Efficient editing of human iPS cells using gesicle-mediated delivery of Cas9-sgRNA protein complexes

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Abstract

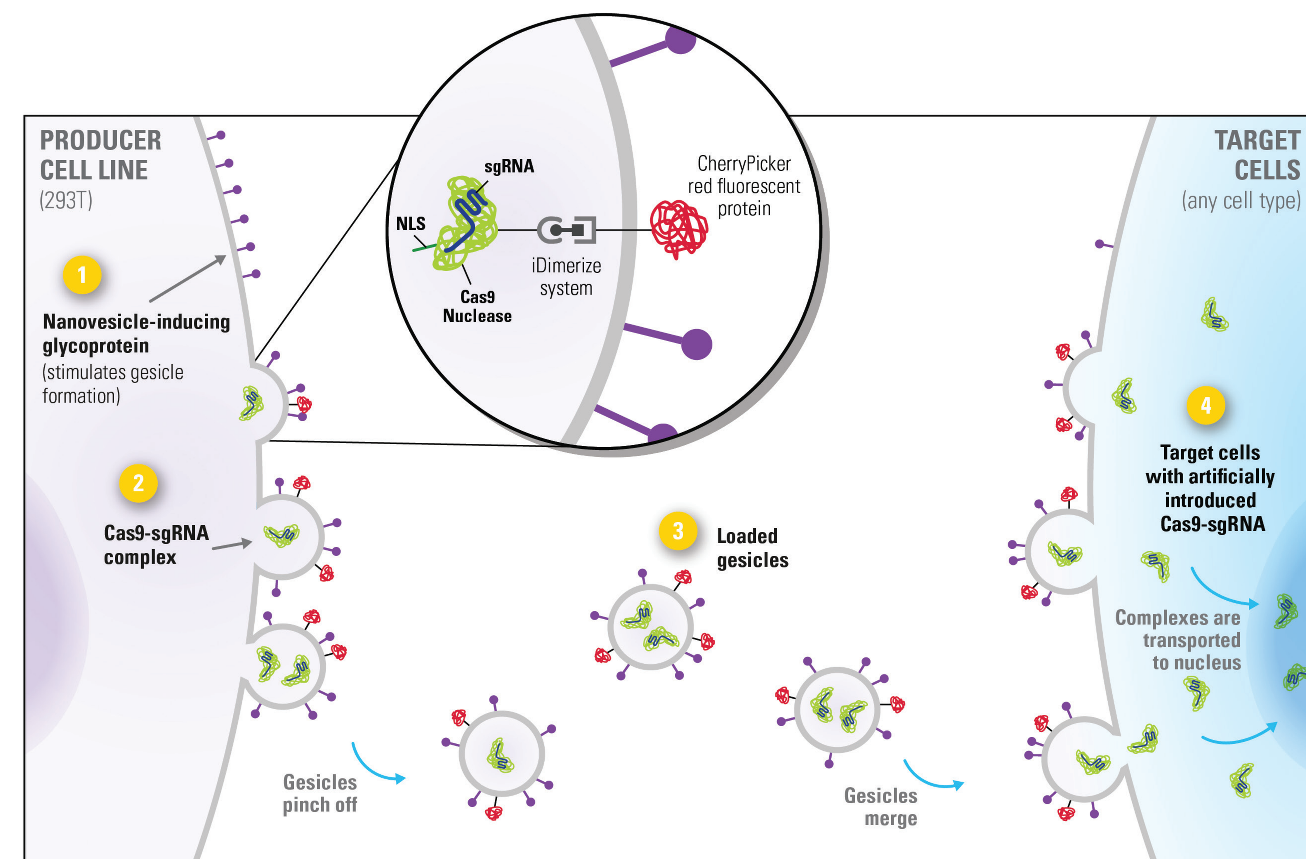
CRISPR/Cas9-based gene editing has revolutionized the field of cell biology. However, two significant challenges remain: obtaining efficient delivery of Cas9 to all cell types and achieving fewer off-target effects. It has been previously demonstrated that genome editing via direct delivery of Cas9-sgRNA ribonucleoproteins (RNPs) has the added benefit of decreased off-target effects due to the short duration of the RNP in the cell.

