Genetic Modification of Target Cells by Direct Delivery of Active Protein

Thomas P. Quinn[1], Montse Morell, Lily Lee, Mei Fong, Michael Haugwitz & Andrew Farmer
[1]Corresponding Author
Thomas_Quinn@clontech.com

Abstract

Precise modification of the human genome has been a goal of researchers for over two decades. Currently, genome modification is performed by either transient or stable delivery of nucleic acids that encode genome modifying components to target cells. This nucleic acid-based approach has a number of drawbacks, including low efficiency, toxicity, prolonged expression, off-target effects, and potential delay in modification due to transcription and translation post-delivery. An alternative approach is direct intracellular delivery of genome-modifying proteins to live cells. This method allows both the timing and dose of target protein delivery to be tightly controlled, thereby improving efficiency. Consistent with this, it has been reported that direct Cas9 protein delivery leads to higher levels of on-target editing and fewer off-target effects (1). Direct protein delivery, however, is limited by the need to express and purify protein in *E. coli*, where issues with protein yield, proper folding, and/or lack of post-translational modification may ultimately reduce activity. The use of recombinant protein is further complicated by a lack of efficient and consistent delivery methods into target cells of interest. Improved methods, combining mammalian-based protein production and efficient packaging into particles into one step can address these shortcomings. Here we report cellular delivery of DNA-modifying proteins using VSV-G-induced microvesicles (Gesicles). Gesicles are produced by co-overexpression of the spike glycoprotein of VSV-G with a protein of interest (POI), within a mammalian packaging cell. This leads to production of Gesicles containing active POI that can be delivered to mammalian cells. Based on this principle, we have developed a method for actively packaging genome-modifying proteins into the Gesicles via ligand-dependent dimerization. This approach allowed us to package a POI containing a nuclear localization signal (NLS) efficiently into this particle. Analysis of the physical properties of these Gesicles demonstrated that they are highly stable over multiple freeze-thaw cycles, are consistent in size, and demonstrate minimal aggregation. Functionally, these Gesicles could efficiently deliver genome-modifying proteins to a variety of cells, ultimately leading to genomic alterations. This effect has been demonstrated in over a dozen different cell lines; in all cases, cells maintained high viability and the results closely mimicked those obtained with viral transduction. Taken together, this work suggests that Gesicles can be considered a novel and universal tool for genome modification, providing a direct, rapid, and transient method for delivering active genome-modifying proteins to target cells.

Introduction

The CRISPR/Cas9 system has revolutionized the field of genome modification; through the action of a relatively robust RNA-guided endonuclease, targeted modification can be achieved at virtually any genomic locus. Current methods utilize transient or stable delivery of plasmid constructs or viral vectors to deliver the Cas9 and sgRNA components to target cells. However, evidence suggests that prolonged expression of these components can lead to undesirable cleavage events at off-target sites (2, 3). Several groups have achieved direct delivery of the Cas9/sgRNA ribonucleoprotein complex (1, 4), however this required potentially labor-intensive *in vitro* production of both the Cas9 protein and sgRNA. In this study, we present an alternative delivery method using extracellular vesicles, termed Gesicles (5), to produce and efficiently deliver active Cre recombinase or Cas9 to target cells and to achieve genome modification.

Conclusions

- Gesicles provide a highly efficient means of producing, packaging, and directly delivering active genome-modifying proteins to any target cell with minimal effects on cell viability
- Delivery of Cas9 via Gesicles leads to levels of genome modification similar to plasmid delivery, but with the added benefit of less off-target activity
- Gesicles offer tight control over protein dose and time of delivery, a level of control that is typically not possible with nucleic-acid-based methods

