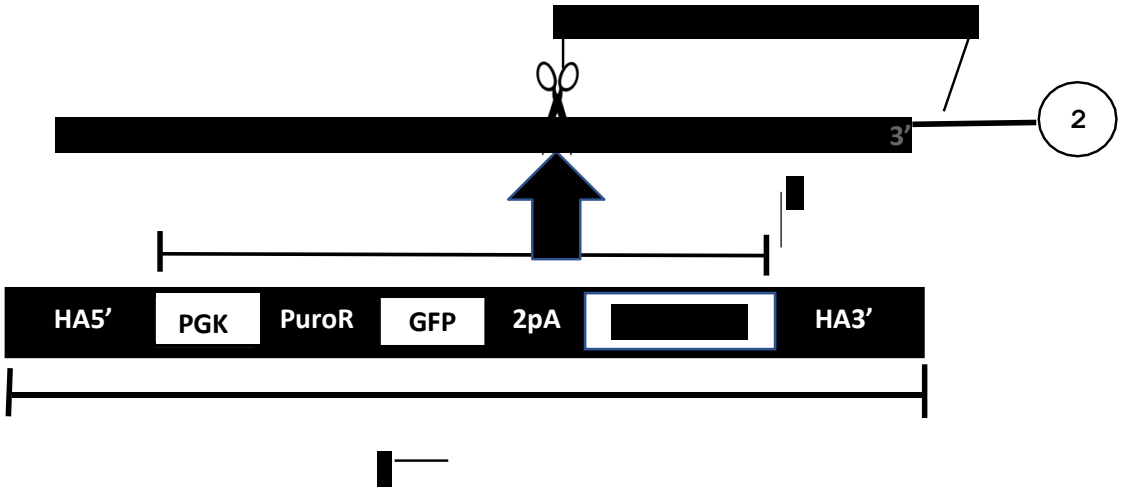
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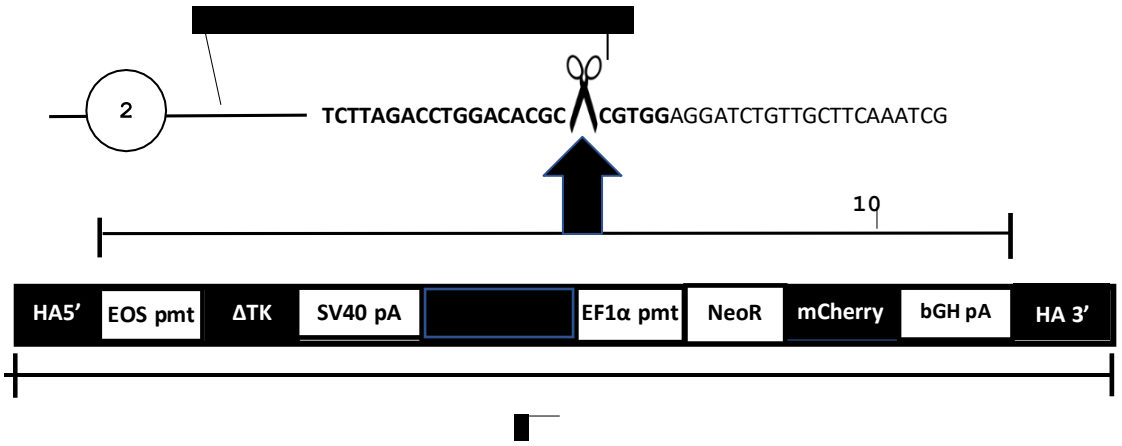
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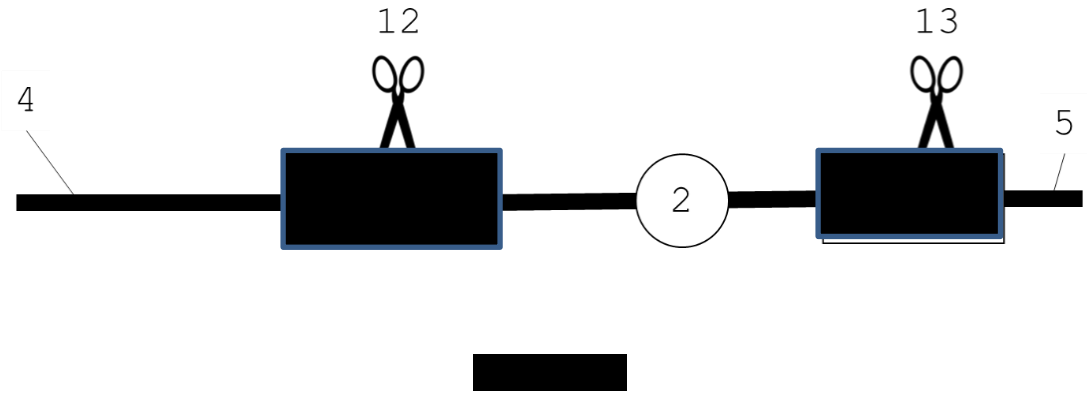