Here we report on a new RNP delivery method for footprint-free genome editing in a broad range of cell types, including human induced pluripotent stem cells (hiPS cells). Cas9 containing a nuclear localization signal (NLS) and an sgRNA of interest are simultaneously expressed in a mammalian packaging cell and actively packaged as an RNP complex into nanoparticles that originate at the plasma membrane. These cell-derived nanoparticles are called "gesicles". Gesicles are produced by the packaging cell due to the expression of a nanoparticle-inducing glycoprotein. We have adapted gesicle technology for the packaging of Cas9-sgRNA RNPs via a ligand-dependent dimerization system (iDimerize™ technology). This method enriches RNPs at the plasma membrane where they are incorporated into newly forming gesicles that are later harvested from the media. When added to target cells, gesicles will deliver the active RNP complexes into the cells.

In this work, we were able to demonstrate that gesicles carrying RNPs can mediate target-specific gene knockout in a broad range of cell types, including hiPS cells, without the toxic effects of other methods. In addition, this nanoparticle-based method allows for tight control of the timing and dose of the Cas9-sgRNA complexes, which decreases off-target effects. Due to the need to do genome editing in hiPS cells for the study of human biology and disease, we have optimized the use of gesicles to knock out genes in hiPS cells without affecting pluripotency.

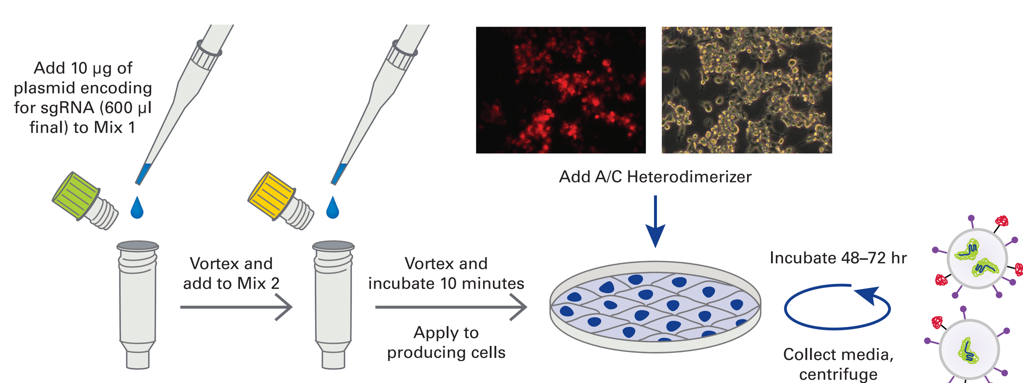
Overall, gesicles can be considered of high interest for genome editing, providing a direct, rapid, and transient method for delivering highly active Cas9-sgRNA RNPs into target cells.