References

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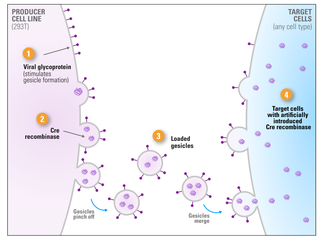
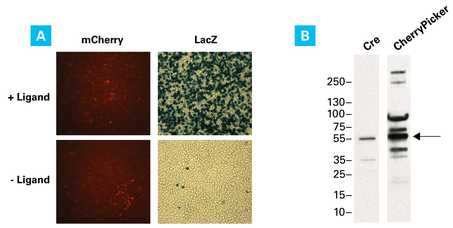
1 Production of exosome-like vesicles (Gesicles) to deliver active proteins



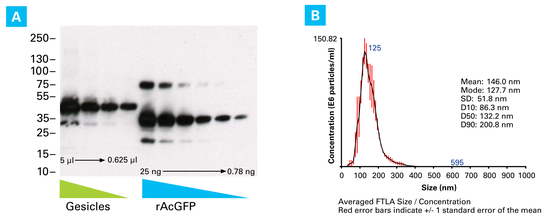
Overview of Gesicle production. (Step 1) In this example, expression constructs for Cre recombinase, CherryPicker™ red fluorescent protein, and VSV-G are co-transfected into the HEK 293T-based packaging cell line. (Step 2) Overexpression of these proteins in the presence of a ligand for induced dimerization leads to the formation of Cre protein-containing Gesicles, which are then collected from the media (Step 3). Addition of these Gesicles to a target cell results in delivery of active Cre protein to the nucleus, and labeling of the membrane with CherryPicker protein (Step 4).

2 Efficient protein packaging into Gesicles



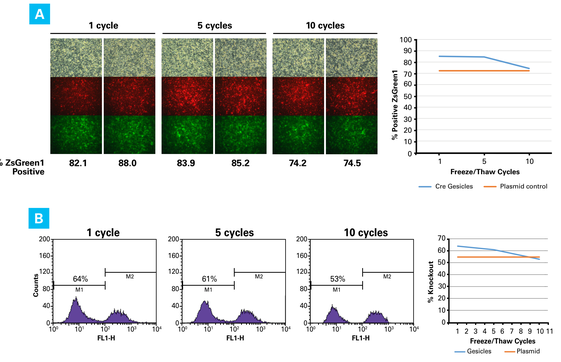
Efficient packaging of active Cre recombinase into Gesicles. Panel A. Both Cre and CherryPicker vectors were cotransfected into the Gesicle packaging cell line. Gesicles were collected, and a loxP-LacZ reporter line was treated with each concentrated preparation. After 24 hr, cells were imaged for red fluorescence and then stained with X-gal. **Panel B.** Cre recombinase Gesicles were analyzed by Western blot for the amount of Cre (left) and CherryPicker (right) contained within the preparations.

3 Gesicle Characteristics



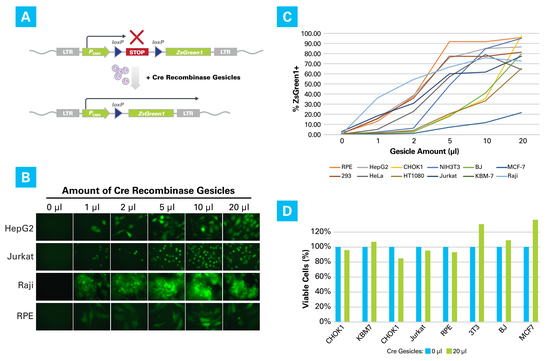
Protein content and particle characteristics of Gesicles. Panel A. Gesicles containing AcGFP1 were produced and were analyzed by Western blot (anti-AcGFP1) to analyze the relative amount of AcGFP1 contained within the particles. Known amounts of recombinant AcGFP1 were used to approximate the amount of AcGFP1 contained within the Gesicles. **Panel B.** Nanoparticle tracking analysis of Cre recombinase Gesicles.

