Genome editing of Mesenchymal Stem/Stromal Cells (MSCs) by CRISPR/Cas9 technology for azurin-based anticancer therapies

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Abstract

The conventional systemic therapies applied in cancer treatments cause several side effects in patients. Therefore, more specific targeted therapies are in need to a more safe and precise treatment. MSCs present the ability to be vehicles for drug delivery to the tumour sites due to their unique immunomodulation and migration potential towards tumours. Azurin is a bacterial protein produced by *Pseudomonas aeruginosa* that has been strongly explored considering its antitumoral activity. Thus, the strategy of the present work includes the genetic edition of MSCs to establish these as potential carriers of azurin into tumoral sites.

The initial steps of this strategy were performed in this work, namely the design and test of four gRNAs to produce DSBs in a genomic safe harbour, the first intron of *PPP1R12C* gene, and the design of a donor template that will allow the incorporation of the azurin encoding sequence into this locus via CRISPR/Cas9 mechanism through homology repair. The guides were designed using bioinformatics tools and chosen considering the lower number and site of off-targets in exonic regions and the higher score between all. The efficiency in HEK293T cells was assessed with the GeneArt™ Genomic Cleavage Detection Kit (Invitrogen™). Of the guides tested, two were successful in producing the desired cuts with efficiencies of 2.72 and 3.00.

Concerning to the construction of donor template, all parts were obtained, being that the next steps are the ligations between them to produce a donor template capable of repairing the DSB in future experiments using MSCs.

Keywords: Mesenchymal Stem/Stromal Cells; Azurin; Anticancer therapies; Genome editing; CRISPR/Cas9 mechanism; Homology direct-Repair.

1. Introduction

Cancer is a result of mutations that interfere with oncogene and tumour suppressor gene function, leading to uncontrollable cell growth, where an oncogene is a gene encoding a protein that promotes cell division whereas a tumour suppressor gene is one that reports to cells when not to divide [1].

The available systemic therapies cause side effects in treated patients, from nausea to damaged tissues, as well as in cancer survivors, namely iatrogenic consequences like genomic mutations and their consequences. Thus, personalized and targeted therapies are necessary and urgent [2].

MSCs are self-renewing and multipotent adult stem cells of mesodermal origin with an important ability to differentiate into several cell types like osteoblasts, chondrocytes and adipocytes. These cells are also involved in processes like immunosuppression and have capacity to migrate towards sites of inflammation and tumours, which is referred to as tropism or homing capacity. Thus, MSCs can be used as vehicles to deliver drugs and anti-tumour agents to tumour sites [3].

Azurin is a cupredoxin type of electron transfer low molecular weight redox protein from the opportunistic pathogenic bacteria *Pseudomonas aeruginosa*. It is reported that azurin selectively induces the apoptosis in several human cancer cell types, entering effectively in cancer cells but not in normal cells, showing lower cytotoxicity concerning to the latter. The cytotoxicity is based on the internalization of the protein, which creates a complex with the tumour suppressor protein p53 and stabilizes it, inducing apoptosis or cell cycle arrest in the G1 [4].

Genome editing with programmable nucleases has been strongly explored, opening new avenues for several applications from basic research in disease processes via cellular and animal models to clinical therapy. Clustered regularly interspaced short palindromic repeats (CRISPR) make part of the prokaryotic adaptive immune system, being a distinctive feature of the genomes of most Bacteria and Archaea and involved in resistance to bacteriophages [5]. There are six CRISPR systems that work according to different mechanisms in order to ensure DNA recognition and cleavage. These systems are grouped in two classes: Class 1 (types I, III, and IV), and Class 2 (types II, V, and VI) [6]. Class 2 systems have been considered for genome engineering owing to their simplicity, and only type II, derived from *Streptococcus pyogenes* [7], has been utilized for RNA-guided engineered nucleases. The effector single protein of type II is Cas9 [6]. This endonuclease acts in sync with two guide RNAs: CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA) [7, 8]. Moreover, in order to simplify the endogenous

system for laboratorial use, the two guide RNAs (tracrRNA and crRNA) have been combined into a single chimeric RNA sequence, named by single guide RNA (sgRNA) [7], which directs the CRISPR-associated protein Cas9 to introduce a sequence-specific DNA cleavage by double-strand breaks (DSBs) in the target DNA. This acquired Cas9-DNA recognition requires both base-pairing complementarity between the crRNA and the target DNA and a short-conserved sequence named by proto-spacer adjacent motif (PAM) [9]. Thus, the system constituted by bacterial CRISPR repeats associated with protein Cas9 (CRISPR/Cas9) consists in a complex of a sgRNA with Cas9 nuclease from *Streptococcus pyogenes* (SpCas9), which recognizes a variable 20-nucleotide target sequence adjacent to a 5'-NGG-3' PAM and introduces a DSB in the target DNA three base pairs upstream of the PAM sequence [10].

This induced DSB is a potentially lethal event for the cell and, therefore needs to be repaired by the DNA repair machinery [11], via one of two main mechanisms: the non-homologous end joining (NHEJ) and the homology-direct repair (HDR) pathways [12].

The mechanism of NHEJ repair consists in the joining of the free DNA ends through a homology-independent and mechanistically flexible process, which often generates random small insertions or deletions (indels) [7, 12].

The HDR pathway of DNA damage repair consists in an accurate strand-exchange process based on existing homologous DNA templates [12], which contain homology to sequences flanking the DSB named by homology arms [11] .

An approach strongly considered in gene therapy is the targeted insertion into a locus named by "safe harbour". This is a predefined locus unrelated to the disease-causing gene being a safe site to introduce genetic material [13]. A promising safe harbour is the AAVS1 locus that is the most common integration site for AAV, a type of virus without a known associated pathology. This locus is located on chromosome 19 within intron 1 of the *PPP1R12C* gene [13, 14].