Mechanism of gesicle production for delivery of Cas9-sgRNA protein complex



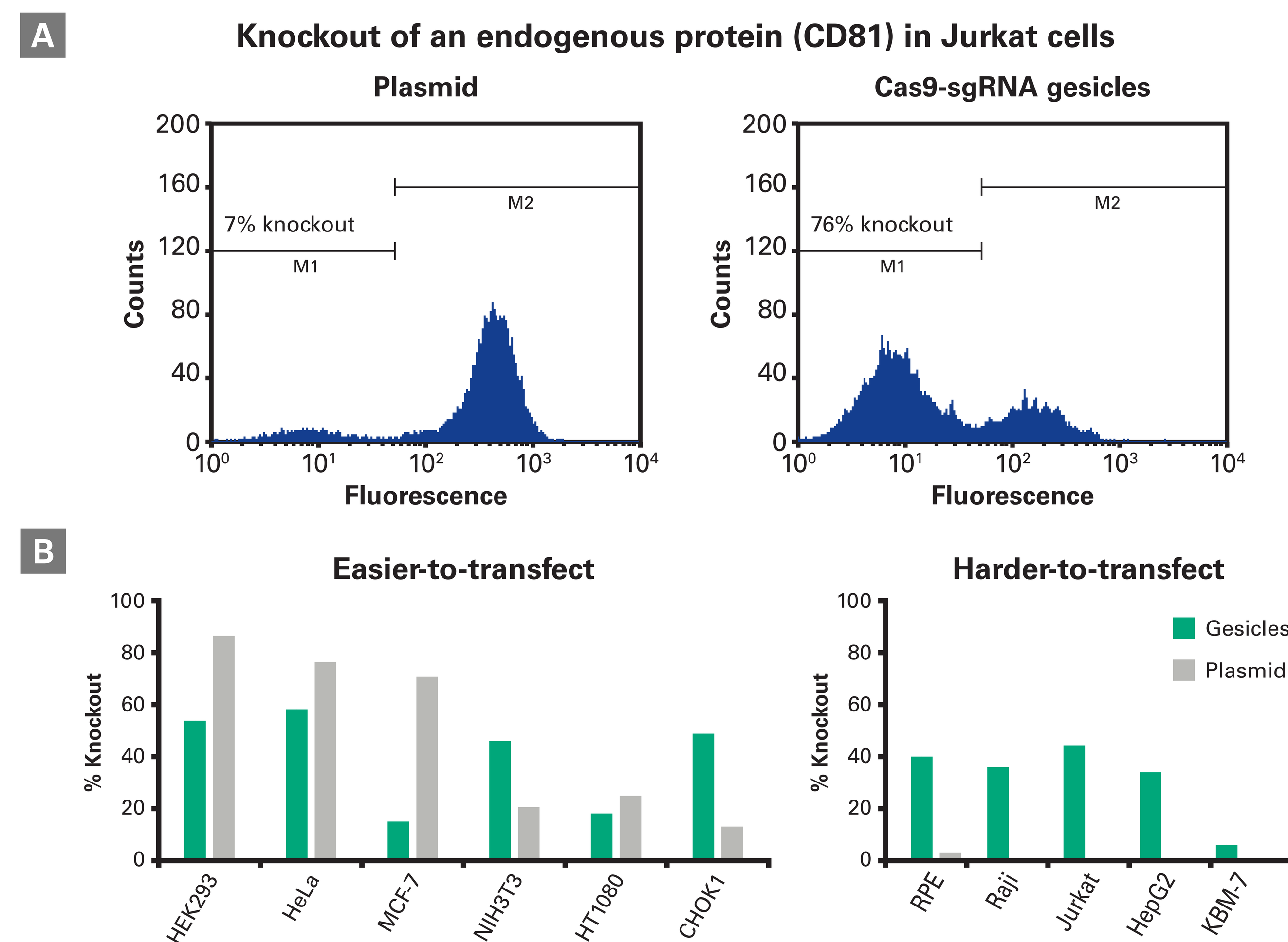
Production of nanovesicles (gesicles) for delivery of Cas9 protein together with sgRNA against a gene of interest. Overview of Cas9 Gesicle production (Cat. #632613). (Step 1) In this illustration, expression constructs for Cas9 endonuclease, sgRNA against a target gene, CherryPicker™ red fluorescent protein, and other elements needed for gesicle production are cotransfected into the HEK 293T-based packaging cell line (Gesicle Producer 293T Cell Line, Cat. #632617). (Step 2) Overexpression of these proteins in the presence of the iDimerize ligand leads to the formation of gesicles containing sgRNA-loaded Cas9 protein, which are then collected from the media (Step 3). Addition of these gesicles to a target cell results in delivery of the Cas9-sgRNA complex to the nucleus and transient labeling of the membrane with CherryPicker protein (Step 4).