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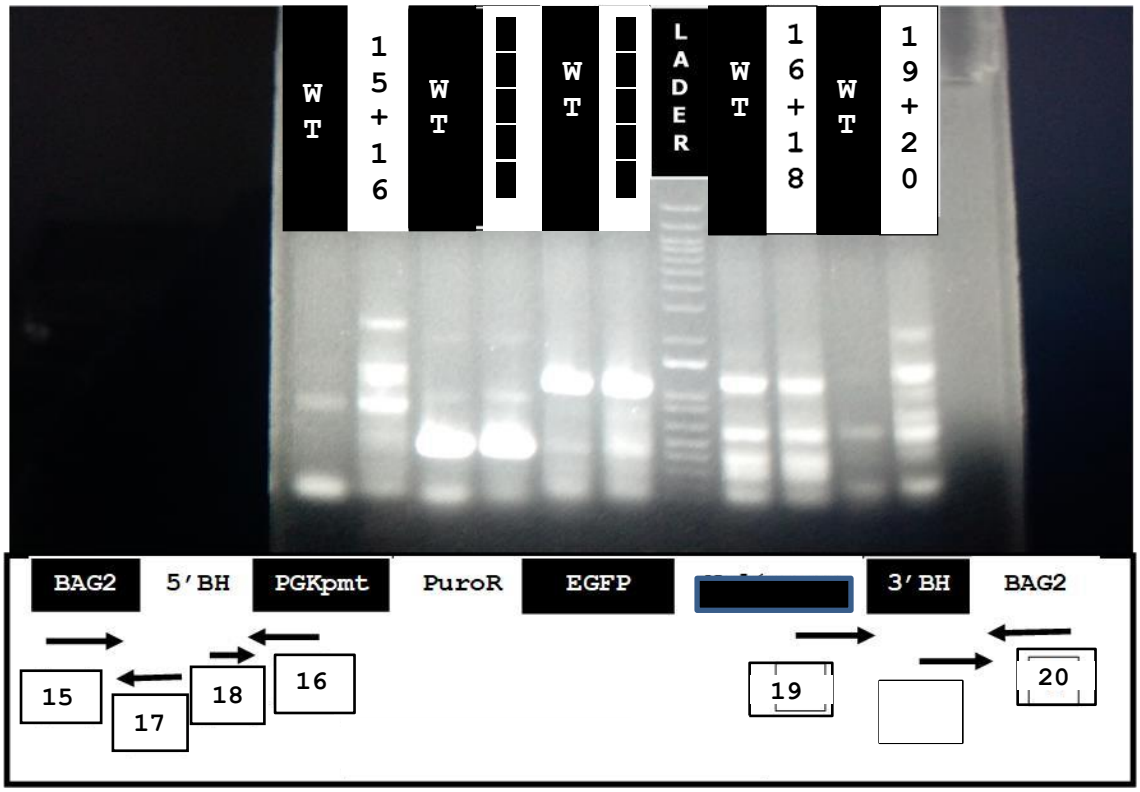
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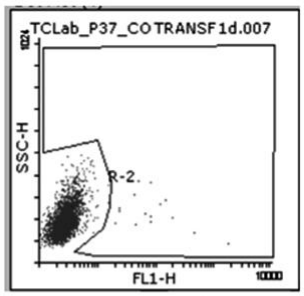

