Expression and purification of the *Streptococcus pyogenes* Cas9 protein for ribonucleoprotein transfection strategy and CRISPR/Cas9-based in AAVS1 safe harbor locus.

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Abstract

Mesenchymal Stem Cells (MSCs) present low immunogenicity and tumor tropism, properties essential to turn them anti-cancer drug delivery vehicles by gene editing. Azurin is a bacterial protein capable of reducing tumor proliferation and inducing apoptosis without known effects in normal cells. If engineered to produce azurin, engineered MSCs could became a tool against tumorigenesis with potential for clinical use. The most efficient and specific genetic engineering tool is CRISPR-Cas9. However, when cells' genome is exposed to this tool for long periods, the chance of off-targeting increases, therefore, an approach to produce *in vitro* a CRISPR-Cas9 complex was developed and optimized for efficient gene edition in AAVS1 safe-harbor, with low lifetime inside cells. Cas9 production and purification optimized, resulted in higher and purer Cas9, using *E. coli* culture at 25ºC with 0.2mM IPTG for overexpression induction. Cleavage efficiency of RNP complex formed with the purified protein was tested *in vitro* with DNA from MSC, HEK and HeLa, and in lipo-transfected HEK cells with successful cleavage detected using GeneArtTM Genomic Cleavage Detection Kit. This strategy may represent a more suitable approach for MSCs since all-in-one plasmid approaches present very low levels of success, particularly due to high sensitivity of these cells to the selection with puromycin. Despite optimization of microporation of MSCs with an all-in-one plasmid (optimal conditions: one pulse, 1400V and 1300V, 30ms), the cells died after puromycin selection, reinforcing the need of higher Cas9 protein levels for future studies to access the donor-to-donor variability and tissue source' influence of MSC.

Keywords: CRISPR-Cas9, RNP, Azurin, MSCs, Microporation, Lipofection

1. Introduction

Recent advances in genetics had led the medical field to enroll in the development of gene and cell therapies that show great potential in treatment of genetic, viral, degenerative and oncogenic diseases, comparing with the present standard treatments available to public. The highest enhancer of this field was the discovery of CRISPR-Cas9 system, a ribonucleoprotein complex founded in bacteria and archaeal with the ability to cleave DNA in pre-determined sites of DNA, used in this basis as a unicellular immune system against virus [1]. Using this cleavage capability and high specificity, editing genomes is a reliable reality with the potential to lead to the cure of diseases using genetic material, or engineered cells with drug delivery properties. With a higher prospect for cancer treatment, engineered Mesenchymal Stem/Stromal Cells (MSCs) are a great candidate. MSCs are multipotent stem cells with the capability of self-renewal, without significant changes, and differentiation into all mesoderm lineages and some ectoderm and endoderm cells [2]. Compared to embryonic stem cells (ESCs), MSCs have a minor potential for replication and differentiation, but using them do not exhibit major ethical concerns since MSCs can be isolated from adult bone marrow and adipose tissue or umbilical cord [3]. Induced pluripotent stem cells (iPSCs) would not present ethical issues towards embryos and differentiation potential would be maintained. However, just like ESCs, iPSCs can form teratomas unlike the MSCs [4,5,6]. MSCs have many features with diverse applications, one in concrete is their unique tropism. Signals of the microenvironment of inflammatory nature, chemoattract MSCs for damaged sites in order for them to act as a reparation system. Such signals can be released due to hypoxia, ischemia, radiation, cutaneous cuts and tumors. This tropism for tumor tissue and injurie sites together with gene editing, can lead to cell therapies for drug delivery at tumors and tissue repair [7]. Another promising characteristic of these cells is their immunomodulatory and anti-inflammatory effects that turns it possible to transplant these cells in humans without the need for MSCs to be from the individual or from an immunocompatible patient thus making the attainment of working cells easier. This hypoimmunogenicity is due to the inhibition of proliferation and maturation of immune cells and their reactions, low expression of HLA class I, no expression of HLA class Ⅱ and costimulatory molecules like CD40, CD80, and CD86 [8]. Even though MSCs show great potential in medical treatment of diverse diseases including cancer, negative aspects have also been observed. The community has been divided about how MSCs affect tumor progression, since there is evidence of MSCs inhibiting tumor development, but also evidences of stimulatory effects on tumor pathogenesis [9]. Engineered MSCs could overcome most of the issues of the negative inherent properties of these cells and effectively make them drug delivery tools to treat for example cancer or to be used in tissue engineering and regenerative medicine. Cancer is a disease caused by a series of alterations in genome and epigenome that lead to activation of oncogenes or inactivation of cancer suppressor genes, thus leading to abnormal proliferation of cells [10]. In this context, gene and cell-based therapies are emerging as a promising strategy to tackle cancer more effectively, while minimizing the need of surgery and radiotherapy. Since CRISPR-Cas9 seems to be the genome editing tool with higher

