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Lysosomal Delivery of Bioactive Proteins to Living Human Cells via Engineered Exosomes

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Lysosomal Delivery of Bioactive Proteins to Living Human Cells via Engineered Exosomes

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DISCLOSURE

This experiments of this thesis was performed in parallel with Mai Anh Do. Therefore, many figures and data look similar, although they were individually prepared and analyzed. Figures, and figure legends were prepared individually. The experiments for the following figures were performed by:

Figure 1 prepared by Dr. Biao Lu.
Figures 2, 4, 8 & 9 performed by Mai Anh Do.
Figures 3, 5, 6, 7 & 10 performed by Daniel Levy

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ABSTRACT

Exosomes are naturally secreted nanovesicles derived from mammalian cells that are used for intercellular communication *in vivo*. As a result, they can potentially be used for intracellular delivery of therapeutics for disease treatment. We have developed an exosome pseudotyping approach using vesicular stomatitis virus glycoprotein (VSVG) to produce protein chimeras that optimize production of modified exosomes containing protein therapeutics and facilitate effective delivery to their target cells. To the VSVG transmembrane scaffold, we have fused both fluorescent and luminescent reporters for exosome tracking/visualization and quantification of activity respectively. Through our design, we have shown the biogenesis of VSVG modified exosomes from transfected producer cells through fluorescence imaging and the production of a VSVG-based stable cell line. In addition, we have characterized isolated engineered exosomes and shown that they exhibited the correct size, distribution, and molecular markers, while retaining the bioactivity of their protein cargo. Furthermore, we show that our engineered exosomes and their protein cargo are internalized by multiple cell lines into the endosomal and lysosomal compartments of those cells. Lastly, these modified exosomes can confer their bioactive cargo, either a luminescent reporter or puromycin resistance into these target cells. In summary, this study presents a novel approach to exosome engineering to enhance therapeutic protein loading and delivery, and more importantly, shows the delivery of modified exosomes to intracellular lysosomal compartments. This aspect leads to the assumption that in future studies, these engineered exosomes can be used as a vehicle for delivery of therapeutic proteins for treatment of lysosomal storage diseases.

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INTRODUCTION

1.1 Current Technologies and Motivation

In the past, small-molecule drugs have dominated the pharmacological market around the world. However, as we exhausted the combinations of chemical compounds that can effectively treat disease, it became imperative that more advanced drugs were developed, which lead to the emergence of protein therapeutics [1]. Protein drugs offer numerous advantages over their small-drug counterparts, mostly stemming from their increased specificity to their drug target and thus, reduced off-target effects [2]. Although they offer great promise, there are still several limitations of protein therapeutics, highlighted by our inability to reach intracellular targets via current delivery methods [3, 4]. Included in protein drug limitations are their susceptibility to degradation *in vivo* and inability to cross the blood-brain barrier in the case of neurological diseases [5].

In an attempt to remedy some of these limitations such as delivery of therapeutics to intracellular targets, many nanoparticle-protein drug conjugates have been investigated such as silica or gold nanoparticles [5, 6]. However, as these nanomaterials are foreign to the body, they can potentially cause a robust immune response, limiting their therapeutic use *in vivo* [7, 8]. Another limitation of foreign nanomaterials is the lack of degradability that many of these materials hold, leading to concerns about accumulation of foreign substances and potential for toxicity [8]. By themselves, gold or silica nanoparticle-protein drug conjugates also lack the ability to target specific cell types, which is often desired in disease treatment and can lead to higher dosages being utilized to gain a therapeutic response. To remedy these limitations, we present a solution in the use of exosomes as a nano-carrier of therapeutic proteins for use in intracellular drug delivery [9, 10].

1.2 Background

Exosomes are lipid based nanovesicles that shuttle proteins, DNA, RNA, and other lipids between cells [11-13]. They are roughly 50-150 nanometers in diameter and are formed within the endosomal compartments in mammalian cells where they are known as multiple vesicular bodies (MVB) before being released upon endosomal fusion with the cellular membrane, thus becoming an exosome (**Fig 1C**) [11, 12, 14, 15]. When the endosome invaginates to form MVBs, cytosolic proteins, DNA, and RNA are enclosed within the MVBs, which then exist within exosomes upon MVB secretion [11]. In mammalian cells, exosomes are secreted by nearly all cell types and as a result, are used in intercellular communication via the transport of proteins, DNA, and RNA [16, 17]. Exosomes deliver their cargo and enter cells via the endocytic pathway using interactions between the exosomal and cellular transmembrane proteins; as exosomes are naturally used as a transport vehicle in cellular communication, it is hypothesized that they can be engineered for use in the delivery of desired therapeutic cargo [18, 19]. Because exosomes are naturally occurring nanovesicles *in vivo*, they can circumvent many of the limitations of synthetic nanoparticles for protein drug delivery such as a lack of immune response, ability to cross the blood-brain barrier, lack of toxicity, and the ability to attach ligands for targeted cell delivery [5, 9]. As an example, via electroporation techniques, DNA, RNA and small molecule drugs can be loaded into isolated exosomes for increased small-molecule drug delivery or gene therapy techniques [9].