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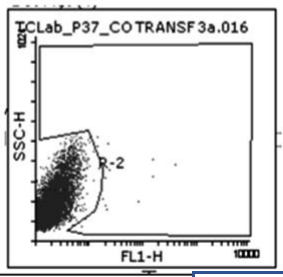

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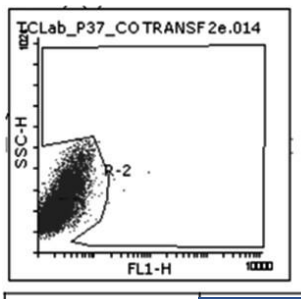
R-2 (GFP+)	35	0,36%



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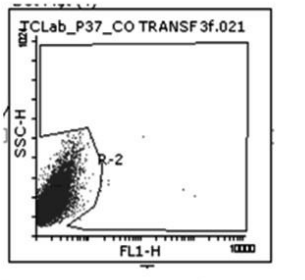
R-2 (GFP+)	49	0,50%



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R-2 (GFP+)	30	0,31%

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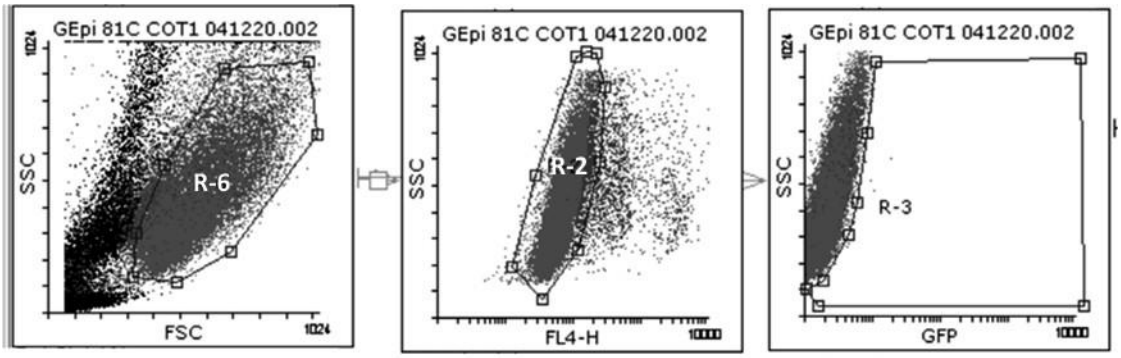

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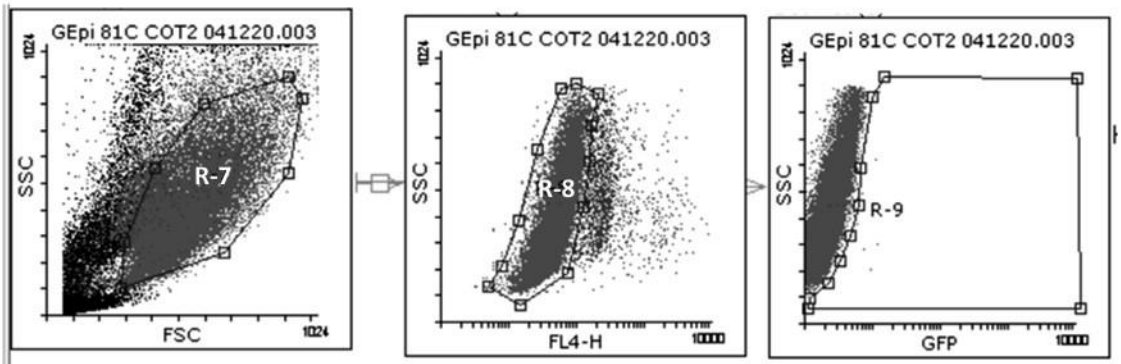
All events	20.320	100,00
R-3 (GFP+)	23	0,11%



	Events	% Visualization
All events	26.415	100,00
R-9 (GFP+)	65	0,25%

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ANNEX 2

DECLARATION

AUTHOR DECLARES THAT THE CONTENT OF THIS ANNEX 2 IS NOT TO BE DISCUSSED WITH THE THESIS

ETHICS IN ALLO-TRANSPLANTATION, AND IN STEM CELL RESEARCH AND CLINICAL TRANSLATION.