potential, several attempts are being made in using CRISPR to correct mutated genes or edit cells to use in cell-based therapies [11]. In the last decade, CRISPR-Cas9 system (Clustered Regularly Interspaced Short Palindromic Repeats) has emerged as a programmable tool for site-specific gene editing for prokaryotes and eukaryotes that due to lower price, easier application and higher efficiency [12], has become the favorite nuclease dependent system for gene editing, when compared with TALEN (Transcription activator-like effector nuclease) and ZFN (Zinc finger nuclease) [13]. Alteration of gene expression by RNAi also has disadvantages [14]. CRISPR-Cas9 is easily engineered, reproducible and affordable. Unlike the other two mentioned editing systems that need a new engineering protein for every single target, CRISPR-Cas9 only needs a sgRNA (single guide ribonucleic acid) sequence of 20 nucleotides. The DNA–RNA interactions are highly predictable, and construction is highly feasible and affordable [15]. CRISPR-Cas9 system consists in a complex composed of a ribonucleoprotein nuclease Cas9 (the most used is originally from type II CRISPR system of *Streptococcus pyogenes* - SpCas9), which is the element that enhances the cut of the DNA, and a synthetic single guide RNA sequence, containing both CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA) components , that is bonded to Cas9 and connects to its complementary DNA target sequence in order for the Cas9 perform the cut in the specific site. The sgRNA of 20 nucleotides binds in its 5′-end to the target DNA sequence site while its conserved 3′-end scaffold binds to Cas9 due to the presence of a stem-loop structure in this end, thus making possible the targeting. The Cas9 itself also needs to bind to the DNA, and for that to happen, a PAM (protospacer-adjacent motif) sequence has to be present downstream of the target site. PAM sequence (5′-NGG-3′ in case of SpyCas9), highly occurs in genomes, thus, CRISPR-Cas9 can targeted virtually any gene. Firstly, Cas9 binds to the sgRNA. Secondly, it searches for a functional PAM sequence, dissociating rapidly from mutated PAMs. Once bound to an effective PAM, Cas9 induces the melting of the PAM-adjacent nucleation site by RNA strand invasion to form an RNA–DNA hybrid and a displaced DNA strand (R-loop) from PAM-proximal to PAM-distal ends. If the complementarity is 100%, between sgRNA and target DNA, Cas9 will cut the targeted DNA. The same can happen with high complementary, lower than 100%, thus the possibility of mistargets. To recognize and cut the two strands of DNA substrates, the nuclease domains, HNH and RuvC are needed [16]. The cleavage of the DNA creates a double strand break (DSB) that is naturally repaired by the cell in two possible ways. Usually it is repaired by error-prone non-homologous end joining (NHEJ) pathway, which introduces a small insertion or deletion (InDel) at the site, thus knocking out the gene. The other pathway of repair, called homology-directed repair (HDR), uses, if existent or added, a donor DNA fragment with homology to the flanking sequence that is integrated into the genome at the DSB site, thus repairing the broken DNA [17]. One of the limitations of CRISPR-Cas9 system is the efficiency of integration in the living cells and possible cell viability loss. In order to realize permanent Cas9-mediated modifications in cells, introduction of the complex (Cas9 + sgRNA) inside the cell is needed. This can be achieved by introducing plasmids capable of expressing Cas9 and sgRNA genes in the transformed/transfected cells or by transfecting directly transcripts or the ribonucleoprotein (RNPs) complex previously build *in vitro*. In the case of plasmids, two can be used, one expressing the nuclease and the other sgRNA, or an all-in-one plasmid, capable of expressing both. The transformation can be achieved with electroporation, lipofection, polymeric nanoparticles, cell-penetrating peptides or virus like retroviruses and lentiviruses, but disruption of the cell membrane is an aggressive procedure that results in many cell´s casualties, liposomes and virus are limited by efficiency and size and *in vitro* complexes can be toxic to the cells duo to the exogenous nature [18]. Depending on the model and objective, the approach to edit the genome can shift, but the highest nuclease activity is achieved with RNPs and stable expression in cultured cells. However, RNP approach is reliable not only in cells, but also in embryos and *in vitro* assays, being most convenient and functional since is synthesis is not dependent of expression by the cells to be edited. Also, it is the best method to avoid off-targeting due to the shorter exposure to the genome [19]. Genomic safe harbors (GSHs) are genomic sites in which integration of new genetic material doesn't affect function predictably and doesn't cause alterations in the host that pose any risk in their survival. The AAVS1 site on chromosome 19 previously mentioned, has gained popularity since it is easier to target with the current editing tools and because it can support transgene expression in multiple cell types. Unfortunately, it fails in some cell types and insertions can be silenced by methylation. Also, edition in this site disrupts the PPP1R12C gene (phosphatase 1 regulatory subunit 12C) [20]. Unfortunately, the effective edition of cells' genome using Cas9 RNP, even though, higher than using plasmids, is very low, as it has been showed in the work of Brock Roberts and Amanda Haupt, in which the repaired human induced pluripotent stem cells (hiPSC) with HDR were <0.5-4%. However, most of the resultant cells developed good morphology, stable genome and gene expression and right location of the edition product [21]. In order to produce a CRISPR-Cas9 RNP complex *in vitro*, *E. coli* can be used to produce Cas9 protein with the right expression plasmid, and gRNAs can be construct and amplified. C. Anders and M. Jinek were able to produce and purify Cas9 protein, using *E. coli* strain Rosetta 2 DE3. Cell are lysed in cell homogenizer and Cas9 is purified with consecutive chromatography and concentrations, more precisely IMAC, Dialysis and 6His-MBP cleavage, IEX and SEC [22]. Since the CRISPR-Cas9 is capable of targeting and cutting pre-determined genetic sequences, it is expected that it would be used

in gene therapy for example, for disruption of proliferative genes in cancer cells, genes for viral proteins, mutated genes that provoke genetic diseases or even being used with donor vectors for effective repair [23]. Azurin, a blue copper protein of redox nature of *Pseudomonas aeruginosa* [24], can interact with molecular pathways of tumor cells, leading to apoptosis of the targeted cancer cells. Capable of entering in the cell´s nucleus, azurin stabilizes p53, increasing is activity and release of cytochrome c into the cytosol, activating caspase cascade that leads to cellular apoptosis. [25] Not only has natural cytotoxicity to cancer, it also shows preferential entry into cancer cells, thus showing potential as tool against cancer [26]. For all previous mentioned reasons, azurin would make a great anti-cancer drug and if expressed by cells with tropism for cancer, could became an efficient treatment against cancer.

In this study, we aim to produce and purify Cas9 protein to edit MSCs genome efficiently with an RNP transfection approach and test efficiency of cleavage in HEK, HeLa and MSC cells *in vitro* and in HEK cultured cells for future integration of an azurin template in order for these engineered cells to become an anti-cancer cell therapy due to tumor cells tropism of the MSCs.

2. Materials and Methods

2.1. Transformation of *E. coli BL21(DE3)*

E. coli DH5α cells containing the plasmid pMJ923 (Addgene plasmid # 78313, a kind gift from Martin Jinek) were grown overnight in LB medium containing ampicillin (150 µg/mL), at 37 ºC and constant agitation of 250 rpm. For plasmid extraction, NZYMiniprep kit (NZYTech) was used, following the manufacturer's instructions. After extraction, purified plasmid DNA concentration was determined with Nanodrop™ (ND-1000 Spectrophotometer). 10 ng of plasmid were used to transform competent *E. coli* BL21 (DE3) by electroporation (25 µFD capacitance, 400 Ω resistance, 2.5 kV; Gene Pulser™, Biorad). After the shock, cells recovered in LB medium for 1h at 37 ºC with constant agitation of 250 rpm to recover, after which they were plated in LB agar plates containing ampicillin (150 µg/mL).

2.2. Cell Culture and Overexpression of Cas9

Transformed *E. coli BL21(DE3)* was cultured in SB medium with ampicillin (150 µg/mL) at 37 ºC with constant agitation of 250 rpm until it reaches exponential phase (OD between 0.6-0.8). Samples were taken at this point for SDS-PAGE and Western blot analysis (Time zero samples). IPTG (Isopropyl-β-D-thiogalactopyranoside) was added at a final concentration of 0.2 mM for the induction of Cas9 expression and incubated at 25 ºC with constant agitation of 250 rpm, for 5-6h. Samples were taken at the end of this time for SDS-PAGE analysis and Western blot. Cells were centrifuged at 4º C, 8000 rpm for 10 min (Beckman J2-MC Centrifuge) and the pellet was resuspended in Binding/Start buffer (20 mM Tris-Cl, pH 8.0, 250 mM NaCl, 10 mM imidazole, pH 8.0) and conserved at -80 ºC. 8% SDS gels and Western blots were made to visualize Cas9 expression. The same was applied for each step of purification.

