

Abstract

Historically, phenotypic control of live cells has been achieved through transient or stable delivery of nucleic acids into primary or transformed cells. However, this approach has a number of drawbacks, including low efficiency, toxicity, genome disruption at the integration site, and a potentially significant delay in phenotypic response due to the requirement for transcription and translation post-delivery. Another approach is to use an inducible system which allows for tight regulation of expression—but inducible systems still depend upon efficient delivery of DNA to function properly. In contrast, direct intracellular delivery of recombinant proteins into live cells allows tight control over both the timing and dose of the desired polypeptide, but is limited by the need to express the protein prior to delivery (typically in *E. coli*). This can lead to issues with protein yield, proper folding, and post-translational modifications, and likely to a reduction in activity. Alternative methods of protein delivery could provide new and effective tools. Here we report on a number of applications that utilize VSV-G-induced microvesicles (Gesicles)¹ to deliver functional proteins directly to target cells. Co-overexpression of the spike glycoprotein of VSV-G with the protein of interest (POI), within a mammalian packaging cell, leads to the production of VSV-G coated microvesicles containing active amounts of the POI. Expanding upon previous work¹, we have developed a number of Gesicles carrying the receptors Pit2, mCAT1, and CAR to alter the susceptibility of primary cells and cell lines to various viruses, including retroviral and lentiviral vectors pseudotyped with either amphotropic and ecotropic envelopes as well as type 5 adenovirus vectors. In all cases, transduction of receptor Gesicle-treated cells resulted in an increase in transduction efficiency, including rendering receptor-negative cells positive for transduction as measured by fluorescence microscopy and FACS. We were also able to deliver other functional membrane proteins such as GPCRs and a cell surface marker, with both demonstrating activity in as little as 3 hours post delivery. To our knowledge, no other current delivery method is capable of delivering membrane proteins in this way. Other experiments confirmed that we could deliver fluorescent proteins and other molecules to the cytoplasm. All activities were found to be due to direct transfer of the POI contained within the Gesicles and not a byproduct of indirect nucleic acid transfer. This work suggests that Gesicles can be considered as another efficient means to direct the phenotype of a cell of interest through the rapid and direct delivery of active protein to a chosen target cell.

Introduction

For phenotypic control of a cell, typical nucleic-acid delivery methods such as transfection of plasmids or viral transduction have a number of drawbacks including low-efficiency delivery, toxicity from the chosen transfection reagent, genomic disruption from integration, and delays in phenotypic response due to the need for transcription and/or translation post-delivery. Direct protein delivery to a cell has many advantages over nucleic acid delivery methods, including speed, efficiency, and tight control of timing and dosage. Currently, direct protein delivery is performed using a number of methods including transfection by either making a complex of the protein with a lipid, polymer, or peptide-based reagent; electroporation; or direct addition by including a cell penetrating peptide (CPP) on the protein of interest. These methods rely on the production of recombinant protein using *E. coli* or other standard methods, which can cause problems with solubility, yield, protein folding, and post-translational modifications. Many or all of these factors are important because they directly relate to the activity of the protein to be transfected. In contrast to biochemical methods for phenotypic control, with direct delivery of a protein, the protein's activity is the most important factor required to observe a phenotypic effect in the target cell. Direct delivery of active proteins using protein-carrying Gesicles is a superior method in that it does not require time for prior protein production and purification and does not suffer from the drawbacks of the previous delivery methods. The protein to be delivered is produced in mammalian cells and packaged simultaneously, thereby maintaining as much native activity as possible. Gesicles are capable of highly efficient delivery, and effects on a cell can be observed in less than one hour post-delivery. This work describes a few of the many applications amenable to this approach.