ETHICS IN ALLO-TRANSPLANTATION, IN STEM CELL RESEARCH AND CLINICAL TRANSLATION, and RELATED TO OUR PROPOSAL

1. - General Bioethics Remarks in Transplantation

Bioethics Principles from the work of: Schröder-Bäck P. *et al.* (2014)[1],

Non-maleficence; Beneficence; Health maximization; Efficiency; Respect for autonomy; Justice; Proportionality. Those are the seven main principles of ethical conduct in the field of human health care, that are also available to be applied in many more fields. No principle is in it-self absolute, but it stands for the boundaries that shall be respected, whenever responsible human actions are to be created.

Non-maleficence: *Primum nil nocere*, (first of all do not harm), is the most ancient Hippocratic principle to be used on health care. Some kind of low-level harm may be tolerated if a greater harm is to be prevented or best if a concrete benefit may arise. Transplantation is a painful and dangerous procedure however it aims in the large majority of the cases for patient's salvation. Creation of a better solution on transplantation, allowing for reduction or annihilation of rejection syndromes, optimizes non-maleficence principle's fulfillment in the field.

Beneficence: all actions shall be produced to the benefit of the patients. This is the second Hippocratic principle. Intimately related to the non-maleficence principle is in it-self different because the obligation is to achieve improvement in the patient's situation, specifically related to the disease as well as related to the patient's overall social environment, (Family, Job, etc., see also the next Principle).

Health maximization: This principle has a broader field of action in the sense that it comprehends a requirement for a social benefit. In many ways "social" health maximization may collide with the individual interests. At least at a first glance. However, real wisdom is represented by the just equation and balance of individual and communitarian needs. This defines the best governance among all governances. The wider the population involved the harder is health maximization.

Efficiency: In Health Services in general and in Transplantation particular needs, available resources will always be overcome. From research to the exact moment of transplantation and beyond, efficiency optimization may improve results and spread them to a wider population. A moral principle of efficiency will require a permanent evaluation of scientific evidence as well as cost-benefice analyses. Such a conduct will allow for more and better results in transplantation.

Respect for autonomy: This principle sustains that everyone's capacity to make decisions must always be respected. However, the autonomy of children and patients disabled by serious diseases may be compromised. Surrogate decision makers, (parents, judges, ethical committees), have huge relevance in the field of transplantation, helping for a balanced decision. Every individual has high value in his or her-self, and never shall be treated as a means for other's goals.

Justice: Most of the time humans are in possession of full autonomy and this implies equal moral worth for everyone. Justice demands equality in opportunities. This principle balances the egoism that frequently arise when someone needs a lifesaving transplant. Institutions other than, but also including associations of patients, must work collegially to prevent unjust decisions. A burden of proof is on the shoulders of the ones that may attempt for unequal opportunities in transplantation.

Proportionality: proportionality is normative. It requires that all decisions must happen in a proportionate way weighing and balancing individual and social decisions. As any coin always have two faces, apparent positive features and outcomes must always be balanced against apparent negative ones.

Proportionality has also a methodological feature. It embodies the casuistic basis reasoning for proportionate decisions when balancing individuals' interests to communitarian benefits.

Until now main ethical issues in transplantation relate to the shortage of solid organs and HSPC to allo-transplant. As patients in need for a lifesaving allo-transplant largely surpasses the number of organs and Bone Marrow donations available, ethical problems

arise related to the choices about organ allocation. Medicine can maintain organs alive for some time in a brain-dead human being, waiting for HLA compatibility tests, Covid-19 screening, and preparation of patients for allo-transplantation. However, technical, surgical, and intensive care issues must be considered aiming to optimize transplant and consequent patients' survival. Organ transplants donated by young donors are more prone to be successful than organs collected from donors aged 40 or more years old, depending on the type of transplants. Donations from live donors, only possible for double organs like kidney, or in liver donations, where only part of the donor's liver is transplanted, and HPSCs CD34+, where only part of the total donor's pool of CD34+ cells is donated, rise specific immediate and long-term safety donor concerns as much as about patient's survival. Anonymous donations are crucial to prevent any contacts between donor and recipient, avoiding any future legal, psychologic, or financial issues. All information must be protected by encryption and several password protections. Ethical Committees in each Hospital must be available day and night to help in those transplant difficult allocation decisions.

Nevertheless, when a wide and efficacious production of transplants may be technically dispensed for a wide population of patients in need, new of the "old" dilemma will arise. Again, those seven principles of Bioethics will define the boundaries of possible actions and the Institutions already in place or new ones will help in the hard work of who to choose to be saved first and who will not. In the very initial steps of transplant production, a huge disproportionality will exist between the number of patients in need and the available capacity to produce the transplants. High ethical criteria shall be in place.