2.3. SDS-PAGE and Western bolt

The polyacrylamide gels were made with a resolving layer of 8% or 12% and a stacking layer of 5%. Samples of cell and protein suspensions were lysed or denatured, respectively, with loading buffer (Tris 1M pH 6.8, SDS 2.0%, glycerol 10%, bromophenol blue 0,0006%, DTT 0.1M) in a dry bath at 95-100 ºC for 5 min in order to run in electrophoresis. In bacterial cell lysates, a centrifugation at 9000 rpm for 1 mi. was also made to remove debris. SDS-PAGE was run with Electrophoresis Buffer 1X for 10 min at 75 V and 120 V until the end of migration. BlueSafe or Comassie was used to dye the gel in order to observe the protein´s migration. To continue Western blot procedure, transfer of the proteins of the gel to a membrane of nitrocellulose was done using TransBlot Turbo and Transfer Buffer 1X at 15 V for 15 min. Ponceau 4R was used to confirm correct transfer. Blockage of the membrane was made with non-fat dry milk 5% (w/v) in PBS Tween for 1h with smooth agitation, followed by incubation overnight at 4º C with smooth agitation with anti-His anti-bodies (His-probe (H-3) sc-8036 mouse monoclonal IgG_1 from Santa Cruz Biotechnology) diluted 1:1000 (v/v) in the milk solution. Membrane was washed with PBS Tween for posterior incubation with secondary antibodies coupled with HRP (goat anti-mouse IgG-HRP sc-2005 from Santa Cruz Biotechnology), diluted 1:2000 (v/v) in PBS Tween for 1h. Membrane was washed for posterior addition of ECL reagent and consequent protein detection in Fusion Solo VILBER LOURMAT with exposure between 3 and 10 min.

2.4. Cas9 purification and MBP-6His cleavage

Per purification, 6-8L of cell suspension were resuspended in Binding Buffer for sonication. The parameters used in BRANSON SONIFIER 250 were the following: Duty cycle of 50 % and Output of 10, 9 cycles, 15 pulses each cycle, with intervals of 5-10 min. After the sonication, the lysate was centrifuged at 4 ºC, 17,600xg for 5 min., and the resulting supernatant was centrifuged again in the same conditions for 1h. The supernatant was applied in IMAC column (Immobilized metal affinity chromatography) in which the His tag will bind to the nickel from the column HisTrap™ FF 5 mL (GE Healthcare). IMAC was performed in AKTA start (GE Healthcare) after washing the system with dH_2O and equilibrated with Binding Buffer A (20 mM Tris-Cl, pH 8.0, 250 mM NaCl, 10 mM imidazole, pH 8.0). After multiple sample applications and washouts to avoid column clogging, the protein was eluted with Elution Buffer B (20 mM Tris-Cl, pH 8.0, 250 mM NaCl, 250 mM imidazole) in a linear gradient from 0-100% of Elution Buffer.

Table 1: Parameters of IMAC program for Cas9 purification.

Column volume	5.027 mL
Pressure limit	0.30 MPa
Flow rate	5 mL/min.
Equilibrium with	1 CV
Sample volume	~20 mL
Wash column with	6 CV
Elution type	Gradient
Start %B	0.0 %
Set target concentration	100% B
Gradient volume	20 mL
Equilibrate with	5 CV

Protein purity along the purification was analyzed with 8% SDS-PAGE gels. The fractions containing higher levels of the target protein were concentrated with Amicon Ultra Centrifugal (Milipore), with a molecular weight cut-off of 10,000 Da, at 4000 rpm, 4 ºC (Epp. Centrifuge) with a final

volume of 3 mL. Imidazole was removed by dialysis with Dialysis Buffer (20 mM HEPES-KOH, pH 7.5, 150 mM KCl, 10 % (v/v) glycerol, 1 mM dithiothreitol (DTT), 1 mM EDTA). The Cas9 fusion protein produced has a TEV sequence for MBP-6His cleavage and in order to do both steps together, the purified Cas9 is inserted in a Slide-A-Lyzer™ G2 Dialysis Cassette (ThermoFisher Scientific), 10K MWCO, 3 mL with 1.5 µM of TEV and left with smooth agitation at 4 ºC. Dialysis was extended for 2-3 days for higher cleavage efficiency. After dialysis, Cas9 was further purified by IEX using HiTrap™ 5 mL SF HP (GE Healthcare) that was first washed with dH_2O and IEX Buffer A (20 mM HEPES-KOH, pH 7.5, 100 mM KCl), before protein application. Elution is performed with a linear gradient of IEX Buffer B (20 mM HEPES-KOH, pH 7.5, 1 M KCl) form 0-50%. Fractions containing Cas9 were pooled and concentrated with Amicon Ultra Centrifugal (Milipore), with a molecular weight cut-off of 50,000 Da, at 4000 rpm and 4 ºC until a final volume of 500 µL.

Table 2: Parameters of IEX program for Cas9 purification.

Column volume	5.027 mL
Pressure limit	0.30 MPa
Flow rate	5 mL/min.
Equilibrium with	2 CV
Sample volume	2 mL
Wash column with	5 CV
Elution type	Gradient
Target %B concentration (0-100%)	50%
Volume 0-100 (CV)	12
Start %B concentration	0%
Equilibrate with	5 CV

The last step for Cas9 purification was SEC (Size Exclusion Chromatography). The column Superdex 200™ 10/300 was previously washed with dH_2O and SEC Buffer (20 mM HEPES-KOH, pH 7.5, 500 mM KCl, 1 mM DTT) and the parameters used in the program are represented in Table 3.

Table 3: Parameters of SEC program for Cas9 purification.

Column volume	24 mL (30 cm x 100 mm)
Pressure limit	1.5 MPa
Flow rate	0.5 mL/min.
Equilibrium with	1 CV
Sample volume	500 µL
Wash column with	5 CV

After SEC, concentration of the enriched fractions was made with Milipore 50,000 MWCO Amicon and Cas9 quantification was made with spectrophotometer at 280 nm and an extinction coefficient of 120,450 M^{-1} cm^{-1}, according to C. Anders and M. Jinek [42]. The resultant Purified Cas9-mCherry protein was divided into 300 µL aliquots for further flash freeze with liquid nitrogen and storage at -80 ºC, until is use.

2.5. Cell Culture and Overexpression of TEV

E. coli BL21(DE3) cells were transformed with pRK793 plasmid (Addgene plasmid # 8827) and cultured in SB medium with ampicillin (150 µg/mL) at 37 ºC, 250 rpm until it reaches exponential phase (OD between 0.6-0.8). Samples were taken at this point for SDS-PAGE and Western blot analysis (Time zero samples). IPTG (Isopropyl-β-D-thiogalactopyranoside) was added at a final concentration of 1 mM for TEV expression induction and incubation was made at 30 ºC with constant agitation of 250 rpm, for 4-6h. Samples were taken at the end of incubation for SDS-PAGE analysis and Western blot. Cells were centrifuged at 4º C, 8000 rpm and for 10 min (Beckman J2-MC Centrifuge) and the pellet was re-suspended in Binding/Start buffer (20 mM Tris-Cl, pH 8.0, 250 mM NaCl, 10 mM imidazole, pH 8.0) and conserved at -80 ºC. 12% SDS gels and Western blots were made to visualize TEV expression. The same was applied for each step of purification.

2.6. TEV purification

2L of cell suspension were re-suspended in Binding Buffer A (50 mM sodium phosphate, pH 8.0, 200 mM NaCl, 10% glycerol, 25 mM imidazole). Sonication was used to lyse the cells, in BRANSON SONIFIER 250 equipment with the following conditions: Duty cycle of 50 % and Output of 10, 9 cycles, 15 pulses each cycle, with intervals of 5-10 min. After sonication, cell lysate was centrifuged at 4 ºC, 17,600xg for 5 min., and the resulting supernatant was once again centrifuged with the same conditions for 1h. The supernatant was applied to an IMAC (Immobilized metal affinity chromatography; column HisTrap™ FF 5 mL, GE Healthcare). IMAC was performed in AKTA start (GE Healthcare) after washing the system with dH_2O and equilibrated with Binding Buffer A. The protein was eluted with Elution Buffer B (50 mM sodium phosphate, pH 8.0, 200 mM NaCl, 10% glycerol, 250 mM imidazole) in a linear gradient from 0-100% of Elution Buffer.