Figure 1: Production of Cas9-sgRNA gesicles using the Guide-it™ packaging set



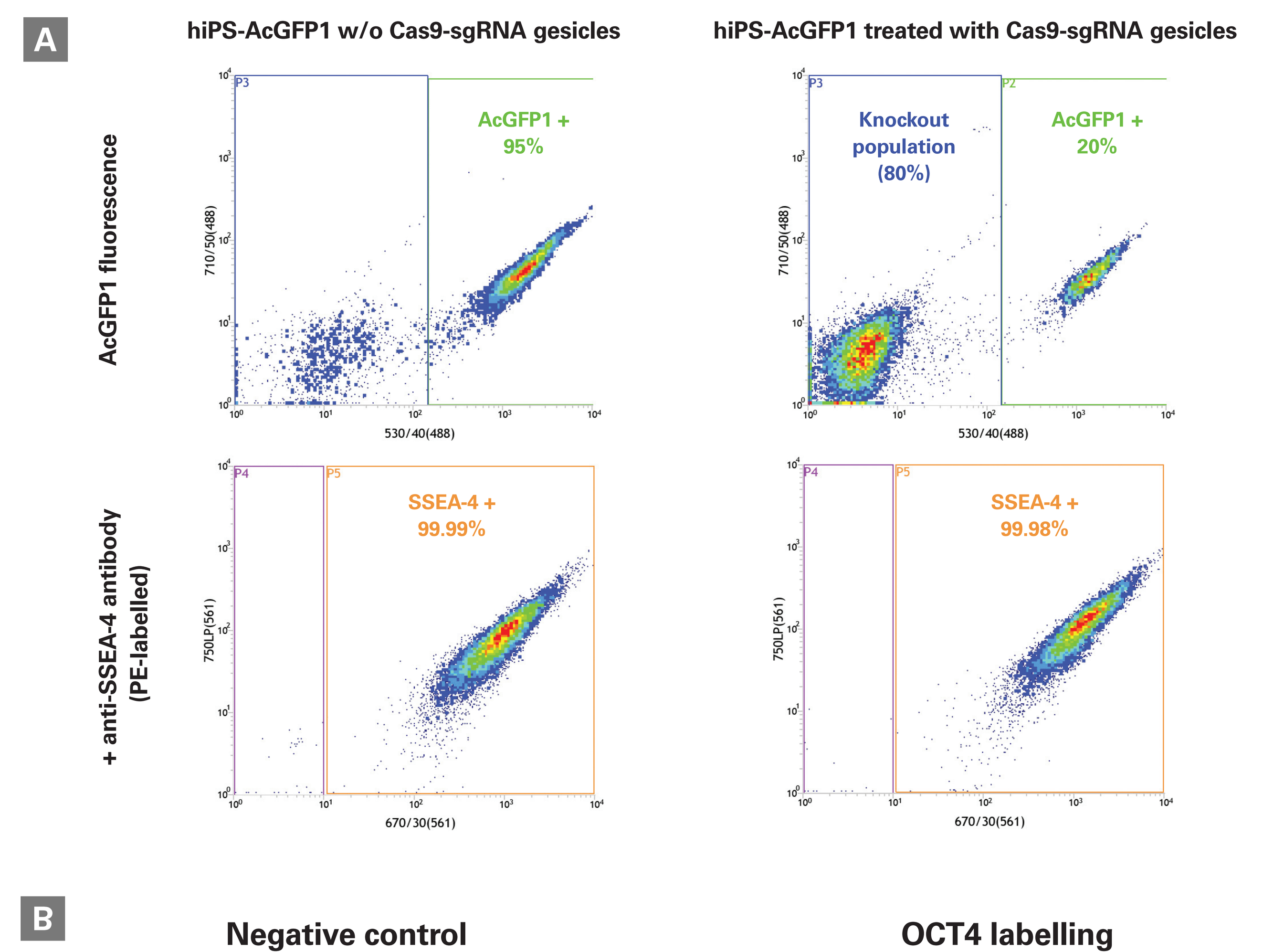
Production of Cas9-sgRNA gesicles using the Guide-it packaging set. The Guide-it gesicle packaging mix (Cat. #632616) contains lyophilized Xfect™ transfection reagent premixed with an optimized formulation of plasmids encoding for all the elements needed for gesicle production. Simply add your pGuide-it-sgRNA designed against your gene of interest, and then apply to Gesicle Producer 293T cells together with the iDimerize ligand. After 48–72 hr, centrifuge the cell media overnight at 7,000 rpm. Decant the supernatant and resuspend the pellet (gesicles containing Cas9-sgRNA complexes). These Cas9 Gesicles are then ready to be added to target cells.