As research evolves aiming to solve unmeet diseases or to bring improvements in actual protocols of incurable diseases those new "old" dilemmas will again be addressed.

2. - Ethics in Stem Cell Research and Clinical Translation

As in respect to Human Stem Cell Research and Clinical Translation, Bioethics principles exposed above may translate as **in:** GUIDELINES FOR STEM CELL RESEARCH AND CLINICAL TRANSLATION issued by International Society for Stem Cell Research (https://www.isscr.org/docs/default-source/all-isscr-guidelines/guidelines-2016/isscr-guidelines-for-stem-cell-research-and-clinical-translationd67119731dff6ddbb37cff0000940c19.pdf.

Principle of Integrity of The Research Enterprise: "*The primary goals of stem cell research are to advance scientific understanding and to generate evidence for addressing*

unmet medical and public health needs. This research should be overseen by qualified investigators and coordinated in a manner that maintains public confidence and that ensures that the information obtained will be trustworthy, reliable, accessible, and responsive to scientific uncertainties and priority health needs. Key processes for maintaining the integrity of the research enterprise include those for independent peer review and oversight, replication, and accountability at each stage of research". Integrity is a *sine qua non* principle that ensures for patients, health authorities, and all community high standards of confidence all along the Research & Development processes. It is on the interests of all involved that each step could be traced and independently controlled to lower the risk of human and machine error.

Principle of Primacy of Patient Welfare: "*Physicians and physician-researchers owe their primary duty to the patient and/or research subject. They must never unduly place vulnerable patients at risk. Clinical testing should never allow promise for future patients to override the welfare of current research subjects. Application of stem cell-based interventions outside of formal research settings should be evidence-based, subject to independent expert review, and serve patients' best interests. Promising innovative strategies should be systematically evaluated as early as possible and before application in large populations. It is a breach of professional medical ethics to market and provide stem cell-based interventions to a large patient population prior to rigorous and independent expert review of safety and efficacy.*" This principle evidences the need for extensive safety experimentation and testing before any stem cell-based therapy could be available for the population.

Principle of Respect for Research Subjects: "*Researchers, clinicians, and clinics should empower human research participants (human subjects) to exercise valid informed consent where they have adequate decision-making capacity. This means that participants—whether in research or care settings— should be offered accurate information about risks and the state of evidence for novel stem cell-based interventions. Where individuals lack such capacity, surrogate consent should be obtained, and human subjects should be stringently protected from nontherapeutic procedures that involve greater than minor increase over minimal risk. In addition, the principle of respect for research subjects should be interpreted broadly to include other entities whose interests are directly implicated by research activities, including tissue providers and researchers or their support staff who harbor conscientious objections to certain aspects of human*

stem cell research." This principle calls for maximum respect toward research subjects implying that no one can be subjected to more than low and improbable known dangers.

Principal of Transparency: "*Researchers and clinicians pursuing stem cell research should promote timely exchange of accurate scientific information to other interested par ties. Researchers should communicate with various public groups, such as patient communities, to respond to their information needs, and should convey the scientific state of the art, including uncertainty about the MAY 2016 5 safety, reliability, or efficacy of potential applications. Researchers and sponsors should promote open and prompt sharing of ideas, methods, data, and materials.*" The wider the availability of information, the better to assure high overall safety standards.

Principle of Social Justice: "*The benefits of clinical translation efforts should be distributed justly and globally, with particular emphasis on addressing unmet medical and public health needs. Advantaged populations should make efforts to share benefits with disadvantaged populations. Trials should strive to enroll populations that reflect diversity in age, sex, and ethnicity. Risks and burdens associated with clinical translation should not be borne by populations that are unlikely to benefit from the knowledge produced in these efforts. As a general rule, healthcare delivery systems, governments, insurance providers, and patients, already overburdened by rising healthcare costs, should not bear the costs of proving the safety and efficacy of stem cell-based interventions. While these par ties may in some cases choose to fund clinical development, such as where there is unmet medical need and insufficient investment from the commercial sector, it is a matter of social justice that the costs of proving the safety and efficacy of a medical intervention be borne by entities that are expressly privileged to profit when such interventions are marketed. Where cell-based interventions are introduced into clinical application, their use should be linked to robust evidence development*." All human beings deserve fair access to innovative treatments. In real life, anyone knows and seem to accept that wealthy people will be treated better and faster when compared to other less favored people. Besides this reality could be difficult to change, the principle must be that anyone in need must be treated in equality.