Table 4: Parameters of IMAC program for TEV purification.

Column volume	5.027 mL
Pressure limit	0.30 MPa
Flow rate	5 mL/min.
Equilibrium with	7 CV
Sample volume	~20 mL
Wash column with	15 CV
Elution type	Gradient
Start %B	0.0 %
Set target concentration	100% B
Gradient volume	20 mL
Equilibrate with	10 CV

Protein purity along the purification was analyzed with 12% SDS-PAGE gels. The fractions containing higher levels of the target protein were concentrated with Amicon Ultra Centrifugal (Milipore), with a molecular weight cut-off of 10,000 Da, at 4000 rpm, 4 ºC (Eppendorf S804R Centrifuge) with a final volume of 2 mL. In the pool, EDTA was added to a final concentration of 2 mM and DTT to a final concentration of 5 mM. Gel filtration was performed in Amersham Biosciences XK16 column of 120 mL. The Gel filtration Buffer used was: 25 mM sodium phosphate (pH 7.5), 100 mM NaCl, 10% glycerol, using the conditions in Table 5.

Table 5: Parameters of Gel filtration program for TEV purification.

Column volume	120.637 mL
Pressure limit	0.15 MPa
Flow rate	0.5 mL/min.
Equilibrium with	0 CV
Sample volume	2 mL
Wash column with	1.5 CV

Enriched fractions of TEV were concentrated with an Amicon Ultra Centrifugal (Milipore), with a molecular weight cut-off of 10,000 Da, at 4000 rpm, 4 ºC. Quantification was made with spectrophotometry at 280 nm and a molar extinction coefficient of 32,290 M^{-1} cm^{-1} according to Joseph E. Tropea and colleagues [27]. The resultant Purified TEV protein was divided into 300 µL aliquots for further flash frozen with liquid nitrogen and stored at -80 ºC until use.

2.7. Microporation of MSCs with All-in-one plasmid

Transfection of MSCs with all-in-one plasmid with Cas9 from *S. pyogenes* with 2A-EGFP (pX458 plasmid Addgene at # 48138) was performed using Neon Transfection System (Thermo Fisher Scientific), according to Madeira *et al*, 2011 [28]. Cells were washed with PBS 1X and detached from plastic surface using TriplE Select. 100 000 cells were resuspended in R Buffer (provided by the manufacturer) and microporated with 0.5 µg of plasmid DNA in a final volume of 10 µL. In this experimented, 24 microporation conditions, varying pulse voltage (mV), pulse width (ms) and pulse number, were tested. After such procedure, cells were immediately transferred to Opti-MEM™ medium (GIBCO™) to increase cell viability after transfection and plated in 24-well-plates previously coated with CELLstartTM and StemPro® MSC SFM XF medium (GIBCO™). 24 hours post-transfection, culture media was replaced, and cells were incubated at 37 ºC with 5% CO_2 for 48 hours.

2.8. Flow cytometry of MSCs

After microporation, cell viability and GFP expression was analyzed with flow cytometry. (FACSCalibur equipment, Becton Dickinson; FL1 filter), where GFP fluorescence intensity was measured after 48h of cell incubation. Cells were washed with PBS 1x, detached using TriplE Select 1x and counted using the trypan blue dye exclusion test. Afterwards, cells were centrifuged in FACS tubes at 1000 rpm for 5 min., fixed in 300 µl of 2% Paraformaldehyde (PFA) in PBS 1x and cells were analyzed within the following hour.

2.9. *In vitro* CRISPR-Cas9 cleavage assay

HEK, HeLa and AT-MSCs (Adipose Tissue-MSCs) were cultured in 6 well-plates for posterior DNA extraction. Medium was removed, and cells were washed with PBS 1X two times. 60 µL of ATL Buffer were added and cell scrapers were used to free lysate. It was added to the lysate 5 µL of RNaseA 100 mg/mL and 20 µL of Qiagen Proteinase K solution. It was incubated for 2-3h in dry bath at 57 ºC. Next it was vortexed for 15 sec. It was added 200 µL of absolute ethanol and 200 µL of AL Buffer. Vortex was repeated, and solution was transferred to DNeasy Mini Spin with a collection tube of 2 mL. It was centrifugated at 8000 rpm for 1 min., discarding the collection tube and switching for a new one, adding posteriorly 500 µL of AW1 Buffer. It was centrifuged at 8000 rpm for 1 min., discarding the collection tube and switching for a new one for posteriorly adding 500 µL of AW2 Buffer. It was centrifuged at 14 000 rpm for 3 min., discarding the collection tube and switching for a 2 mL Eppendorf and 200 µL of AE Buffer were added. The solution was Incubated at room temperature for 1 min. and centrifuged for 1 min. at 8000 rpm. Quantification was made in Nanodrop for DNA concentration and purity. The DNA resultant from DNA extraction was mixed with Na acetate 3 M for a final concentration of 0.1X and with absolute ethanol for a final concentration of 2.5X. The solution was incubated at -80 ºC for 1h. It was centrifuged for 30 min. at 4 ºC at 15 300 rpm and supernatant was removed. It was added 500 µL of ethanol 70% and resuspension was performed. It was centrifuged for 10 min. at 4 ºC, at 15 300 rpm and supernatant was removed. Speed vacuum for 15 min. at 60 ºC was made and for further resuspension in 20 µL of dH_2O. DNA quantification was once again made using Nanodrop. Firstly, the double-stranded DNA template as cleavage substrate was generated through PCR amplification of the target region. DNA samples were diluted to 3 ng/µL in RNA-free water. 2 µL of the resultant DNA solution were mixed with 1:1 (Fw/Rev) mix of primers, 25 µL of AmpliTaq Gold ® 360 Master mix to a final volume of 50 µL. The following PCR program was used:

Table 6: PCR program for DNA substrate amplification.