4 Gesicles and their protein cargoes are stable



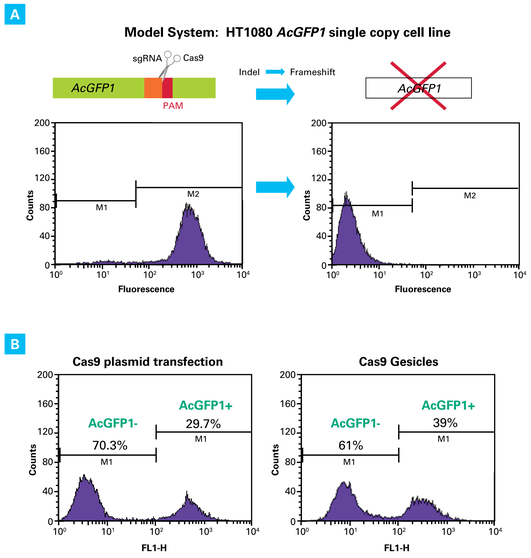
Cre recombinase and Cas9 Gesicles are stable for up to ten freeze-thaw cycles. Panel A. To test the stability of the Cre protein-containing Gesicles, the particles were produced as described (Fig. 1) and then subjected to multiple rounds of freezing (-80°C) and rapid thawing to room temperature (25°C). After thawing, the preparations were added to an HT1080 ZsGreen1 reporter cell line (*loxP*-conditional *ZsGreen1*, refer to Fig. 5A). As a positive control, a Cre-expressing plasmid was transfected into the cells using Xfect™ Transfection Reagent (plasmid control). After 48 hr, the cells were imaged by fluorescence microscopy and analyzed by flow cytometry. **Panel B.** Cas9 protein-containing Gesicles were produced and subjected to freezing and thawing as described above. After thawing, the Cas9 Gesicles were added to an HT1080 AcGFP1 cell line that was previously transfected with an sgRNA targeting *AcGFP1*. Six days post-treatment, the cells were analyzed for the loss of green fluorescence (a measure of AcGFP1 knockout) by flow cytometry. As a positive control, Cas9 was delivered by plasmid transfection (plasmid).

5 Using Gesicles for Cre recombinase-mediated genome modification

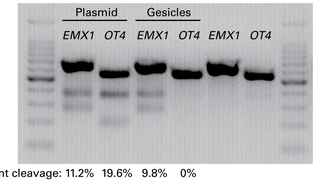


Efficient delivery and genome modification with Cre recombinase Gesicles in various cell types. Panel A. Cell lines were created that contain an integrated, *loxP*-conditional *ZsGreen1* expression cassette. In this system, successful Cre-mediated recombination can be measured by ZsGreen1 expression. These cell lines were treated with various amounts of Cre Recombinase Gesicles and then analyzed by fluorescence microscopy (**Panel B**) and flow cytometry (**Panel C**) 48 hr post-treatment. Cell viability was assessed using trypan blue dye exclusion (**Panel D**).

6 Successful genome modification using Gesicle-delivered Cas9



Mutation detection assay



Percent cleavage: 11.2% 19.6% 9.8% 0%

Successful genome modification using Gesicle-delivered Cas9. Panel A. Schematic of the AcGFP1 knockout assay used to test Cas9 Gesicle functionality. HT1080 AcGFP1 cells were transfected with a plasmid encoding an sgRNA against *AcGFP1* using Xfect Transfection Reagent. A Cas9 plasmid was either co-transfected with the sgRNA, or Cas9 protein was delivered via Gesicles. The cell population was assayed 6 days post-transfection for the loss of fluorescence by flow cytometry as a measure of cleavage efficiency (**Panel B**). To further test the functionality of the Cas9 Gesicles, HEK 293T cells were either simultaneously transfected with plasmids encoding Cas9 and a sgRNA against *EMX1*, or the sgRNA-expressing plasmid was transfected and then Cas9 Gesicles were added after 8 hr. After 72 hr, the Guide-it™ Mutation Detection assay was performed (**Panel C**). The *EMX1* gene and a potential off-target locus (*OT-4*) were amplified by direct PCR (6). The amplicons were then melted and hybridized, and mismatched targets were cleaved using Guide-it Resolvase. A control sample that lacked Resolvase was included for comparison (Control). The percentage of DNA cleavage was determined by densitometry. For the Gesicles, no off-target effect could be detected.

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