3. - Specific Ethical Issues Related to Our Proposal

3.1. - Investors' reimbursement

Our proposal represents a huge potential jump forward in relation to human allo-transplantation. For every patient in need for a transplant, as technical and scale issues

are addressed, reliance on human donations will fall to zero. This represents a new hope for all patients in need for an allo-transplant. However, technology always have own limitations. One can envision situations where multiple patients will be in need for an allo-transplant to be created and Cell-Therapy Labs only will be able to prepare one in time. Mainly in the initial steps of the technology development, when Laboratories will be short in staff, space, finance, and other requisites, ethical issues will rise related to who will be saved and who will not. Again, Ethical Committees will help in making such difficult choices.

During my ImmunoHemotherapy Internship, I studied costs related to transplantations of HSPCs, (also known as Bone Marrow Transplantations). Having into consideration all intricate and complex issues involved, related to Hematopoietic Stem and Progenitor Cell collection from donors, actual HLA compatibility issues, immunosuppression protocols and related complications, disease relapses, and overall patient survival, I am convinced that our proposal will be able to start investors' reimbursement not too long after Regulatory Authorities may give approvals. This must also be a relevant ethical issue in the mind of all Cell Therapy entrepreneurs, because the greater the respect we can have toward investors the larger the number of patients we will be able to treat.

3.2. - Freeing Donors From Second Donations in Relapses

In actual Bone Marrow transplants, ethical and medical issues related to more than one donation from the same donor - relevant issue to be considered whenever a relapse happens- are not present anymore with our proposal. HLA full-match transplants will be created the number of times a patient may present with a relapse. Also, today not all donations contain the optimum amount of Hematopoietic Stem and Progenitor cells a patient may be in need. This is a terrible problem. Blood cancer eradication protocols (remission, induction, and consolidation steps), leave patients without any capacity to create blood cells, and totally dependent on a donation to survive. Without a reasonable amount of HSPCs, patients may not be in condition to recover.

Donation is always performed after the bone marrow ablation protocol is initiated, and thereafter, patient will not be able to recover from bone marrow ablation without a Bone Marrow transplantation. Once treatment is started a HSPC transplant is mandatory. Synchronization in a very short-time scheduling, do not give any opportunity to find any other donor in-time if a donation is not performed or in the case collected cells are not in the right amount, between 2 x 10^6 to 5 x 10^6 CD34+ cells/Kg patient's weight, Yamamoto C. *et al*. (2018)[2].

This issue is solved by our proposal, because from day one the optimum number of needed cells will be expanded for transplantation and samples stored for eventuality of relapses and for the construction of a wide Stem Cell Bank. The same reasoning applies in transplantation of artificially-build solid organs when it becomes possible. Since we will be able to create HLA full-match solid organ, it will be possible to create a copy. This is a major technical and ethical advantage of our proposal when compared to the reality today.

3.3. - Clinical Trials

One of the most important ethical and Regulatory Authorities issues related to our proposal, people may pose, is: How can we have approval for our transplants if what is the foundation and main advantage in our proposal is HLA full-match? This being such a specific human feature, that we do not have any animal model to experiment in, as well as we cannot make experiments in human beings. How can we get approval to transplant it ever?

People forgot that today, any Hematopoietic Stem and Progenitor Cell transplant performed all over the world has the exact same ethical and technical issues, and no one is denning approval for them to be performed. Moreover, actual HPSC allo-transplantations are, for the most part, performed without full HLA-match! Our solution takes Bone Marrow allo-transplantation to the level of full HLA-match, what is a relevant safety advantage for patients and for Ethical Committees and Regulatory Authorities to consider and rely on. A major advantage for approvals by Ethical Committees and Regulatory Authorities will be that any transplant performed in accordance with our proposal, always represents an HLA full-match allo-auto-transplant (a transplant where auto Chromosome 6 pair is in cohabitation with all the other 22 chromosome pairs that are from healthy allogeneic origin). The same is to say that it will always be safer than all the other HSPC allo-transplants based on non-related donations or even from half-match paternal, maternal, or half-match sibling donations. HSPC transplants prepared in accordance with our proposal are much closer to a twin or HLA full-match sibling donation. Twin and sibling HLA full-match donations are the best possible allo-transplants to perform today, (2022).