Enzyme activation	15 ºC	10 min.	1X
Denaturation	95 ºC	30 sec.	40X
Anneal	60 ºC	30 sec.	
Extend	72 ºC	30 sec.	
Final Extend	72 ºC	7 min.	1X
Hold	4 ºC	Hold	1X

PCR products were run in a 1.3% Agarose gel at 90 V to assure correct amplification. The Alt-R CRISPR-Cas9 System (Integrated DNA Technologies) was used to generate synthetic guide RNAs (crRNA and trancrRNA) to test the in *vitro* cleavage efficiency of the *in house* obtained Cas9 protein. Lyophilized RNAs were re-suspended with IDTE Buffer (Tris 10 µM pH 7.5, EDTA 1 mM, not provided) to a final concentration of 100 µM. To generate the RNA duplex (crRNA:trancrRNA; gRNA), both oligos were mixed in equimolar concentrations to a final duplex concentration of 10 µM, and incubated in dry bath at 95 ºC for 5 min. The solution was cooled to room temperature before preparing the RNP complex. Cas9 was added to the gRNA duplex prepared, to a molar ratio of 1:1 inCas9 dilution buffer (30 mM HEPES, 150 mM KCl, pH 7.5). It was further incubated for 10 min at room temperature for RNP formation. Finally, the *in vitro* digestion reaction was performed by mixing 1µM of RNP, with 100 nM DNA substrate in Cas9 Nuclease Reaction Buffer (200 mM HEPES, 1 M NaCl, 50 mM $MgCl_2$, 1 mM EDTA, pH 6.5 at 25°C), at room temperature followed by 1h incubation at 37 ºC. To release the DNA substrate from the Cas9 protein, 20mg/mL of Proteinase K was added and incubated at 56 ºC, for 15 min. Cleaved DNA substrate was verified by agarose gel electrophoresis in a 1.3% agarose gel at 90 V. The primers were purchased from Stab Vida, with the following sequences:

Forward primer: 5' CAGGTTCCGTCTTCCTCCAC 3'

Reverse primer: 5' AAGAGGATGGAGAGGTGGCT 3'

2.10. CRISPR-Cas9 cleavage assay in HEK cells

HEK cells, were cultured in 24 well-plates for posterior transfection of the CRIPSR-Cas9 complex, using Lipofectamine 2000 (Invitrogene). The 100 µM solutions of crRNA and trancrRNA prepared in *In vitro* CRISPR-Cas9 cleavage assay, were used to form the gRNA duplex, by adding to a PCR tube, 8 µL of nuclease-free water, 1 µL of crRNA and 1µL of trancrRNA and consequently incubated for 5 min. in dry bath, at 95 ºC. The duplex was diluted with RNase-free water to a final concentration of 1 µM. The stock solution of Cas9 was also diluted to reach 1 µM. It was mixed 3 µL of gRNA, 3 µL of Cas9 and 44 µL of simple medium (DMEM). It was prepared CRISPR-Cas9 complex with our guide, a positive control and a negative control. In another Eppendorf, 2 µL of lipofectamine 2000 were diluted in 48 µL of simple medium (DMEM). Both these mixes were incubated for 5 min. in room temperature and afterwards mixed together and incubated at the same temperature, for 20 min. To finalize, it was added to the mix, 100 µL of simple medium (DMEM) and the solution was added to the cells and incubated at 37 ºC for 7h. After 7h the medium was replaced by complete medium (DMEM medium, 10% FBS, 1% PENSTREP (GIBCO™)) and 48h were needed for CRISPR-Cas9 complex to act. Cells were washed with PBS 1X, detached with 0.05% trypsin, and incubated at 37 ºC for 3 min., cells before being released from the surface. 200 µL of

medium were used to resuspend the cells before centrifugation at 1200 rpm for 5 min. Supernatant was removed and pellet of cells was conserved at -80 ºC for future use with GeneArt kit. Following GeneArt step, it was mixed 50 µL of cell lysis buffer and 2 µL of protein degrader and 50 µL of such mix was used to resuspend cell's pellet for further transfer to PCR tubes. In the thermocycler, the following program was used:

Table 7: Cell lysis and protein denaturation program.

68ºC	15 min.
95ºC	10 min.
4ºC	Hold

The lysate was vortexed for further mix in a PCR tube, 2 µL of cell lysis, 1 µL of each primer (10 µM), 21 µL of Nuclease-free water and in the end, 25 µL of AmpliTaq Gold ® 360 MasterMix. The following amplification program was used:

Table 8: PCR program for DNA substrate amplification.

Enzyme activation	15 ºC	10 min.	1X
Denaturation	95 ºC	30 sec.	40X
Anneal	68 ºC	30 sec.	
Extend	72 ºC	30 sec.	
Final Extend	72 ºC	7 min.	1X
Hold	4 ºC	Hold	1X

An agarose gel of 1.2 % was made to verify the correct amplification. 2µL of PCR product was mixed with 1 µL of 10X Detection Reaction Buffer and Nuclease-free water was added to reach 9 µL. The following re-annealing program was used:

Table 10: Re-annealing program.

95 ºC	5 min.
95-85 ºC	-2 ºC/sec.
85-25 ºC	-0.1 ºC/sec.
4 ºC	Hold

1 µL of Detection Enzyme was added and the solution was incubated at 37 ºC for 1h. An agarose gel of 1.2 % was made to verify the correct if there was cleavage in the appropriated locations, by the band's sizes.

3. Results

3.1. Optimization of the microporation conditions in all-in-one Cas9 plasmid

Previous results from our group had demonstrated that MSC cells transfected with pX459 (pSpCas9(BB)-2A-Puro, Addgene plasmid #62988) where a custom-designed guide RNA sequence targeting the AAVS1 locus has been cloned, were highly susceptible to selection to puromycin. Therefore, an attempt to optimize the parameters for microporation was performed with pX458 (Addgene at # 48138), using the EGFP as a reporter for the success of the transfection. For that, 100 000 cells were used for each condition. Different voltages were used ranging from 850 to 1600 V, with pulse widths ranging from 10 to 30 ms in 1 to 3 pulses. After microporation, cells were left to recover for 48h. The condition with highest number of fluorescent cells was that of cells transfected with a voltage of 1400 V, a pulse width of 30 ms in 1 pulse. In a similar condition with 1300 V, fluorescence was the second highest (Figure 1). With the resultant cells, flow cytometry was performed for relative quantification of fluorescent and cell viability. The previously top condition was once again the one with highest fluorescence (31%), but the cell viability was really reduced, surviving just 5000 viable cells. Condition 1300 V, 30 ms, 1 pulse showed second best fluorescence levels and greater number of cells viability (16,6 % and 20 800 viable cells). Unfortunately, these statistics aren't trustworthy since flow cytometry was run with a number of events inferior to the minimum advised (100 000 cells).

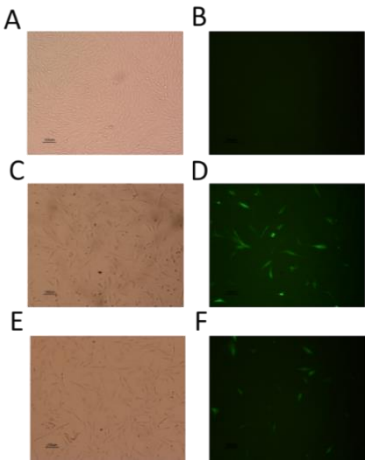

Figure 1: Fluorescence microscopy of MSCs microporated with all-in-one plasmid. **A:** Control (not microporated) in white light. **B:** Control in fluorescence microscopy **C:** Conditions 1400 V for 30 ms in 1 pulse in white light. **D:** Conditions 1400 V for 30 ms in 1 pulse in fluorescence microscopy. **E:** Conditions 1300 V for 30 ms in 1 pulse in white light. **F:** Conditions 1300 V for 30 ms in 1 pulse in fluorescence microscopy.