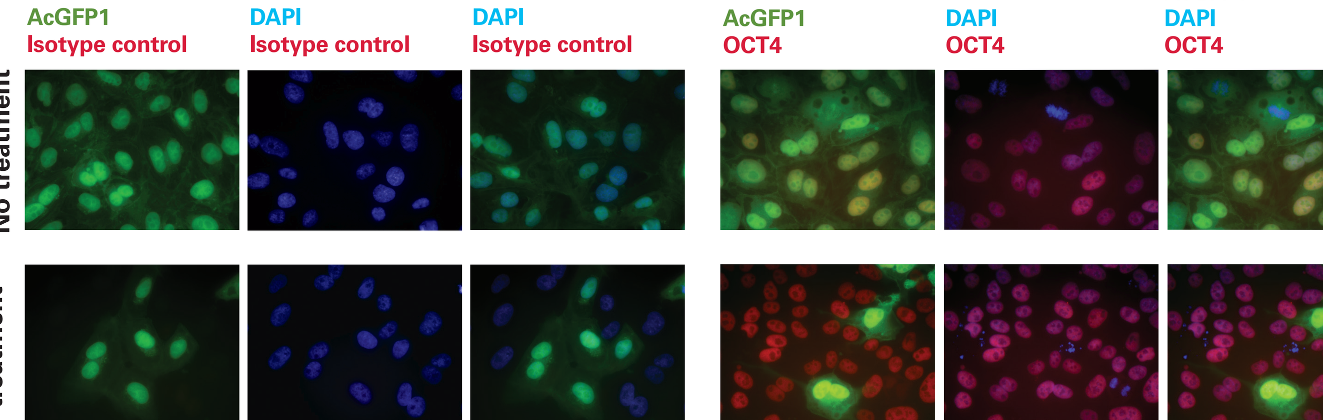
Figure 2: Use of Cas9-sgRNA gesicles as a genome editing tool



Use of Cas9-sgRNA gesicles as a genome editing tool to knock out genes in multiple cell lines. Panel A. The cell-surface protein receptor CD81 was knocked out in Jurkat cells using either plasmid cotransfection of Cas9 DNA and sgRNA, or Cas9-sgRNA gesicles (with the sgRNA against *CD81* in both instances). Knockout efficiencies were measured six days later via antibody labeling of CD81 followed by flow cytometry. The knockout efficiency obtained via gesicles was significantly higher than the results achieved with plasmid transfection. **Panel B.** Cas9-sgRNA gesicles outperform plasmid transfection in harder-to-transfect cell types. Cell lines were created that contained an integrated *ZsGreen1* fluorescent protein expression cassette. In this system, gene editing of *ZsGreen1* can be monitored by a loss of green fluorescence. These cell lines were treated with Cas9-sgRNA gesicles (with the sgRNA against *ZsGreen1*), and then analyzed by flow cytometry. Cas9-sgRNA protein complex delivery and ZsGreen1 knockout via gesicles was efficient and comparable to plasmid-based delivery in easier-to-transfect cell types (left graph) and surpassed the results achieved via plasmid-based delivery in harder- to-transfect cell types (right graph).