Taking into account the above information, the CRISPR/Cas9 method may be a strong potential "bench-to-bedside" tool. Moreover, considering the properties of azurin as an anticancer protein and the ability of the MSCs to be vehicles for drug delivery to the tumour sites as described above, a strategy that includes genetically modifying MSCs to carry azurin to the target site may be a promising approach that should be carefully accessed as an anticancer therapy. The initial steps of this strategy, namely the designing and testing of gRNAs to produce DSBs in a genomic safe harbour, and the design of a donor template that leads to the incorporation of the azurin gene that encodes this protein into this locus via CRISPR/Cas9 is the scope of this master thesis.

2. Materials and Methods

2.1 Cell culture

HEK293T cell line was grown in a humidified atmosphere at 37ºC with 5% CO_2 (Binder CO_2 incubator C150). HEK293T cells were obtained from ECACC and cultured in T-flasks with DMEM medium (GIBCO™) supplemented with 10% of heat-inactivated FBS (GIBCO™), 100 IU/ml penicillin, and 100mg/ml streptomycin (PenStrep, GIBCO™), D. For their maintenance, cells were passaged 3 times per week by chemical detaching with Trypsin 0.05%.

2.2 Guide design, cloning and testing

2.2.1 Guide Design

The guide RNA (gRNA) sequence was designed with the help of three online CRISPR Design Tools (Wellcome Trust Sanger Institute (WTSI) Genome Editing, CRISPOR [15], and CRISPR Design from Zhang Lab, MIT) based on the highest score and the lowest number of predicted off-targets in exonic regions. The genome location selected to target was the first intron of *PPP1R12C* gene known as AAVS1 locus. The pairs of oligonucleotides corresponding to both strands of each guide sequence were synthetized by Sigma-Aldrich©.

2.2.2 Guide Cloning

The oligonucleotides were resuspended in Tris.HCl pH8 10mM to a final concentration of 1mM by incubation for 15min at room temperature with shaking. The oligonucleotides were diluted to 100µM with ultrapure water, and phosphorylated and annealed in a reaction containing 10µM of both sense and antisense oligonucleotides, 1x T4 DNA Ligase Buffer (ThermoFisher), 1mM of ATP, 10U of PNK (ThermoFisher), and ultrapure water to a final volume of 10µl, incubated for 30-40min at 37ºC and for 5 min at 95ºC. Then, the reactions were cooled down on the bench (~2,5h) to the room temperature.

Prior to the ligation step, all annealed oligonucleotides were diluted 1:250 with ultrapure water. The mixes were prepared in 0.5ml microcentrifuge tubes containing in a final volume of 10µl: 50ng of vector px459 [pSpCas9(BB)-2A-Puro (PX459, Addgene plasmid # 62988) [16]] previously digested with *Bbs*I restriction enzyme; 1µl of oligo duplex (1:250); 1x T4 DNA Ligase Buffer (ThermoFisher); 0.5mM of ATP; and 5U of T4 DNA Ligase (ThermoFisher). As a control, 50ng of vector px459 were ligated without an insert in the same conditions. All tubes were incubated overnight at 8ºC.

Competent *E.coli* DH5α bacteria were used to transform with ½ of ligation products on the next day: 5µl of each ligation were incubated on ice with 100µl of competent bacteria in 2ml microcentrifuge tubes during 15-30min; after 1min30s at 42ºC cells were cooled down on ice for 1-2min. 900µl of LB medium were added to tubes, and these were left for 1h at 37ºC with shaking (220rpm). Bacteria were pelleted by brief centrifugation, supernatant was discarded, and the pellet resuspended in the remaining liquid and plated onto LB agar plates with 100µg/ml of ampicillin. Cells were incubated overnight at 37ºC and isolated colonies were picked in 3ml of

ampicillin-containing LB medium and grown overnight at 37ºC and 220rpm. Plasmid DNA was extracted using NZYMiniprep Kit (NZYTech) [17], following the manufacturer's instructions. Sanger sequencing was performed at GATC Biotech. After the sequence confirmation, the correct cells were stored in 15% glycerol at -80ºC.

2.2.3 Cleavage efficiency test of the guides

Approximately, 130000 HEK293T cells/well were plated in a final volume of 1ml in a 24-well plate and incubated until reaching 70-80% of confluence. After that, cells were transfected using Lipofectamine® 2000 Transfection Reagent (Invitrogen™). The px459 plasmids with the guides (gRNA plasmids) and the control empty px459 plasmid were diluted to final concentration of 200ng/µl and ~500ng were transfected per well. 50µl of Opti-MEM™ medium (GIBCO™) were mixed by pipetting "up and down", with 2µl of Lipofectamine® 2000 (A), or with 3µl of diluted plasmid (B) and incubated for 5min at room temperature, before mixing both A and B, in dropwise manner and incubating for more 15min at room temperature. The final 100µl were added to the cells dropwise to the correspondent well containing 1ml of medium and further mixed by pipetting "up and down".

The selection of transfected cells with puromycin started 24 hours after transfection by replacing the medium in each well with medium containing puromycin (2µg/ml) with exception of the control well that has only cells. This selection was performed during 48h, so after 24h the medium containing puromycin was renewed. After selection, cells were collected from all wells except the well with no transfected cells to which medium containing puromycin had been added since all cells were dead, as expected. Cells were washed with PBS and collected with trypsin, pelleted by centrifugation and stored at -80ºC for further analysis.

The cleavage efficiency of the guides was tested with GeneArt™ Genomic Cleavage Detection Kit (Invitrogen™) [18], following the manufacturer's instructions, being that, in the conditions used in the thermocycler, the annealing temperature was 61ºC.