After establishing these conditions, MSC cells were microporated with pX459 where the custom-designed guide RNA had been cloned, using the conditions 1400 V for 30 ms in 1 pulse and 1300 V for 30 ms in 1 pulse. Several attempts were made to improve cell survival upon selection with puromycin, however, all failed. Both microporation conditions were tested and cells were cultured with the following puromycin concentrations: 0.1, 0.25 and 0.5 µg/mL. Unfortunately, cells died or the time of culture needed would lead to a not observable cut, having the different conditions, identical results.

3.2. Optimization of 6-His-MBP-Cas9-mCherry production in *E.coli* BL21(DE3)

Firstly, to edit the genome of the MSCs, the CRISPR-Cas9 tool must be produced in quantity, in order to later transformation of the complex in the cells. *E.coli* BL21(DE3) was used for over-expression of

Cas9 protein, by transformation of this strain through electroporation with pMJ923 plasmid (Addgene plasmid # 78313, a kind gift from Martin Jinek, Annex 1), a vector that encodes for Cas9 protein in which overexpression is induced by with IPTG. In this plasmid, Cas9 is fused with mCherry, MBP (for higher solubility) and 6 His (tag for purification). This plasmid was extracted from *E.coli* DH5α, quantified with Nanodrop and transformed into competent *E.coli* BL21(DE3) by electroporation. Cultures in selective medium (with ampicillin) were made with the resultant transformed bacteria, and one colony was further cultured in petri diches for cell stock. To test the expression of Cas9 at different temperatures, cell cultures were induced by IPTG and incubated at different temperatures: 37, 30, 25, and 20 º C. Samples were acquired at 0, 2 and 4 hours after induction, depending on the tested temperatures. The protein samples were separated in SDS gels and visualized by Western blots (Figure 2), to see the over-expression of Cas9.

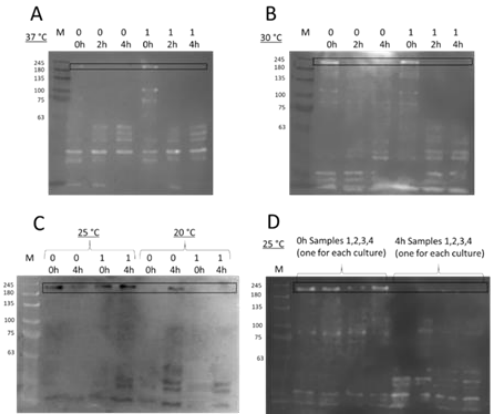

Figure 2: Western blots made with antibodies for 6-His of Cas9 produced. 0 represents no IPTG and 1 represents addition of IPTG (1mM). The molecular weight of mCherry-Cas9-6His-MBP is approximately 230 kDa (6His = 1kDa, SpCas9 = 158, MBP = 42 kDa, mCherry = 29 kDa) and the dark sections indicate the location of the Cas9 fusion protein. **A:** Samples of 0h 2h and 4h of culture at 37 °C; **B** Samples of 0h 2h and 4h of culture at 30 °C; **C:** Samples of 0h and 4h of culture at 25 °C and 20 °C; **D:** Samples of 0h and 4h of culture at 25 °C.

The results of samples taken after different hours of culture showed an apparent decrease of Cas9 production through time which was initially thought to be due to toxic effects of Cas9 overexpression in bacteria. Therefore, lower IPTG concentrations were tested: (0, 0.2 and 0.5 mM) in culture for Cas9 production and 18 ºC or 25 ºC temperatures were also tested in longer periods after induction, namely of 0, 6h and overnight (O.N.). The results showed over-expression of Cas9 in all conditions at 25 °C with the higher productivity present in the culture where 0.2 mM of IPTG were used. At 18 °C, the higher production was also showed in concentration 0.2 mM.

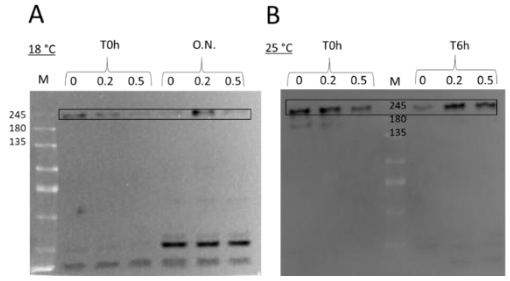

Figure 3: Western blots of Cas9 production with different concentrations of IPTG (no IPTG, 0.2mM and 0.5 mM). **A:**Tested at 18 °C at time 0h and after overnight growth. **B:** Tested at 25 °C at time 0h and after 6h of growth.

With all the previous experiments testing different temperatures and IPTG concentrations, we concluded with effect, that the best conditions for Cas9 production in this *E.coli* strain, is at 25 ºC, using 0.2 mM of IPTG. After reaching the conclusion that 25 °C was the best temperature, 8.250 L of culture were produced for further sonication and purification.

3.3. Production of TEV

In the process of Cas9 purification, a step needed to maintain the functionality of the protein is the cleavage of 6 His-MBP out of the Cas9 fusion protein. For such purpose, the Cas9 fusion protein has a TEV recognition sequence in which TEV protease will cleave. TEV protease had to be therefore, produced and purified for use in Cas9 purification. *E. coli* BL21(DE3) transformed with pRK793 plasmid (Addgene plasmid # 8827) was used for over-expression of TEV with IPTG 1mM induction. The fusion protein is expressed as MBP-His6-TEV(S219V)-Arg5. The MBP is present in this fusion protein in order to increase solubility, maintain active state of the protease and inhibit formation of inclusion bodies. Incubation of 2L of cell suspension were performed at 30 ºC, at 250 rpm, for 5h and 12% SDS gel was made in the end with samples taken at 0 and 5 h after induction. The resultant gel, after 5h of induction with IPTG (1mM), shows the appearance of two new bands, one of 42 KDa that represents MBP self-cleaved *in vivo* by the presence of TEV sequence in the fusion protein, and a band close to 29 KDa representing the His6-TEV(S219V)-Arg5 protease.

3.4. Purification of TEV

The cells from 2L of culture were lysed by sonication before IMAC (Immobilized Metal Affinity Chromatography) was performed in a HisTrapTM FF 5 mL, (GE Healthcare). As it was seen in the chromatogram of IMAC purification, one big spike in the absorbance at 280nm is observed in the elution step, which should represent TEV protease. Samples of the enriched fractions were taken and a 12% SDS gel was made to observe if the spike corresponded effectively to the TEV protease. TEV protease was further purified in a gel filtration. The resultant chromatogram showed a big spike, assumed to be TEV protein. 12% SDS-PAGE gels were run with the

samples of the spike to verify the purity of the protein. Strong bands appeared close to 29 KDa, the size of TEV in IMAC and Gel filtration gel, showing that effectively TEV protease was in monomers. The enriched fractions were once again concentrated, reaching 9µM of TEV protease in the end (3.78 mg/mL).

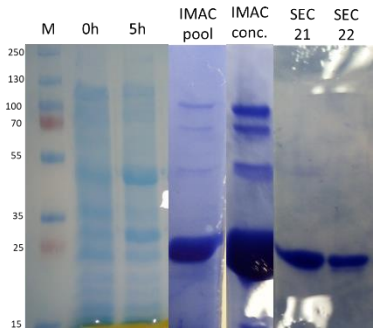

Figure 4: 12% SDS gels of each steps of TEV purification.