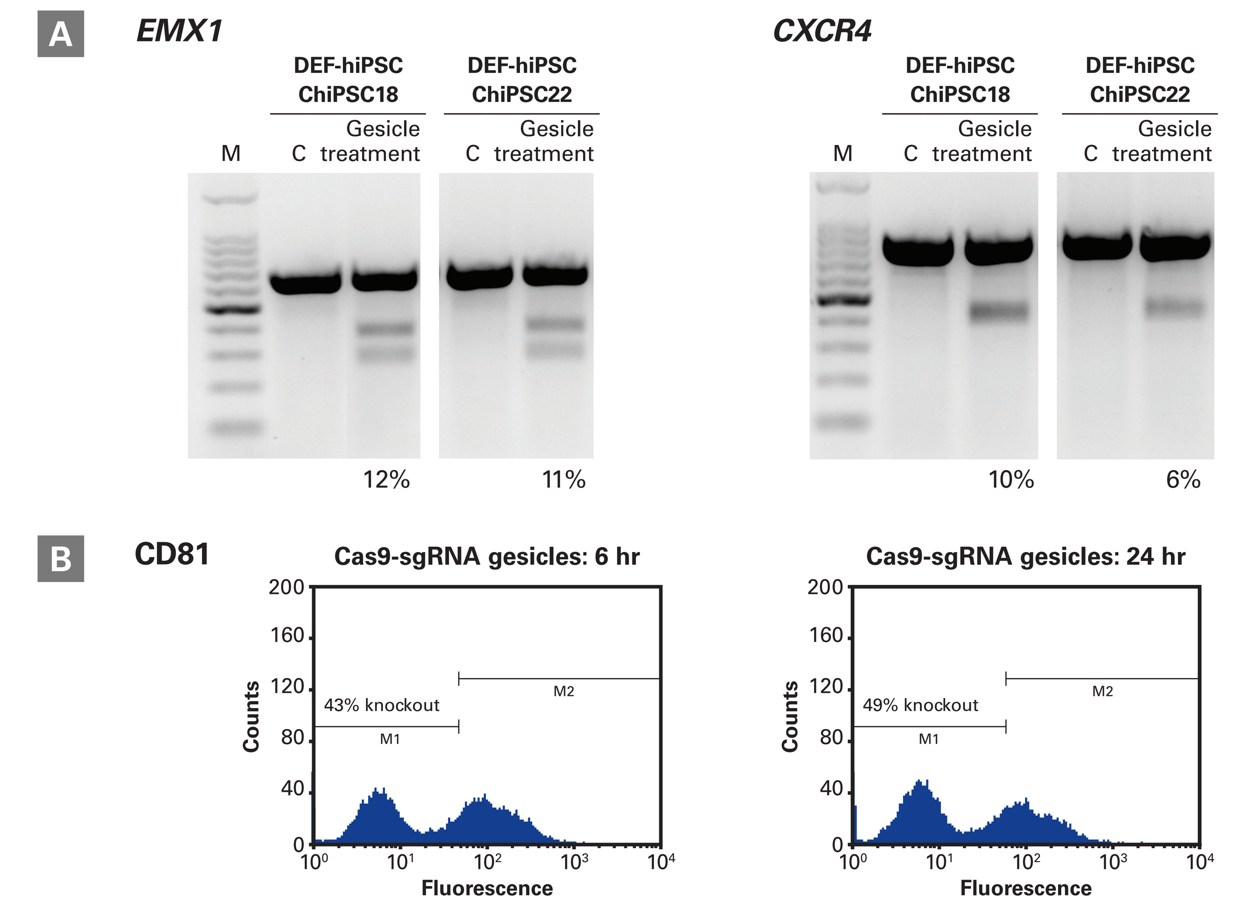
Figure 3: Knockout of a fluorescent protein in hiPS cells