Another great advantage is that the twin-like situation achievable by our proposal is better than a twin-transplant *vis-a-vis* the patient has a congenital disease. Transplant will be healthy not carrying patient's mutation. Only if the patient's disease is on patient's chromosome 6 dependence, will not be addressed by our proposal. But any genetic

disease a patient may complain of, on the dependence of any of the other 22 chromosome pairs but the chromosome 6, will be addressed and potentially cured.

3.4. - Major Ethical Issues Related to Safety of Donors.

Another major ethical and clinical advantage is that no donors-related ethical considerations, and clinical and risk evaluations shall be made anymore. This fact will speed up the transplantation process as well as is a relevant contribution in lowering costs. With experience accumulated in Bone Marrow transplants, arguments will arise that will enable to safely allo-auto-transplant other cell, tissue, or solid organ, created on the bases of our proposal.

3.5. - "Human experimentation"

Considering the issues that human experimentation may raise, we will not be the first to be confronted with and obtain approval for. Others have already been pioneering it, even in the most difficult-for-approval field of Pediatric allo-transplantation[3]. For instance, our product will be always safer than the solution already approved to address inherited urea cycle disorders[3].

Anyway, in addition to our commitment to provide for the highest standards of cGMP products, several Ethical Committees and Regulatory Authorities will always evaluate every step before, during and after any transplantation as is always required by Ethics and Law.

PATENTS IN SCIENTIFIC RESEARCH

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1. - Why Are Patents Relevant in Science?

Everyone involved in research all over the world are seriously committed to the advancement of knowledge. Usually, secondary utilitarian considerations that may or may not emerge from the actual work are not main subject for discussion or concern. Most of the time, people search explanation for complex or apparently simple questions, without caring about future uses for those discoveries or even for the money taxpayers invested in their work.

However, everyone should be also able to consider this other side of the coin. Economic resources are always scarce, as also are the available reagents, and all other materials to pursue our goals[4,5].

It seems that for many of the world research institutions relying mainly on national budgets is enough to fulfill all goals and overcome all limitations.

Of course, this is in-itself a narrowing limitation in scientific research.

May be unconsciously or may not, we are all losing many opportunities by not reasoning all the way around.

What if our work could feedback to the community, (meaning paying it back), an important part of the invested money? What if, a relevant part of the invested money may return to the community embodied in enterprises and jobs? What if those jobs could be for unqualified people but most importantly for highly differentiated PhD holders?

Our Bioengineering PhD program: Cell Therapies and Regenerative Medicine, included an important curricular step where useful entrepreneurship tools were given to each one of us. Almost an MBA program was taught, mainly by Prof. Frederico Ferreira. I am deeply grateful to Prof. Frederico Ferreira by the important knowledge he taught us. So, the seeds were seeded. It is up to anyone to grow as much and as better as we can, the seeds invested in our education. But in the stress for publication, people may be losing the opportunity to create an even better world for themselves and for many others. People can and must make publications of their achievements, but if a careful timing is programmed, publications can also be done after a patent application is filled. And this is exceptionally relevant as the opposite is not possible anymore.

During my PhD I attended many intellectual property events by Propriedade Intelectual/IST and Transferência Tecnológica/IST, and it was easy to conclude that too many researchers were there, seeking to fill an impossible patent. The subject of their PhDs could have been novel, have industrial application, and were inventive… but because there was a previous publication, subject lost novelty. And novelty is a *sine qua non* requirement for patentability, EPO- Guidelines for Examination - https://www.epo.org/law-practice/legal-texts/html/guidelines/e/g_i_1.htm and Instituto Nacional da Propriedade Industrial – INPI)

European Patent Office, (https://documents.epo.org/projects/babylon/eponet.nsf/0/B415FE40DAEEEC60C125864600479CB3/$File/EPC_17th_edition_2020_en.pdf.