3.5. Purification of Cas9-mCherry

Initially the process of purification of Cas9 is very similar to the TEV purification, in which the resultant cells of the 8.250 L of culture were lysed by sonication and IMAC (Immobilized Metal Affinity Chromatography) was performed in a HisTrapTM FF 5 mL, (GE Healthcare). The chromatogram of IMAC shows a sharp spike assumed to be the Cas9 fusion protein. Confirmation of the identity of the spike was made in an 8% SDS-PAGE gel of the enriched fractions of the spike. Furthermore, after purification the fractions associated to this spike presented a strong pink color in the glass tubes suggesting the presence of mCherry released light by the fusion protein. The gel showed strong bands of 230 kDa in the samples corresponding to the spike, representing 6-His-MBP-Cas9-mCherry. These rich fractions (4-10) were concentrated with 10,000 MWCO Amicon (Milipore) for further Dialysis to remove imidazole from the purification and combined 6 His-MBP cleavage with TEV in a Dialysis cassette (ThermoFisher). The concentrated sample was divided for two dialysis cassettes for a better ratio between Cas9 sample and TEV. In one, 3 mL of Cas9 were cleaved with 900 µL of TEV produced, while in the other, 3.7 mL of Cas9 were cleaved with 600 µL of TEV. The two tested quantities of TEV showed in both, almost 100% efficiency in cleavage after 40h to 64h of incubation at 4 ºC, while dialysis was occurring for imidazole removal. This phenomenon was visualized by the shift of Cas9 band of 230 to 187 kDa in the 8% SDS gels. The two resultant solutions of cleaved Cas9 were concentrated together in order to reach the volume indicated for IEX (Ion Exchange) Chromatography. The resultant chromatogram of IEX, showed two spikes at approximately 20% and 30% of Buffer B while a final manual run with 100% elution buffer shows another spike. 8% SDS gels of the enriched fractions of each spike allowed the verification of protein purity. The manual run was executed since pink color was observable even after the end of the program, showing thus, that some Cas9 was still trapped inside the column. The 8% SDS gels demonstrated no Cas9 protein in the first spike, while in the second spike showed high Cas9 concentrations with high purity in most fractions in the 187 kDa bands. The samples 4 and 5 of the manual run showed also enrichment in Cas9 with less but still good level of purity. The samples 28 to 38 and EM 5 and 6 were concentrated together until 500 µL for further purification with SEC (Size Exclusion Chromatography) too remove mostly the small proteins still present in the sample. Quantification of Cas9 in the concentrated sample of IEX rich fractions, was made with spectrophotometry using a wavelength of 280 nm and an extinction coefficient of 120,450 M^{-1} cm^{-1} [42] to estimate protein concentration. The quantification was done with dilution 1/10 in order to maintain inside of the range method and for minimal waste of Cas9. The graph showed two spikes as predicted, one at 280 nm, describing Cas9 and another one at 587 nm, the excitation spike of mCherry fluorescence. After calculations with the absorbance at 280 nm, the concentration determined for the sample of concentrated Cas9 without dilution, was 15 µM, using the Lambert-Beer Law. SEC was performed, resulting in a chromatogram with two great spikes that correlated with pink elution from column. After 8% SDS-PAGE gel, the two spikes were revealed as being effectively Cas9 plus some bands of close size with chances of being Cas9 degraded or protein contaminants of similar sizes and properties, that we were unable to separate. Rich fractions (13-21) were then concentrated and a new 8% SDS gel and Cas9 quantification was made, reaching 6 µM of Cas9-mCherry.

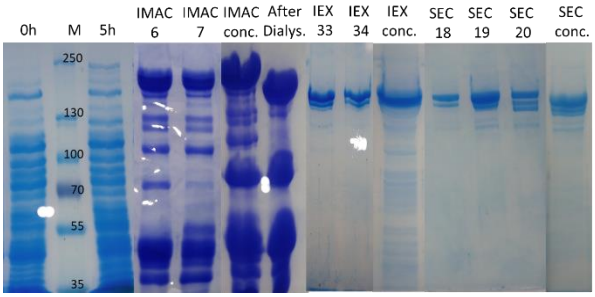

Figure 5: 8% SDS gels of each steps of Cas9 purification.

3.6. *In vitro* cleavage assay of CRISPR-Cas9 complex

After production and purification of Cas9 protein, a cleavage test *in vitro* is needed to confirm the functionality of the produced protein. HEK, HeLa and AT-MSCs (Adipose tissue-derived MSCs) were cultured for DNA extraction and consequently, amplification by PCR of the target region for CRISPR-Cas9 edition, the first intron of the *PPP1R12C* gene (phosphatase 1 regulatory subunit 12C), located in a recognized genomic safe harbor of chromosome 19. The PCR was performed, and a 1.3% agarose gel was

run with the PCR product. The PCR product showed only one band in all PCR products of the three types of human cells as predicted, with a size of 372 pb, the size of the targeted region meant to be amplificated. After the amplification of the DNA target region, an *in vitro* cleavage assay was performed with purified Cas9 protein. The gRNA duplex was assembled with specific crRNA and trancrRNA from IDT (Integrated DNA Technologies) before being complexed with the Cas9-mCherry to produce the RNP complex. The results showed efficient cleavage of the DNA substrate, close to 50%. However, a band of 400 bp representing the DNA substrate uncleaved, and two bands close to 175 and 197 pb, the size of the fragments produced by cut in the right place are seen. It is observable that the uncleaved band after cleavage is higher in the gel. This is possibly due to a not 100% release of the RNP complex from the DNA substrate, thus increasing the molecular weight of the fragments.

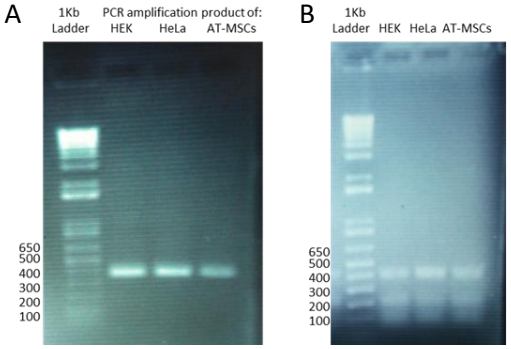

Figure 6: 1.3% Agarose gels of: **A:** PCR product of target region of HEK, HeLa and AT-MSCs **B:** CRISPR-Cas9 cleavage assay in the PCR product of target region of HEK, HeLa and AT-MSCs.

3.7. Cleavage assay of CRISPR-Cas9 complex in HEK cells

Upon observing successful DNA cuts *in vitro* we continued the study in cultured cells *in vitro*, to observe if the same efficiency would be observed within the cells. HEK cells were first tested, since they are of human origin, and are easily transfectable. The cells were transfected with the *in vitro* assembled RNP complex, using Lipofectamine 2000. Three different complexes were construct, one with the guide2 for targeting the safe harbor and positive and negative control kits from IDT (Alt-R™ CRISPR-Cas9 Control kit Human). Cells were transfected and after 48h, were pelleted for lysis with GeneArt kit lysis buffer (Invitrogen). Like in the *in vitro* test, the target region was amplified with PCR and separated on an agarose gel. A band close to 372 bp for guide 2, and a band close to 1083 bp in both controls were visualized as predicted, showing effective amplification of the target regions. Within the cells, after cleavage, the DNA is repaired most of the times by NHEJ, therefore InDel mutations are added to the DNA sequence. By denaturating and re-annealing, both wt and mutated sequences are put together in the final DNA product, which after incubating with the cleavage detection enzyme (not disclosed in the kit), results in a cleaved PCR product, producing two bands of the predicted sizes. It is possible that due to the similarity in sizes of the fragments produced by guide2, only one band is observable due to low resolution of the gel. The band of PCR amplification was also well observed in the three samples. The positive control appears also with two new bands (827 and 256 pb) with the predicted size, showing effective cleavage. The negative control maintained only the PCR amplification band as predicted.