Previously, our group has demonstrated that the naturally occurring exosome surface protein, CD63 could be utilized to shuttle desired proteins into exosomal compartments by creating a chimeric CD63 scaffold-based protein chimera with a reporter protein attached to either

the N or C-terminus of CD63 [20]. Other naturally occurring exosome surface proteins have also been used for protein loading such as CD81 or CD9 [21]. However, to optimize loading of the protein, and uptake of the modified exosomes by various cell lines, we will be using a pseudotyping approach.

Pseudotyping is a method to repackage viruses with new viral envelope proteins [22-24]. This process is typically used to alter and ideally optimize the infectivity of the original virus [25, 26]. One of the most commonly used proteins in pseudotyping is the G glycoprotein of vesicular stomatitis virus (VSVG), due to its high infectivity to a large range of cell types [27]. In addition to this, the VSVG envelope is generated by inward budding of the cell membrane and endosomal compartments, which mimics the mechanism of natural exosome biogenesis [28]. Due to the effective loading of proteins into exosomes and high level of infectivity, rather than using a naturally occurring exosomal protein such as CD63, we will be using VSVG to create desired protein chimeras loaded into, now modified exosomes for therapeutic usage as a proof-of-concept.

The proteins that we have attached to the VSVG exosome transmembrane scaffold are either a green or red fluorescent protein, and either *Gaussia* luciferase or a puromycin resistance protein. The *Gaussia* luciferase protein, a luminescent reporter allows us to quantify the activity of our engineered exosomes, while the puromycin resistance protein allows us to generate a VSVG based stable cell line for continuous production of modified exosomes which can confer puromycin resistance to target cells upon uptake [29, 30]. The fluorescent proteins allow us to track the biogenesis, and uptake of the engineered exosomes [30].

1.3 Project Goals

Ultimately, the goal of this project is a proof-of-concept that exosomes can be used as an effective delivery vehicle for bioactive proteins in disease treatment. To achieve this, we first needed to show that VSVG could effectively shuttle and anchor our bioactive protein chimeras into extracellularly secreted exosomes. Thus, we needed to track the biogenesis and bioactivity of our modified exosomes within transfected producer cells. Secondly, to achieve our goal, we needed to characterize the isolated exosomes, showing that they had the appropriate size, protein cargo activity, and surface markers that would be expected/necessary. Lastly, we wanted to demonstrate that the VSVG-modified exosomes could be effectively taken up by various mammalian cell lines via the VSVG surface protein, and cellular surface proteins. In addition, the modified exosomes needed to deliver the bioactive protein chimeras to the correct cellular compartments within these target cells. From these three achievements, we believe that will be able to replace our reporter cargo with therapeutic proteins in the future, allowing us to develop an effective delivery system for disease treatment.

MATERIALS AND METHODS

2.1 Design and Construction of Expression Vectors for VSVG Fusion Proteins Chimeric protein constructs were configured from the 5'→3' end with the following components: a cytomegalovirus promoter, the VSVG signal peptide and full length VSVG, which consists of the extracellular portion, juxtamembrane, proximal, transmembrane, and cytosolic regions of VSVG. Following the full length VSVG, an in-frame insertion of either red fluorescent protein (RFP) or green fluorescent protein (GFP) is attached, followed by either *Gaussia* luciferase (Gluc) or a puromycin resistance gene (puro) (**Fig 1A**). At the 3' end, a polyadenylation site was added to finalize the protein construct. The gene expression vectors for the chimeric protein constructs were synthesized by gene synthesis techniques by Genscript (Piscataway Township, NJ, USA).

2.2 Cell Culture Human embryonic kidney cells (HEK 293) were purchased from Alstem (Richmond, CA, USA). Human hepatocellular carcinoma cells (HEPG2), human glioblastoma cells (U87), and mouse adipose tissue fibroblast cells (L929) were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco; MA, USA), with 10% Fetal Bovine Serum (FBS) (GE Healthcare Life Sciences; Issaquah, WA, USA), and 1% Penicillin Streptomycin (Pen Strep) (Gibco; MA, USA). At ~80% confluence, cells were treated with 0.25% trypsin-ethylenediamnietraacetic acid (Trypsin-EDTA) (Gibco; MA, USA) for cell dissociation and passaged. All cells were incubated at 37°C and 5% CO_2.