Conclusions

- Gesicles provide a highly efficient means of producing and directly delivering an active protein to any target cell
- Active proteins can be delivered to membranes, cytoplasm, or other subcellular locations such as nuclei
- Gesicles offer tight control over protein dose and time of delivery -which is not possible by delivery of nucleic acids
- Protein is easily expressed and packaged in mammalian cells in one step. No recombinant protein expression in *E. coli*, etc. is required.
- Gesicles facilitate a broader application of ecotropic pseudotyped lentivirus, providing safer application of lentiviral technology in a wide variety of cell types, including primary cells.
- Gesicles also allow functional testing of a GPCR in under 4 hours from time of delivery to completion of the functional assay.
- Gesicles successfully deliver proteins for genome editing

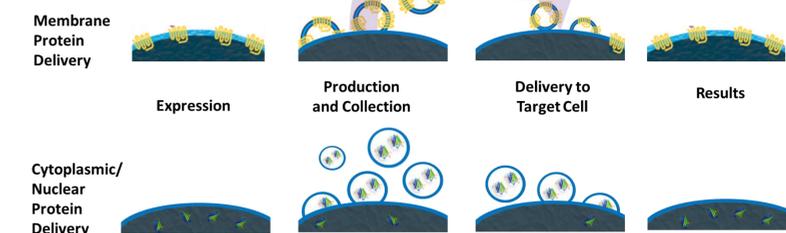
References

1. Mangeot, P-E *et al.* (2011) Mol. Ther. 19:1656-1666

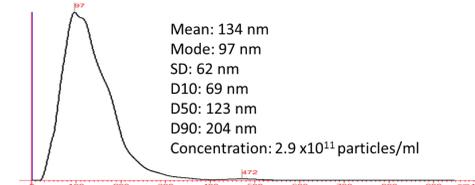
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1 Gesicle Production and Characterization

A. Gesicle Based Delivery Process



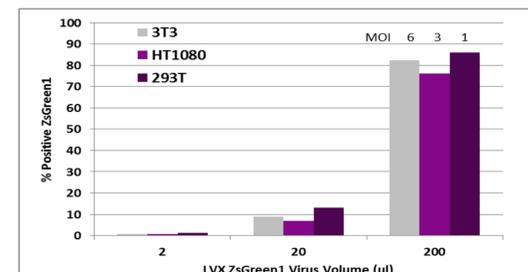
B. Particle Characterization



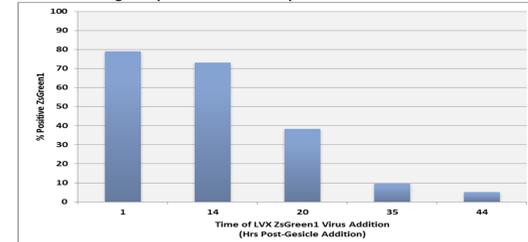
Gesicle Production, delivery, and characterization. Panel A. Schematic showing the process of Gesicle production and delivery. Cells are co-transfected with plasmids expressing VSV-G and the protein of interest. Gesicles are formed and released into the media where they are collected and concentrated. Protein delivery occurs when the Gesicles are placed in contact with the target cell. Panel B. Nanoparticle tracking analysis was used to characterize an mCAT1 Gesicle preparation. A number size distribution was generated using a NanoSight instrument on a diluted suspension of mCAT1 Gesicles. A mean particle size of 134nm was observed with a concentration of ~3x10¹¹ particles/ml.

2 Gesicles Deliver Functional Quantities of Viral Receptor Rapidly and Transiently to Target Cells

A. Comparison of mCAT1 Gesicle-treated Human Cell Lines to NIH3T3 Cells



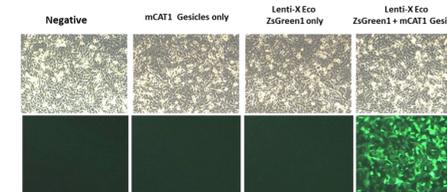
B. Time Course Demonstrating Lifespan of mCAT1 Receptor On Cell Membrane



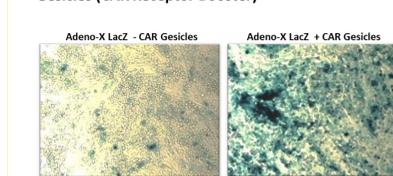
Efficient, rapid, and transient delivery of viral receptors to target cells. Panel A. Human cells treated with mCAT1 Gesicles exhibit transduction efficiency similar to 3T3 cells. 3T3, HT1080 and 293T cells were plated 24 hr hours prior to transduction. mCAT1 Gesicles (10 µl) were added to HT1080 and 293T cultures and incubated for 2 hr. Ecotropic Lenti-X ZsGreen1 lentivirus was then added to all cultures. Cells were assayed for fluorescence by FACS 48 hr post transduction. Panel B. Time course of receptor duration on target cells. HT1080 cells were treated with mCAT1 Gesicles, incubated for 2 hr, then transduced with ecotropic Lenti-X ZsGreen1 lentivirus at the times indicated. Cells were assayed for fluorescence by FACS flow cytometry 48 hr post transduction