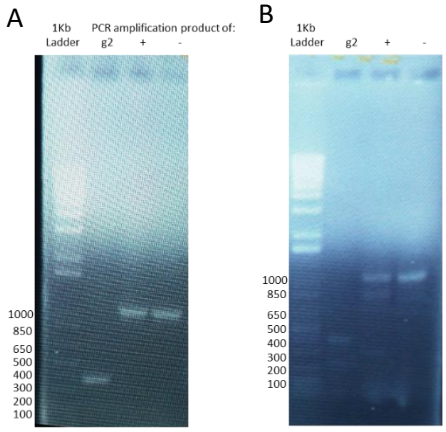

Figure 7: 1.3% Agarose gels of: **A:** PCR product of target regions of HEK. **B:** CRISPR-Cas9 cleavage assay in the PCR product of target region of HEK.

4. Discussion

In the development of this novel cell-based therapy against cancer, MSCs will be engineered with a transfectable RNP CRISPR-Cas9 complex assembled *in vitro* and a donor template, to express and secrete azurin protein. The inherent tropism to cancer cells of the MSCs together with the anti-cancer properties of azurin, have the potential to generate a new therapy against cancer.

The best conditions for the microporation of MSC were observed to be at a voltage of 1400 V, with a pulse width of 30 ms in 1 pulse and a similar condition with the variant of 1300 V instead of 1400 V. The first showed highest fluorescence, thus higher efficiency in transfection, however, the viability was extremely poor. The second condition even though it had less fluorescence, it had greater viability. Even though, the microporation conditions were optimized to MSCs with the all-in-one plasmid, selection with puromycin showed negative results, thus clarifying that the problem was not in the aggressiveness of microporation, but in the puromycin. It also shows one more time that RNP transfection is a better solution, since it thus not need selection with antibiotics.

The second step of this study was the effective production of Cas9 in *E. coli* BL21 (DE3). Due to the exogenous nature of this protein, different proteins are expressed in response. When comparing SDS gels of Cas9 and TEV proteins, it is clear that there are

different protein expressions even though the strain is the same and culture conditions are identical. After IMAC it is possible to observe that the purification of TEV is highly efficient when compared to IMAC of Cas9. This suggests that the proteins expressed by response of Cas9 are richer in histidine, making purification harder. This is also consequence of the nature of proteome of *E. coli* BL21 (DE3). Recombinant His-tagged proteins expressed in this strain are commonly coeluted with native *E. col*i proteins in IMAC and this effect is increased specially when the expression of the recombinant protein is low, like the Cas9 protein. The native *E. col*i proteins have clustered histidine residues with metal binding sites. Such problem could be fix with engineered *E. coli* BL21(DE3) where the most dominant contaminants have alternative tags or mutations for affinity loss to nickel. [29] Alternatively, other strains can also be used like Rosetta strains since in some cases this strain is better suited for the expression of proteins of eukaryote origin. In previous purifications, after dialysis and 6 His-MBP cleavage, IMAC was tried to run manually to separate cleaved from uncleaved Cas9 protein. Unfortunately, both forms had high affinity to the nickel, revealing that Cas9-mCherry is rich in histidine or other resides with affinity for nickel. A possible reason is that Cas9 HNH nuclease domain is most likely to employ a one-metal-ion mechanism for target-strand DNA cleavage, that is a conserved general base histidine. RuvC likely uses a two-metal-ion catalytic mechanism for cleavage of the nontarget DNA strand, based in conserved aspartate residue. [30] Due to the toxicity of Cas9, it was also used an induction system with IPTG in order to limit exposure of cells and decrease suppressor mutations that inactivate Cas9. [31] The ideal conditions for Cas9 protein in this system were culture at 25 ºC with 0.2 mM of IPTG. IPTG at high concentrations showed inhibition of Cas9 expression. This could be consequence of the reduction in growth rate and saturation due to higher IPTG concentrations. Also, the reduction in growth rate is higher in early exponential phase when compared to late exponential phase. [32] Along purification, mCherry have emitted pink light even without laser excitation, and without even being exposed to almost no light. This is due to the fact that mCherry is from the group of red fluorescent proteins, also called permanently fluorescent proteins. [33] This transition begins when exposed to light in purification. Strangely, the color only appears later when storage in almost no light environment at 4 ºC, showing a possible slow activation maybe due to low temperatures. It is possible that imidazole of the elution buffer also influences since it is a key constituent of the fluorophore system of red fluorescent proteins such as mCherry, that is responsible for their pH sensitivity. [34] However, it has been already showed that mCherry is not very sensitive to pH variations, in terms of emitted fluorescence. [35] After finishing purification, only contaminants with same afiinity, ionic perfil, and size have been maintain, sugesting that the possible contaminants may be Cas9 degraded.

The cleavage assay *in vitro* of the PCR product of HEK, HeLa and AT-MSCs, using the constructed RNP CRISPR-Cas9 showed maintenance of functionality even after defrost and refrost of the complex and the efficiency in DNA cleavage in the target site was close to 50% in all cases despite the DNA origin. By the size of the bands generated in agarose gel, it appears that the cuts were performed in the right location. The 50% percentage efficiency is assumed due to the same intensity of the bands of uncut DNA and cut DNA. The cleavage assay performed in cultured HEK cells reveals a weaker efficiency compared to *in vitro* as expected, since the complex needs to enter the cells and reach the target DNA Still promising results were showed in the agarose gel of the cleavage assay using guide2. A weak band close to 200 bp was seen in this gel. Due to low resolution of the gel, the two supposed band most likely became undistinguishable owing to their close size (175 and 197 bp). Comparing the intensity of the PCR amplification band, with the cleaved, it is possible to assume an efficiency of cleavage close to 40%. Also, the possibility of CRISPR-Cas9 being still attached, could alter and increase the molecular weight of the bands. The positive control emerged with two smaller bands, besides the uncleaved, having the right sizes of 1083 bp (uncleaved), 827 bp and 256 bp. The negative control maintained the PCR amplification band only, as predicted.

Cleavage assay of MSCs was also performed using the same method used in HEK, with 8h of incubation with RNP guide2, using 4 µL of Lipofectamine 2000 instead of 2µL used in HEK, and with incubation overnight with 2 µL of Lipofectamine 2000. Unfortunately, results were not adequate due to low efficiency of transfection in MSCs, using Lipofectamine 2000. Due to malfunction of the microporator, Lipofectamine 2000 strategy had to be used to test cleavage efficiency in HEK and MSCs. In case of MSCs, it is known that the best method for transfection is microporation as documented [36].

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