2.3 Cloning into pBluescript II KS plasmid (pKS)

20µg of pKS (Stratagene) DNA were hydrolysed with 150U of *EcoR*V enzyme (ThermoFisher) in a final volume of 200µl of 1x Red Buffer (ThermoFisher) for 1h at 37ºC. After that, 5U of Fast AP (ThermoFisher) were added for another 1h at 37ºC. The DNA was precipitated with ethanol overnight, run on agarose gel and purified with NZYGelpure kit (NZYTech) [19], following the manufacturer's instructions.

Prior to ligation step, PCR fragments were phosphorylated and blunted in a reaction containing 20µl of DNA fragment, 1x T4 DNA Ligase Buffer (ThermoFisher), 0.5mM of dNTPs, 0.5mM of ATP, 1.5µl of T4 DNA polymerase (5U/µl) or Klenow polymerase (10U/µl) (ThermoFisher), and 15U of PNK (ThermoFisher) in a final volume of 30 µl. This mix was incubated at 37ºC for 1h and 15min at 75ºC. Ligation was carried out in a final volume of 10µl of 1x T4 DNA Ligase Buffer (ThermoFisher) containing 50ng of linearized and dephosphorylated pKS vector, 0.5mM of ATP, 5U of T4 DNA Ligase (ThermoFisher) and 6.5µl of purified DNA fragment at 8ºC overnight. Control ligation containing vector alone was made in parallel.

Competent *E.coli* DH5α bacteria were transformed with ½ of ligation products on the next day as described in section 2.2.2. Plasmid DNA was extracted using NZYMiniprep Kit. To identify the clones with correctly inserted DNA fragments, plasmid DNA was hydrolysed by restriction enzymes *Hind*III FD (ThermoFisher) and *Eco*RI FD (ThermoFisher) in a following reaction: 3µl of plasmid DNA and 0.5FDU of each enzyme in 20µl of 1x Buffer FD (ThermoFisher). The mix was incubated for 1h at 37ºC and analysed on 1.2% agarose gel. The positive clones were Sanger sequenced at GATC Biotech and, after the confirmation, stored in 15% glycerol at -80ºC.

2.4 PCR Amplification

2.4.1 Fragments Amplification by PCR

PCR reactions were made to amplify the *IL6*-promoter and homology arms, *hAzurin,* and *eGFP*+SV40polyA genes using as templates genomic DNA of HEK293T cells, pVAX-engineered Azurin plasmid DNA (custom synthesized by NZYTech) and pEGFP-N1 plasmid DNA (Clontech), respectively.

The promoter sequence of *IL6* gene [20] was amplified with 2.5U of NZYLong DNA Polymerase (NZYTech) in a final volume of 25µl containing: 1x Reaction Buffer (NZYTech), 0.4mM of dNTPs, 0.4µM of each primer, and 15ng of correspondent DNA. The conditions used in the thermocycler were: initial denaturation at 95ºC for 2min; 30 cycles with denaturation at 95ºC for 30s, annealing using a temperature gradient 55ºC-58ºC-60ºC-62ºC for 45s, and extension at 68ºC for 1.30min; and a final extension at 68ºC for 5min. This PCR product was purified and consequently reamplified with Phusion, as described below.

The PCR reactions were performed in a final volume of 25µl of 1x Phusion HF Buffer (ThermoFisher) containing: 0.5U of Phusion High-Fidelity DNA Polymerase (ThermoFisher), 0.4mM of dNTPs, 0.5µM of each primer, forward and reverse, and 1µl of correspondent DNA. The conditions used in the thermocycler were: initial denaturation at 98ºC for 2min; 30 cycles with denaturation at 98ºC for 10s, annealing using temperature gradient 55ºC-58ºC-60ºC-62ºC for the *IL6*-promoter and 54ºC-56ºC-58ºC-60ºC for the *hAzurin* and *eGFP*+SV40polyA for 45s, and extension at 72ºC for 1.30min for the *IL6*-promoter and 60s for the *hAzurin* and *eGFP*+SV40polyA; and a final extension at 72ºC for 5min. The promoter sequence of *IL6* gene was purified and cloned into pKS vector as described in section 2.3. The promoter sequence of *IL6* gene in one of the resulting clones was re-amplified with Phusion High-Fidelity DNA Polymerase according to protocol referred before relative to this polymerase but with thirty-five cycles instead thirty cycles including five initial cycles with an annealing temperature of 54ºC and thirty consequent cycles with an annealing temperature of 62ºC. The resultant PCR product was treated with PNK (ThermoFisher) for 1h at 37ºC, diluted 1:2 and purified following the PCR clean-up protocol present in NZYGelpure kit. The purified DNA was cloned into pKS vector. The promoter sequence in one of the resulting clones was re-amplified with Phusion High-Fidelity DNA Polymerase following the protocol referred before relative to this polymerase but with an annealing temperature of 60ºC. The resultant PCR product was purified following the PCR clean-up protocol, digested with *Dpn*I restriction

enzyme (New England Biolabs) adding 0.5µl of enzyme and 0.5µg of DNA in a final volume of 25µl of 1x NEBuffer (New England Biolabs) by incubation for 15min at 37ºC, and purified by gel extraction.

The homology arms were amplified with KAPA2G Fast HotStart ReadyMix PCR Kit (KAPABIOSYSTEMS) in a final volume of 25µl containing: 1x KAPA2G Fast HotStart ReadyMix, 0.5µM of each primer, forward and reverse, and 15ng of correspondent DNA. The conditions used in the thermocycler were: initial denaturation at 95ºC for 3min; 35 cycles with denaturation at 95ºC for 15s, annealing using a temperature gradient 60ºC-62ºC-64ºC for 15s, and extension at 72ºC for 1s; and a final extension at 72ºC for 1min. These PCR products were purified being that the PCR clean-up protocol was applied in the 5'HA purification while the gel extraction and subsequent purification were applied to 3'HA.

2.4.2 PCR ligation

The Ligation I was amplified in a final volume of 25µl containing 9ng and 18ng of *IL6*-promoter and *hAzurin* DNA, respectively, following the protocol correspondent to Phusion High-Fidelity DNA Polymerase (ThermoFisher) but with a gradient temperature in annealing stage of 60ºC-62ºC-64ºC and an extension time of 2.30min. The resultant PCR product was purified.