3 Gesicles Broaden the Application of Multiple Virus Pseudotypes

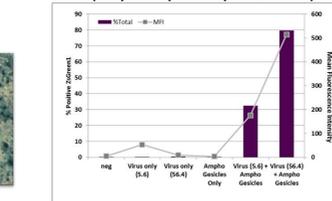
A. Human Cells Transduced using Ecotropic Gesicles (Ecotropic Receptor Booster)



B. CHO-K1 Cells Transduced Using CAR Gesicles (CAR Receptor Booster)



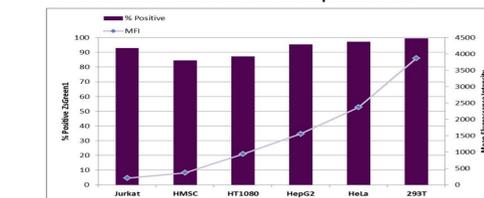
C. CHO-K1 Cells Transduced Using Amphotropic Gesicles (Amphotropic Receptor Booster)



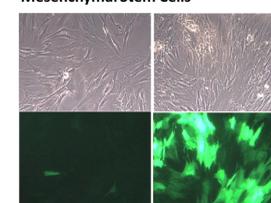
Use of Gesicles to broaden viral applications. Panel A. Transduction of human cells with ecotropic lentivirus. HT1080 cells were plated 24 hr prior to transduction. 10 µl mCAT1 Gesicles (Ecotropic Receptor Booster) were added, the cells were incubated for 2 hr, and then the medium was exchanged for fresh medium containing ecotropic Lenti-X™ ZsGreen1 virus (MOI=15). Cells were imaged using white light (top) and by fluorescence microscopy (bottom) 48 hr post transduction. Panel B. CAR Gesicles facilitate transduction of CHO-K1 cells with adenovirus. CHO-K1 cells were plated 24 hr prior to transduction. 10 µl CAR Gesicles (CAR Receptor Booster) were added, the cells were incubated for 2 hr, and then the medium was exchanged for fresh medium containing Adeno-X™ LacZ virus (MOI=34). Cells were assayed for β-Gal activity 72 hr post transduction. Panel C. Amphotropic Gesicles facilitate transduction of CHO-K1 cells with amphotropic retrovirus. CHO-K1 cells were plated 24 hr prior to transduction. 10 µl amphotropic Gesicles (Amphotropic Receptor Booster) were added, the cells were incubated for 2 hr, and then the medium was exchanged for fresh medium containing amphotropic retrovirus expressing ZsGreen1 (MOIs=5.6 & 56) for 24 hr. Cells were analyzed for fluorescence by FACS analysis 48 hr post transduction.

4 Gesicles Deliver Functional Viral Receptors to Primary Cells and Cell Lines

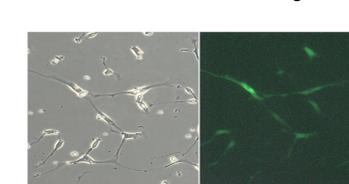
A. Transduction of Different Human Cell Lines with Ecotropic Lentivirus



B. Transduction of Primary Human Mesenchymal Stem Cells



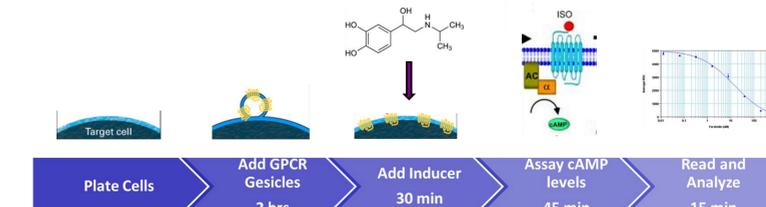
C. Transduction of Human Neural Progenitors