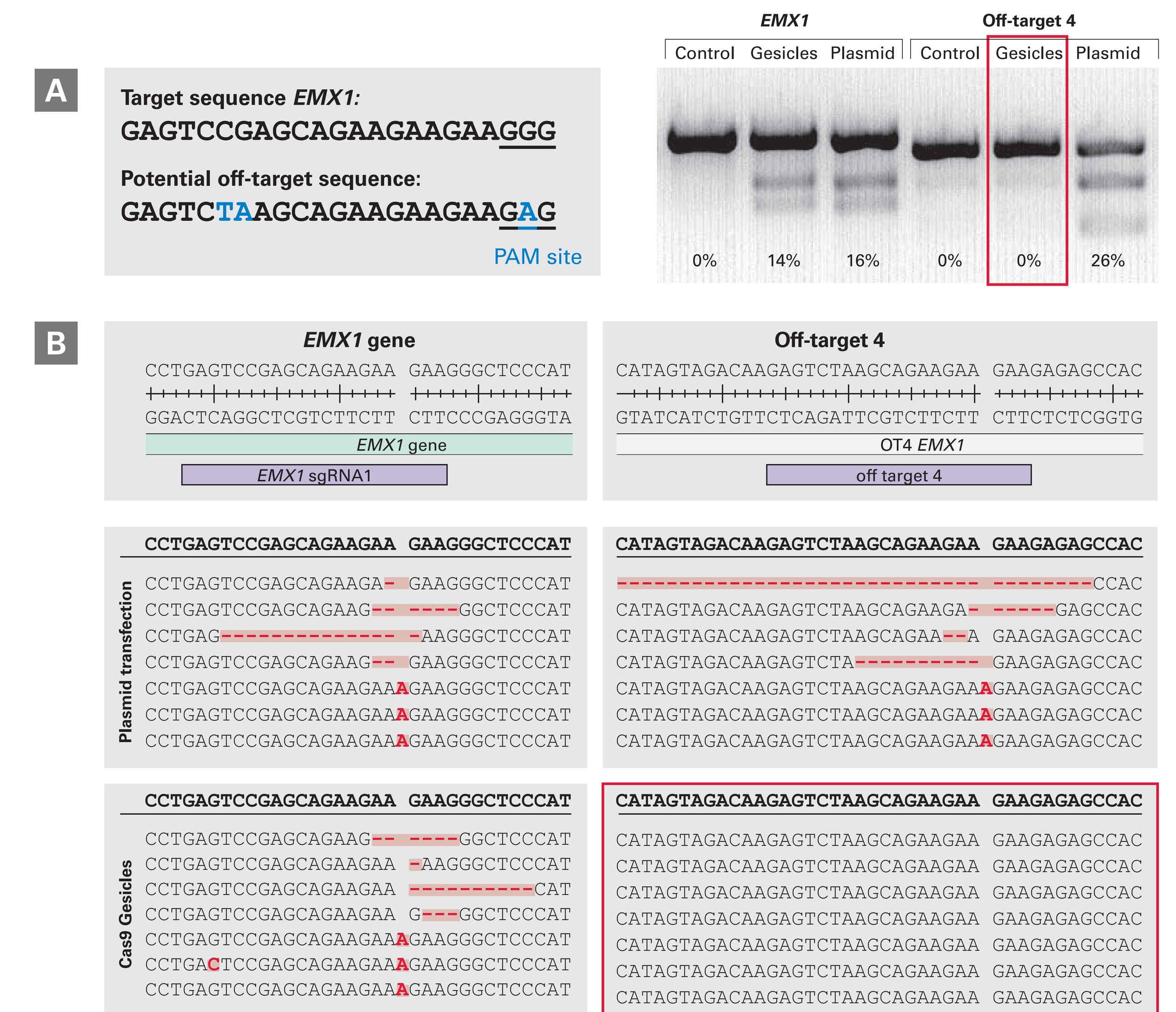
Knockout of a fluorescent protein in hiPS cells. Panel A. Cas9-sgRNA gesicles targeting *AcGFP1* were added to Cellartis® Human iPS Cell Line 22 (DEF-hiPSC™ ChiPSC22, Cat. #Y00325) stably expressing AcGFP1, cultured under non-differentiating conditions using the Cellartis DEF-CS™ Culture System (Cat. #Y30010). After gesicle treatment, AcGFP1 expression was determined via flow cytometry in untreated DEF-hiPSC ChiPSC22-AcGFP1 (top left) and gesicle-treated DEF-hiPSC ChiPSC22-AcGFP1 (top right). Both samples were also labelled with anti-SSEA4 (PE) antibody (bottom). Gesicle-mediated delivery of Cas9-sgRNA complexes induced knockout in about 75% of cells without a loss of pluripotency. **Panel B.** Immunohistochemistry was performed on the DEF-hiPSC ChiPSC22-AcGFP1 cells six days after gesicle addition. Cells were permeabilized and stained with an antibody against OCT4 (eFluor 570-labelled) or an IgG2a isotype control eFluor 570. Cells treated with the gesicles presented a mixed population of fluorescent and non-fluorescent cells (a product of successful gene editing of the *AcGFP1* gene by Cas9 Gesicles), all of them positive for OCT4 expression. Therefore, gesicle treatment left the pluripotency of the edited cells unaffected.