The Ligation II was amplified in a final volume of 25µl containing 1µl of each correspondent DNA diluted 1:10, following the protocol correspondent to NZYLong DNA polymerase (NZYTech) but with thirty-five cycles instead thirty cycles where the annealing temperature was 60ºC and the extension time was 2min. This PCR product was purified and cloned into the pKS vector. The Ligation II was re-amplified from one of the clones obtained with Phusion High-Fidelity DNA Polymerase (ThermoFisher) in a final volume of 25µl containing 15ng of correspondent DNA. In this case, with an annealing temperature of 60ºC and an extension time of 2min. The resultant PCR product was purified following the PCR clean-up protocol.

The Ligation II+3'HA was performed in a final volume of 25µl of 1x PCR Buffer (GRiSP) containing 1U of Xpert HighFidelity DNA Polymerase (GRiSP), 0.8µM of each primer, forward and reverse, and 15ng of each correspondent DNA. The conditions used in the thermocycler were: initial denaturation at 95ºC for 1min; 35 cycles with denaturation at 95ºC for 15s, annealing at 60ºC for 15s, and extension at 72ºC for 1.30min; and a final extension at 72ºC for 3min. The resultant PCR product was purified.

3. Results

3.1 Cas9 Guide construction and test

As stated before, we chose the referred AAVS1 genomic safe harbour to precisely introduce the gene coding for azurin in a stable manner within the genome of human Mesenchymal Stem cells using CRISPR/Cas9 technology. This region is located in the first intron of the *PPP1R12C* gene [13, 14].

The selection of the appropriate Cas9 guide sequence followed the steps described next. First, the bioinformatic design of four different guides, corresponding to four different regions of the first intron of *PPP1R12C* gene, based on two main features (which are dependent on the algorithm of each bioinformatic tool used) namely the overall score and the number of exonic off-targets. The identification of possible off-targets located in coding sequences of other genes allows the verification of which gene can be possibly disrupted, creating a possible mutation into a protein or interruption of gene expression, whereas the probability of this happening in intronic regions is lower. Secondly, the cloning of the selected guides that were previously synthetized by Sigma-Aldrich© into guide RNA and Cas9-expressing vector pX459 (Addgene plasmid # 62988); and lastly the test of the cleavage efficiency of each guide using the GeneArt™ Genomic Cleavage Detection Kit (Invitrogen™) in target cells.

3.1.1 Bioinformatic Design

The three different tools used to design the guides' sequence were: Wellcome Trust Sanger Institute (WTSI) Genome Editing; CRISPOR [15]; and CRISPR Design from Zhang Lab, MIT. The obtained guides are presented in

Table 1. The score and number of exonic off-targets of each guide obtained from each bioinformatic tool referred above are presented in Table 2.

Table 1 Cas9 guides used in this work with respective sequence and PAM.

	Guide 1 (Reverse strand)	**Guide 2 (Forward strand)**	**Guide 3 (Forward strand)**	**Guide 4 (Forward strand)**
Sequence[1] 5'-3'	CGAATTGGAGCCGCTTCAAC	CCAGCGAGTGAAGACGGCAT	CAATCCTATTATAGCCGAAT	CGGCCAGCGGTTTGGTAACG
PAM[2]	TGG	GGG	GGG	AGG

[1] A – Adenine, T – Thymine, C – Cytosine, G - Guanine; [2] proto-spacer adjacent motif.

Table 2 Number of exonic off-targets sites and score value for four guides tested in this work, obtained from three different bioinformatics tools (WGE, CRISPOR, and CRISPR Design).

	Tools	Guide 1	Guide 2	Guide 3	Guide 4
Number of Exonic off-target sites	WGE[1]	0	0	No data available.	1
	CRISPOR	0	1	0	1
	CRISPR Design	2	7	0	3
Score	CRISPOR	94	92	96	97
	CRISPR Design	94	89	92	95

[1] Wellcome Trust Sanger Institute (WTSI) Genome Editing.

3.1.2 Cloning of double-strand guide oligonucleotides into pX459 vector

The next step was the cloning of the selected guides into the pX459 vector with a codon-optimized SpCas9 for expression in human cells and the single guide RNA under the control of U6 promoter. The presence of two asymmetric sites for the *Bbs*I restriction enzyme in pX459 allows the double-strand guide DNA to be cloned into the vector before the sgRNA scaffold. This vector also includes a selection marker of resistance to puromycin antibiotic. The correct insertion of guide sequences into the pX459 vector was verified by Sanger sequencing at GATC Biotech.

3.1.3 Cas9 Cleavage efficiency test

The cleavage efficiency of each selected Cas9 guide was tested in order to choose the guide with the best cleavage efficiency prior the design of the homology arms for the introduction of azurin cassette. This test was performed in HEK293T cells and also attempted in human bone-marrow derived MSCs. HEK293T are widely used for the development of genome-editing approaches as well as the study of DNA repair mechanisms in general [21], and the MSCs were used because they make part of therapeutic strategy proposed in this work.

The efficiency of the guides was determined with the GeneArt™ Genomic Cleavage Detection Kit (Invitrogen™) [18].

HEK293T cells

In this test, the cells were first transfected with pX459 containing each of the guides with Lipofectamine2000 as per standard protocols, upon which, successfully transfected cells are selected with the addition of puromycin to the cell culture media. The puromycin concentration used to select the HEK293T cells was 2µg/ml [22] with 48 hours of exposition. The test uses genomic DNA from positive transfected cells to amplify by PCR loci where the gene-specific double-strand breaks occur.