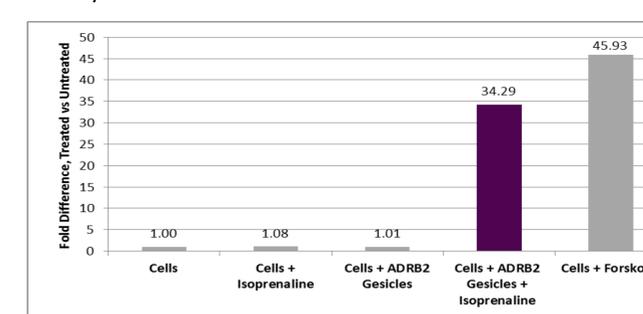
Use of Gesicles to transduce primary cells and cell lines. Panel A. Transduction of different human cell lines with ecotropic virus. Target cells were plated 24 hr prior to transduction. 10 µl mCAT1 Gesicles (Ecotropic Receptor Booster) were added, the cells were incubated for 2 hr, and then the medium was exchanged for fresh medium containing ecotropic Lenti-X ZsGreen1 lentivirus. Each cell line was assayed for Lenti-X ZsGreen1 expression by FACS 48 hr post transduction. Panel B. The CAR Receptor Booster facilitates transduction of primary human mesenchymal stem cells (hMSC) with adenovirus. hMSCs were plated 24 hr prior to transduction. 10 µl CAR Gesicles (CAR Receptor Booster) were added, the cells were incubated for 2 hr, and then the medium was exchanged for fresh DMEM containing Adeno-X ZsGreen1 virus (MOI~190) and incubated for 72 hr. Cells were assayed for fluorescence activity 24 hr post transduction. Panel C. Transduction of normal human neural progenitor cells with ecotropic virus. Neural progenitor cells were differentiated for 12 days and treated with mCAT1 Gesicles (Ecotropic Receptor Booster) and ecotropic Lenti-X ZsGreen1 lentivirus as in Panel A.

5 Direct Delivery and Functional Characterization of a GPCR in Under 4 Hours

A. Timeline of GPCR Production, Delivery and Signaling



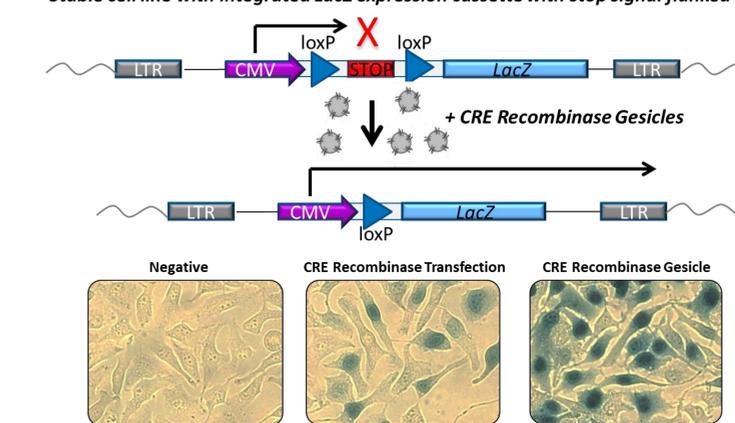
B. Direct Delivery of ADRB2 GPCR in CHO-K1 Cells



Use of Gesicles to deliver the ADRB2 receptor and assay for GPCR activity in less than 4 hr. Panel A. Workflow for ADRB2 receptor delivery via Gesicles, and the downstream assay for receptor activity. After cells were plated, the entire experiment took less than 4 hr from Gesicle addition to functional characterization and analysis. Panel B. CHO-K1 cells were plated at 1x10⁵ cells/well in 96-well format. 24 hr after plating, cells were treated with 1 µl GPCR (ADRB2) Gesicles (~3x10⁸ particles) for 2 hr in the presence of polybrene (4 µg/ml). Isoprenaline (10µm) was added to Gesicle treated cells and incubated for 30 min. Cells were assayed for cAMP levels immediately thereafter.

6 Simplified Genome Modification Using CRE Recombinase Gesicles

Stable cell line with integrated LacZ expression cassette with stop signal flanked by loxP



Genome modification with CRE recombinase Gesicles. A rhabdomyosarcoma cell line harboring an integrated, loxP-conditional LacZ expression cassette was either transfected for 6 hrs with a plasmid expressing CRE recombinase or treated with CRE recombinase-containing Gesicles for 2 hrs. Twenty-four hours after treatment, cells were stained for LacZ expression.