Figure 4: Knockout of endogenous genes in hiPS cells



Efficient knockout of endogenous genes in hiPS cells using gesicles. Panel A. The genes *EMX1* (left) and *CXCR4* (right) were knocked out using Cas9-sgRNA gesicles in DEF-hiPSC ChiPSC18 and DEF-hiPSC ChiPSC22 cell lines. Knockout efficiencies were determined using a Guide-it Resolvase assay (Guide-it Mutation Detection Kit, Cat. # 631443). A control sample that was not treated with Cas9 was included for comparison (Control, C). The percentage of DNA cleavage was determined by densitometry. The knockout could be detected in all the samples. **Panel B.** Successful knockout of CD81 in hiPS cells. Gesicles containing Cas9-sgRNA complexes designed to target human *CD81* were harvested and added to Cellartis Human iPS Cell Line 18 (Cat. #Y00305), cultured with the DEF-CS Culture System for six or 24 hr, and then cultured in gesicle-free DEF-CS culture media for an additional seven days. The surface expression of CD81 on gesicle-treated cells was determined via flow cytometry. Graphs show DEF-hiPSC ChiPSC18 cells after six hr (left) or 24 hr (right) of gesicle treatment, labeled with anti-CD81 (FITC) antibodies. There is an increase in knockout percentage when the incubation time is increased.

Figure 5: Cas9 gesicles reduce off-target effects



The use of Cas9 Gesicles reduces off-target effects compared with plasmid-based treatment. Panel A. Decrease of off-target effects detected via Guide-it Resolvase assay. HEK 293T cells were either simultaneously transfected with plasmids encoding Cas9 and a sgRNA against *EMX1*, or treated with Cas9-sgRNA gesicles. After 72 hr, the *EMX1* gene and a potential off-target locus (off-target 4) were amplified and the percentage of DNA cleavage was determined using a Guide-it Resolvase assay. With the Cas9 Gesicles, no off-target effect could be detected. **Panel B.** Indels generated after Cas9 targeting of *EMX1*. Indels were identified using the Guide-it Indel Identification Kit (Cat. # 631444). Sequencing data for the different clones revealed a range of deletions and insertions (highlighted in red) in both sites (target site *EMX1* as well as off-target 4) in the case of cotransfection with Cas9 and sgRNA plasmids. In the case of the cells treated with Cas9 Gesicles, indels could only be detected at the target site; the off-target site was not mutated.

Conclusions

- Gesicles provide a highly efficient means of producing, packaging, and directly delivering Cas9 protein complexed with sgRNA to any target cell, including hiPS cells
- Delivery of Cas9 via gesicles leads to levels of genome modification similar to plasmid delivery, but with the added benefit of drastically reduced editing of off-target sites
- Gesicles can be used to knock out genes in hiPS cells without any change in cell pluripotency

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