The resultant PCR product is denatured and re-annealed, generating mismatches as strands with an indel re-anneal to strands with no indels or a different indel. These mismatches are then detected and cleaved by the Detection Enzyme of the kit and the resultant bands are revealed by agarose gel electrophoresis (see Figure 1) and analysed by ImageJ to obtain a value for rate of efficiency for each guide.

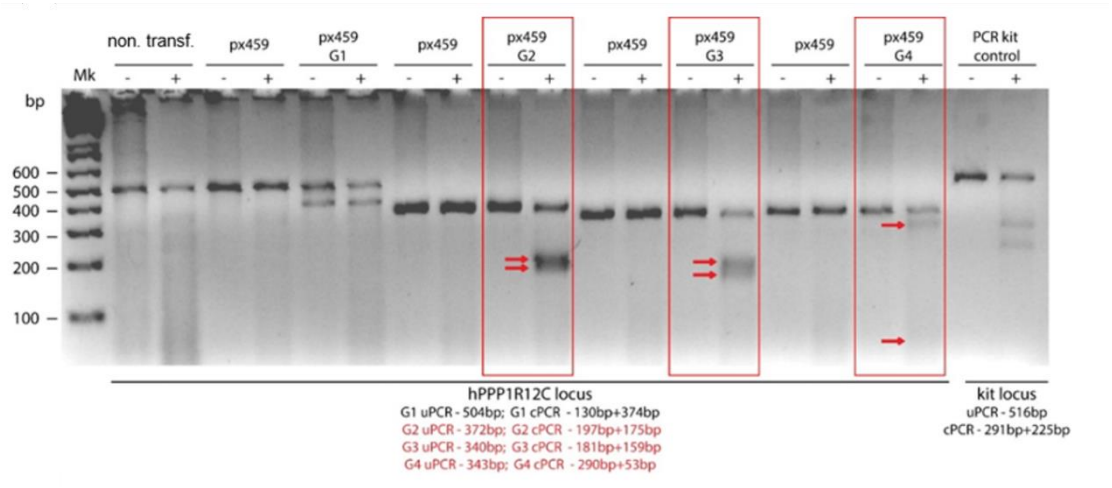

Figure 1 Agarose gel electrophoresis of PCR products digested with Detection enzyme to determine cleavage efficiency of the tested guides. Efficiency ratios are the following: Guide 1: non-cutter; Guide 2: 2.72; Guide 3: 3.00; Guide 4: impossible to observe the second band (ratio of [cut/noncut] measured by ImageJ software). G1 - Guide 1; G2 - Guide 2; G3 - Guide 3; G4 - Guide 4; uPCR – uncut PCR; cPCR – cut PCR; Mk – 1Kb Plus DNA Ladder (Invitrogen™).

The observation of the Figure 1 and the presented ratios for each guide allows to conclude that Guide 3 is the best one, since the intensity of the sum of the bands correspondent to cut fragments is three fold higher than the intensity of the band correspondent to uncut fragments, meaning that the efficiency of this guide is higher, that is in the agreement with the bioinformatic analysis referred previously. Furthermore, it is possible to conclude from Table 2 that the best guide considering the score value and number of exonic off-target sites is the Guide 3, since its score is 96 (CRISPOR tool) or 92 (CRISPR Design tool) and number of exonic off-target sites is 0 in both tools.

MSCs

Taking into account the success rate verified with HEK293T cells, only Guide 3 was tested in MSCs. Two attempts to test the Guide 3 in MSCs were performed.

The first attempt to test the Guide 3 in MSCs was carried out with a cell density of ~38000 cells/cm^2 and a concentration of 2.5µg/ml [23, 24] of puromycin to select the cells.

24h after the microporation, the cells presented a satisfactory morphology and confluence, being that in the conditions where the DNA was microporated to the cells, the number of cells was lower than in control conditions (microporation with no addition of plasmid DNA), suggesting a successful microporation protocol. Thus, at 48h after microporation, selection with puromycin was initiated. After 24h of selection with puromycin there were still some live cells in control, therefore the medium containing puromycin was changed. At the beginning of the second day, the medium containing puromycin was changed again in order to improve the selection. By the end of this day, the cells were mostly dead in the control condition so the medium was replaced by StemPro® MSC SFM XF without puromycin to allow the expansion of the cells that survived. However, after three days of recovery the cell density remained unchanged. 50% (1ml/well) of the medium was changed every two days in order to maintain the essential factors for the cell recovery, however the cells eventually died.

Due to the lack of success of the first attempt, the second attempt to test the Guide 3 in MSCs was realized with a lower cellular density (of ~7400cells/cm^2) and lower concentrations of puromycin (1µg/ml [25] and 0.5µg/ml [26]).

24h after the microporation, the cells presented a satisfactory morphology, being that in the conditions where cells were microporated with plasmid DNA the number of cells was also lower. After 48h the cell density increased, so the selection with puromycin was initiated.

At the concentration of 1µg/ml of puromycin, cells were maintained for 48h, and after 24h the medium containing puromycin was changed. By the end of the second day, the majority of cells in control condition were dead so the medium was replaced by StemPro® MSC SFM XF medium. The level of confluence in other conditions was very low.

At the concentration of 0.5µg/ml of puromycin, the selection was performed during 72h since after 48h there were still some alive cells in control conditions. The medium containing puromycin was renewed every 24h. By the end of the third day, the cells in control conditions were dead so the medium was replaced by StemPro® MSC SFM XF medium. The level of confluence in other conditions was higher than in the previous attempt.

50% (1ml/well) of the medium was changed every two days in order to maintain the essential factors to the cell recovery. However, after one week of a modest increase in confluence the cells ceased to grow, without reaching confluence levels necessary to realize the cleavage efficiency test.

Therefore, it was not possible to obtain in the time course of this thesis a conclusive test of the cleavage efficiency of Guide 3 in bone-marrow derived human mesenchymal stem cells.