Something that could attract investors to help in development of that specific research field, suddenly lost most of the interest, because anyone could explore, take advantage, and profit from someone else hard work. And even in distant countries someone can benefit from non-protected other taxpayers' money! Is not fair!

In my case, I was specifically conducted by Prof. Domingos Henrique, my Supervisor to my PhD program: Cell Therapies and Regenerative Medicine, with the exact aim of making some difference. I was told by my Supervisor that one of the main goals in my PhD Program was to create enterprises, patents, and jobs. We call it: Translation Research. In the specific case of my PhD program, the goal was Translation Medicine. I understood very well the goals to pursue and the need to keep all novelty hidden, otherwise would have been losing patentability[6].

2. - OUR PATENT APPLICATION – Inventors: Prof Margarida Diogo, Prof. Domingos Henrique, Doctor Carlos Rodrigues, Doctor Cláudia Miranda and João Carreira, MD. INPI nº 117101/ U /05-03-2021

AND LAB2MARKET@IST 2020 PROGRAM.

Aiming for a patent as the paving condition to allow for patients' treatment, we developed a team composed by Professor Domingos Henrique (Instituto de Medicina Molecular - Prof. João Lobo Antunes (IMM-Prof. João Lobo Antunes), Professor Margarida Diogo (Instituto Superior Técnico, (IST), Institute for Bioengineering and Biosciences (iBB), Stem Cell Engineering Research Group (SCERG), Doctor Carlos Rodrigues (Instituto Superior Técnico, (IST), Institute for Bioengineering and Biosciences (iBB), Stem Cell Engineering Research Group (SCERG), Doctor Cláudia Miranda (Instituto Superior Técnico, (IST), Institute for Bioengineering and Biosciences (iBB), Stem Cell Engineering Research Group (SCERG) and João Carreira MD, PhD Student at Instituto de Medicina Molecular – João Lobo Antunes (IMM – João Lobo Antunes) and (Instituto Superior Técnico, (IST), Institute for Bioengineering and Biosciences (iBB), Stem Cell Engineering Research Group (SCERG).

With help from Núcleo da Propriedade Intelectual in Instituto Superior Técnico (PI/IST) and Núcleo de Transferência de Tecnologia also in Instituto Superior Técnico (TT/IST) I attended several meetings on the Intellectual Property protection by Patents and other legal possibilities.

As a push forward, in full Covid-19 pandemics, and even before a patent application was filled, an invitation was received for a participation in the program Lab2Market@IST2020. LAB2MARKT@IST2020 was a PI/IST and TT/IST-based program intended to speed up translation from academia to business. From March to June 2020 also with help of I-DEALS and EVERIS FOUNDATION, an intensive preparation for how to contact and explain our invention to the markets was fine tuned.

This was interesting, profitable, and new time for all participants. And a real opportunity to go and find ways to reimburse my country taxpayers by the creation of enterprises and jobs for motivated, intelligent, skilled, and sometimes unemployed highly differentiated PhD holders!

At the end of Lab2Market@IST 2020 Program we got the invitation from a Venture Capital investment group for us to work with, named HOVIONE CAPITAL, today BIONOVA CAPITAL. I will not lose the opportunity to build an enterprise with the help of all patent co-authors, or the ones that may be courageous enough or have time for it!

Moreover, patients shall start to be cured in my lifetime. And that is the most important issue that is motivating me. Overriding difficult Covid-19 pandemic obstacles is a real challenging issue. No experience in a very protected, regulated and submitted to highly intensive investments as is the field of Cell Therapies in particular, and Pharmaceuticals in general is a huge responsibility to share. Finding the best partnerships possible, is also a significant issue.

Patent application nº 117101 U 05-03-2021, was filled in **Instituto Nacional da Propriedade Industrial (INPI), Portugal**

Other Patents will arise soon, as safeguards to protect investors' money. As much as necessary, and possible. In the meanwhile, new discoveries shall be converted into patents. Enterprise survival as well as safeguarding investor's money will be a great challenge to handle in a daily basis. Patents must be the milestones of the process. And that will be great time for all of those that live for discovery. Entrepreneurship, Discovery as (R&D), and Patents are the vertices of an ultra-dynamic triangle that not many people understand and even less can construct and maintain spinning. For sure, the real difference in human development happens whenever the triangle is maintained alive in permanent movement.

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