3.2 Donor Template

The construction of the donor template to be used for HDR of DSB was performed in several steps. First of all, each part of the final construct was amplified from an appropriate DNA template. After that, the attempts to perform the ligations between the parts were realized. All cloned DNA fragments of the donor template referred below were cloned into pBluescript II KS vector.

3.2.1 Construction of the parts of donor template

IL6-promoter

The promoter of the *IL6*-gene was amplified by PCR using genomic DNA of HEK293T cells as a template [20]. Several attempts of PCR amplification and cloning were performed to obtain PCR product without mutations introduced during amplification process, and the best resulting DNA obtained has two nucleotide mismatches in its sequence, for which the verification by Sanger sequencing was not totally conclusive. Therefore, 1µl of this PCR product was re-amplified with Phusion HF DNA Polymerase in order to correct the possible errors. The resultant PCR product of this re-amplification was cloned and the insertion of the promoter fragment was verified by hydrolysis of the vector with enzymes *Hind*III and *Eco*RI as referred in section 2.3. Two clones were verified by Sanger sequencing and one of these presented only one mismatch in region where the reverse primer anneals. The fragment correspondent to the *IL6*-

promoter sequence in this clone was re-amplified with the Phusion HF DNA Polymerase and cloned. The results of the sequencing showed that the mismatch of the initial template remained in the three clones. One possible conclusion is that this can be due to a mismatch in the DNA sequence of the template that can differ from that in the databases to which the sequenced DNA is compared to. It would be important to amplify by PCR the same region from different DNA template sources, e.g, different cell lines to verify if they contain the same mismatch.

Considering that the referred DNA was from a plasmid and was cloned into bacteria, i.e. it was methylated, this was digested with *Dpn*I enzyme, after re-amplification with Phusion HF DNA Polymerase and purification with NZYGelpure kit from one of these three clones, to degrade the template from bacteria and preserve the PCR product, which probably was corrected because the primers used in re-amplification had the corrected sequence. The resultant product of digestion with *Dpn*I was purified with NZYGelpure kit.

hAzurin and *eGFP*+SV40polyA

The *hAzurin* and *eGFP*+SV40polyA were amplified from pVAX-engineered Azurin and pEGFP-N1 plasmids DNA, respectively.

5' and 3' homology arms

The homology arms were designed based on the genomic location of the DSB introduced by the Guide 3 and amplified from genomic DNA of HEK293T cells.

Both were purified with NZYGelpure kit. However, taking into account the specificity of the amplification just a PCR clean-up was applied to 5'HA whereas a gel extraction and subsequent purification were applied to 3'HA.

The scheme of donor template and the amplification of the correspondent portions are presented in Figure 2 (A) and Figure 2 (B), respectively.

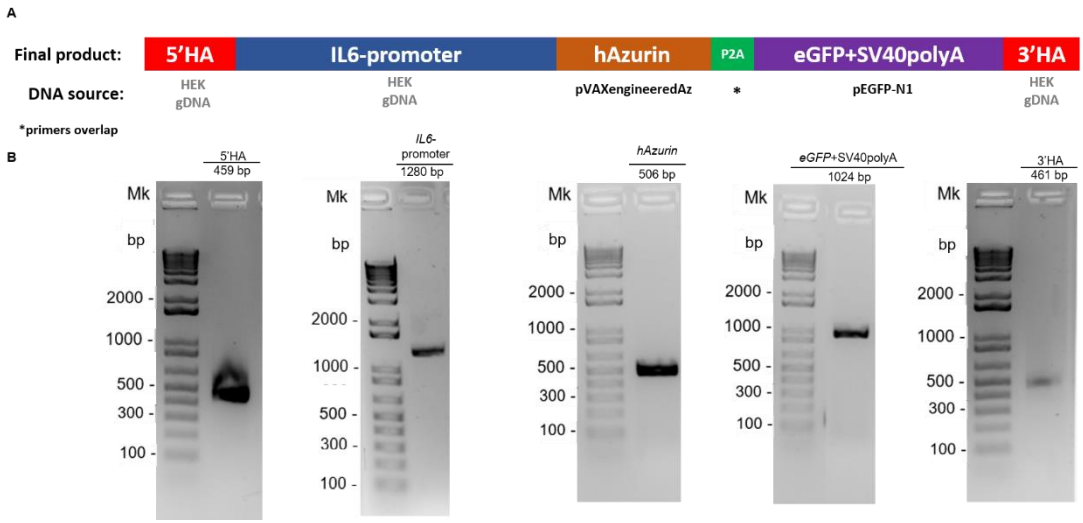

Figure 2 (A) The final donor template constituted by homology arms (5'HA and 3'HA) and *IL6*-promoter amplified from HEK293T genomic DNA, human Azurin (*hAzurin*) gene amplified from pVAX-engineered Azurin vector, *eGFP*+SV40polyA sequence amplified from pEGFP-N1 vector, and P2A sequence resultant from primers overlap. (B) Agarose gels electrophoresis of PCR products correspondent to portions of the donor template: 5' and 3' Homology Arms amplified from genomic DNA of HEK293Tcells, with primers 5HA.G3 Fw and IL65HA.G3 Rev, and polyA3HA.G3 Fw and 3HA.G3 Rev, respectively; *IL6*-promoter amplified from genomic DNA of HEK293Tcells with primers 5HAIL6.G3 Fw and AzIL6 Rev; *hAzurin* and *eGFP*+SV40polyA amplified from pVAX-engineered Azurin and pEGFP-N1 plasmids DNA, with primers IL6Az Fw and P2AAz Rev, and P2AGFP Fw and 3HApolyA.G3 Rev, respectively. Mk – 1Kb Plus DNA Ladder (Invitrogen™).

3.2.2 PCR Ligations

Ligation I

The Ligation I is constituted by *IL6*-promoter and *hAzurin* fragments referred above. This Ligation was obtained by PCR using primers with complementary linkers to the sequences to be ligated, generating a 20-nt overlap between the two sequences. The resultant PCR product corresponding to the expected MW (1748bps) was purified with NZYGelpure kit.

Ligation II

The Ligation II is constituted by *hAzurin* and *eGFP*+SV40polyA fragments referred above, and P2A sequence was included in overlapping primers. This last sequence allows a bicistronic expression which means that both GFP and azurin will be co-expressed but translated as independent proteins rather than a fusion protein.

This Ligation was obtained by PCR as described in section 2.4.2. The resultant PCR product was purified with NZYGelpure kit. The purified PCR product was cloned and the insertion of the

fragment referred previously was verified by digestion of the vector. Two clones were verified by Sanger sequencing. The results of the sequencing confirmed the insertion of the correct Ligation II sequence in the two clones.

Ligation II was reamplified from one of these two clones and purified with NZYGelpure kit.

Ligation II + 3'HA

The Ligation II + 3'HA is constituted by Ligation II and 3'HA fragments referred before.

This ligation was obtained by PCR as described in section 2.4.2. The resultant PCR product was purified with NZYGelpure kit.

The amplification of the Ligations referred before is presented in Figure 3.

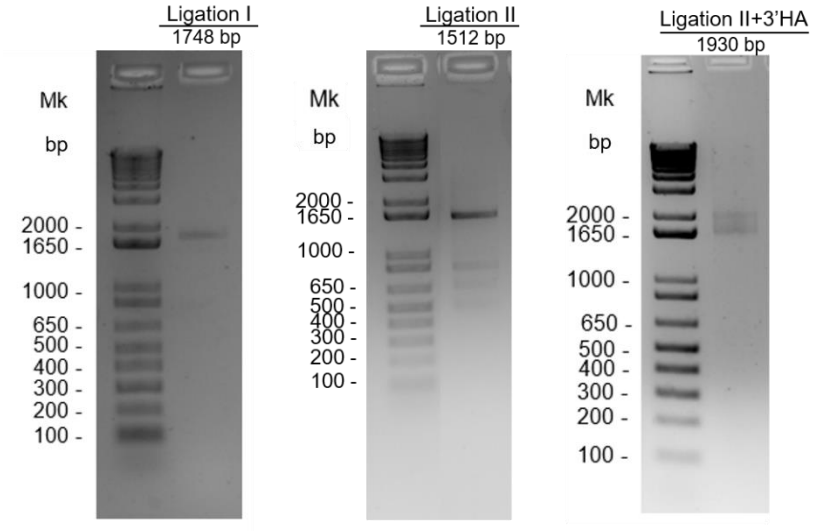

Figure 3 Agarose gels electrophoresis of PCR products correspondent to Ligations: Ligation I amplified from *IL6*-promoter and *hAzurin* DNA fragments with primers 5HAIL6.G3 Fw and P2AAz Rev; Ligation II amplified from *hAzurin* and *eGFP*+SV40polyA DNA fragments with primers IL6Az Fw and 3HApolyA.G3 Rev; Ligation II+3'HA amplified from Ligation II and 3'Homology Arm DNA fragments with primers IL6Az Fw and 3HA.G3 Rev. Mk – 1Kb Plus DNA Ladder (Invitrogen™).

Summary of results

For the construction of the final vector, it was necessary to amplify by PCR six DNA fragments, and ligate them by five PCR ligations. Of these, all individual fragments were obtained and sequence verified the *hAzurin*, P2A, and *eGFP*+SV40polyA DNA fragments.

Due to the lack of time following steps were not yet done:

- Ligation between Ligation I and 5'HA;
- Sequencing verification of Ligation I + 5'HA and Ligation II + 3'HA for posterior ligation between them.

4. Discussion

Taking into account the properties of the azurin as an anticancer protein and the tropism ability of the MSCs towards tumour sites described in section 1, the formulated strategy of this work was to test the feasibility of stably incorporating a gene coding for azurin within the genome of MSCs.

In this work, we chose the described genomic safe harbour AAVS1 locus in the intron 1 of *PPP1R12C* gene [13, 14], and the incorporation of the gene that encodes azurin into this locus via the CRISPR/Cas9 mechanism.

Thus, four guides correspondent to four different regions of the target genome location were firstly designed, using three bioinformatics tools including the Wellcome Trust Sanger Institute (WTSI) Genome Editing, CRISPOR, and CRISPR Design from Zhang Lab, MIT. Considering their score and the number and position of exonic off-targets of the four tested guide sequences, the best guide was the Guide 3 because of its good score and the zero exonic off-targets.

After the design of the guides, these were tested in HEK293T cells showing that the Guide 3 was also the best considering its cleavage efficiency observed in the agarose gel. For this reason only Guide 3 was tested in second cell line.

Regarding the cleavage efficiency test in the MSCs, two attempts were realized to test the Guide 3 in MSCs. The test with MSCs presented several troubles over time, starting with the puromycin selection of the cells since it originated a very slow recuperation.

In the first attempt, higher cell density and puromycin concentration were applied taking into account the prediction of a large cell death due to the stress originated by microporation and puromycin selection. In other words, this attempt was to test if the utilization of a higher cell density could improve the efficiency of the transfection. However, according to the results obtained, it was possible to verify that the cell density may probably

be an extra stress for cells, so the second attempt was realized in order to overcome this.

In this second attempt, the applied cell density was still quite high but more physiological, i.e., it was closer to what they are normally adapted and where they grow better [27]. In the second approach, the tested concentrations of puromycin were also lower than the applied in first attempt since the cells recuperation was very slow, which originated a very low level of cell confluence at the end of the test. This low confluence may difficult the recovery of the cells since these apparently need the cell-to-cell contact to proliferate. Thus, a lower puromycin concentration to select the cells originated a slower selection that, in turn, allowed the maintenance of the cell support during this process.

The passage of the cells to a 12-well plate, in order to put the cells closer, proved to be quite aggressive to a few cells since some cells did not even adhere, and did not recover.

The deficient growth of the cells may be originated by the transfection of a larger plasmid than that used in protocol on which this part of work was based, thus an optimization of microporation parameters may be necessary. As such, current efforts are being taken to optimize the parameters of transfection. A major difficulty in the system used in this thesis was due to the lack of a fluorescence marker like GFP that could be used to quantify the percentage of transfected cells. A new plasmid pCAG-SpCas9-GFP-U6-gRNA (Addgene #79144) will be used to obtain the parameters which may allow the higher transfection rates like the appropriate cell number, pulse voltage, pulse width and pulse number. Positively transfected cells may then be evaluated through flow cytometry. Afterwards, the plasmid already constructed in this thesis containing Guide 3 will be transfected using the optimized parameters to determine the cleavage efficiency of this guide in MSCs.

An alternative to overcome these difficulties may also be the utilization of RNP complex with purified protein and synthetic guides that is a more laborious system but is also a cleaner and more efficient, with a high activity shortly after protein transfection, followed by a rapid degradation and the avoidance of cellular toxicity related with DNA.

The different efficiencies commonly presented by these two cell types reflect the difference in biology and genetic background between established cell lines and human primary stem cells [28].

The next step was the construction of the donor template that will be incorporated into genome by HDR via CRISPR/Cas9 mechanism. This template is constituted by two homology arms designed around the cleavage site for the Guide 3, the promoter sequence of *IL6* gene, the *hAzurin* sequence (i.e. a codon optimized sequence of *azurin* gene from *Pseudomonas aeruginosa*, that also contains in its N-terminal a peptide sequence that confers it the ability to be secreted to the outside of the cells), and the *eGFP*+SV40polyA sequence, with a P2A sequence in between.

The two homology arms were designed near the cleavage site for the Guide 3 to allow the homology between the donor template and the region that was cleaved by Cas9 in order to incorporate this template in this locus. These homology arms were amplified from HEK293T cells and their length is about 440bp each one. Although some studies suggest that large insertions may require longer homology arms namely with >500bp [11], it becomes hard to clone such a large insert.

The promoter sequence from the *IL6 gene* [20] was amplified from HEK293T cells, being the choice between several promoters of the genes that are up-regulated by MSCs in presence of TGF-α [29]. In order to select this gene two main observations were taken into account: the higher fold induction (10.47 [29]) and the smaller region size (about 1200bp [30]). Therefore, considering the secretion of TGF-α by cancer cells, the utilization of this promoter to direct the expression of genes of interest like *hAzurin* in the tumour microenvironment is logical taking into account potential contribution for the field of anticancer therapies. However, a recent study from September 2017 employed just a part of the promoter sequence used in the present work applying a similar strategy based on the utilization of MSCs and the up-regulation of the promoter sequence of *IL6* gene in the cancer microenvironment [31]. This recent study may be considered in future steps of this work since it is easier to work with smaller DNA sequences.

The *hAzurin*, in turn, was amplified from pVAX-engineered Azurin plasmid DNA, which was obtained by our group in a previous work. The *eGFP*+SV40polyA sequence was amplified from pEGFP-N1 plasmid DNA, where *eGFP* sequence is optimized for use in mammalian cells and SV40polyA promotes transcript stability and export to the cytoplasm.

On the other hand, the P2A self-cleaving peptide allows the expression of two separate proteins from the same mRNA, in other words, a bicistronic expression, which means that when there is a GFP there is necessarily a hAzurin, as referred in section 3.2.2, allowing a better control in expression of *hAzurin* gene.

5. Conclusion

To conclude, with this work the initial objectives were almost all achieved.

The Cas9 guides were tested in HEK293T cells and MSCs, allowing the choice of one guide to be

employed in CRISPR/Cas9 technology in order to cleave the selected safe harbour. However, the test in MSCs was not finished.

Despite the fact that the cloning of the full construct was not possible during the time frame of this thesis, it was possible to determine the PCR conditions for the amplification of all components. It should be noted that several attempts to optimize the mentioned protocols in section 3.2 were realized until the referred results were obtained.

Thus, the possible next steps to continue this work may start with the cloning of Ligation I and consequent ligation to 5'HA, and cloning of Ligation II+3'HA and selection of the clones with the correct insert, choosing the one with the band with correct size considering that this ligation after purification presented two bands (see Figure 3). After that, in order to ligate the two resultant fragments (5'HA+Ligation I and Ligation II+3'HA) a possible strategy is the hydrolysis of the both with *Afl*II enzyme that cuts in the end of *hAzurin* fragment and generates sticky ends that allow the ligation by complementarity.

6. Current Challenges and Future Perspectives

There are several challenges, some already mentioned previously, in the way to achieve the final objective of the strategy proposed in this work, being that some alternatives to overcome or at least attenuate these difficulties are referred below.

Concerning to the difficulties in selection of MSCs with puromycin during the test of guides after microporation, it is known that the presence of antibiotics like this can originate the donor integration into unspecific sites. Moreover, considering the proposed alternative in section 4 of the utilization of purified protein and synthetic guides, a way to select the cells after this type of transfection may be through single cell sorting. For the delivery of this RNP complex and the donor template a recent study from October 2017 proposed the usage of gold nanoparticles conjugated to DNA and complexed with cationic endosomal disruptive polymers as vehicle [32].

Another difficulty of this work was the PCR amplification of the homology arms from the selected locus, what means that the choice of other locus or other genomic safe harbour could be employed to overcome this barrier.

The challenge concerning to big size of SpCas9 that sometimes interferes with its performance throughout the process that leads to cleavage of the DNA, namely its delivery, may be overcome with the utilization of an alternative protein as the Cas9 from *Staphylococcus aureus* (SaCas9) that is ~1kbp shorter and can edit mammalian genome with efficiency like that of SpCas9. However, more studies are needed to understand the long-term impact of this Cas9 expression [33].

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