2.3 Transient Transfection At ~70% confluence, in six-well plates, HEK 293 cells were transfected by a solution containing plasmid DNA (1.3-2 μg/well), PEI transfection reagent (10 μL), and Opti-Mem (200 μL) (Gibco; MA, USA).

2.4 Cell Microscopy Images of live cells were using either fluorescence (Olympus; Waltham, MA, USA) or confocal microscopy (Leica; Buffalo Grove, IL, USA). The software used for image capturing was either cellSens Standard (Olympus; Waltham, MA, USA) or Leica Application Suite X (Leica; Buffalo Grove, IL, USA) for fluorescence and confocal microscopy respectively. Adobe Photoshop CS was used for the overlay, cropping, brightness, and contrast adjustment of images.

2.5 Isolation of Exosomes HEK 293 Cells were cultured to confluence in DMEM, 10% FBS, and 1% Pen Strep in 145 mm tissue culture dishes. They were transiently transfected at ~70% confluence with a solution containing 20 μg plasmid, 100 μL PEI transfection reagent, and 2 mL Opti-Mem. After 24 hours at ~80-90% confluence, cell media was changed to serum free media UltraCulture (Lonza; Portsmouth, NH, USA) for 48 hours. The conditioned media was then collected and centrifuged at 1500g for 10 minutes and filtered through a 0.2 μm filter. Exosomes were then isolated from the collected filtered UltraCulture media using the ExoQuick-TC (System Biosciences; Palo Alto, CA, USA) exosome isolation reagent via the manufacturer's protocol, which entails an incubation at 4°C overnight, before centrifugation at 3000g for 1.5 hours. The supernatant was then removed, and isolated exosomes were re-suspended in PBS (Teknova; Hollister, CA, USA). The re-suspended exosome's protein concentration was measured using NanoDrop Lite (Thermo Fisher Scientific; Fremont, CA, USA).

2.6 Luciferase Assay Transfected HEK 293 cells were lysed with 500 µL of 1x reporter lysis buffer (Promega; Sunnyvale, CA, USA) in 6 well plates, and the cell lysate was collected. 20-30 µLof either cell lysate or isolated exosomes were added to a 96-well white bottom plate (Corning; Tewsbury, MA, USA). 100 µL of coelenterazine *Gaussia* Luciferase substrate was added to the wells and luminescence was recorded using an infinite m200PRO (Tecan; San Jose, CA, USA) plate reader and icontrol 1.8 software (Tecan; San Jose, CA, USA).

2.7 ELISA A standard curve for CD63 was established using an ExoELISA-ULTRA protein standard (System Biosciences; Palo Alto, CA, USA). 50 µg of isolated sample exosomes were diluted in a volume of 120 µL of Coating Buffer (System Biosciences; Palo Alto, CA, USA). 50 µL of CD63 primary antibody was added to each well at a 1:100 dilution in Blocking Buffer (System Biosciences; Palo Alto, CA, USA) and incubated. 50 µL of secondary antibody at a 1:5000 dilution was then added and incubated. 50 µL of Super-Sensitive TMB ELISA substrate (System Biosciences; Palo Alto, CA, USA) was then added. 50 µL of Stop Buffer was introduced and quantification was performed using an infinite m200PRO plate reader and icontrol 1.8 software at 450 nm absorbance.

2.8 Nanoparticle Tracking Analysis (NTA) Exosomes were isolated and resuspended as previously outlined. 150 µL of isolated exosome sample was sent to Particle Characterization Laboratories Inc. (Novato, CA, USA) for NTA analysis per the company's protocol.

2.9 Western Blot Exosomes were isolated as previously outlined. 60 µg of each isolated exosome sample were loaded onto a 4-12% ExpressPlus PAGE gel (GenScript; Piscataway, NJ, USA) with Prestained Protein MW Marker #26612 (ThermoFisher Scientific; Fremont, CA, USA) in their respective wells. Gel Electrophoresis was run using a Mini-PROTEAN Tetra Cell (BioRad; Hercules, CA, USA) and Powerpac power source (BioRad; Hercules, CA, USA). Proteins were transferred to a Amersham Hybond P 0.2 µm PVDF membrane (GE Healthcare Life Sciences; Issaquah, WA, USA) with a semi- dry transfer cell (Bio-Rad). The membrane was then incubated with either primary mouse antibody CD81 mAb (Santa Cruz Biotechnology; Santa Cruz, CA, USA) or CD63 (Santa Cruz Biotechnology; Santa Cruz, CA, USA). The membrane was then incubated with anti-mouse HRP-conjugated secondary antibody (ThermoFisher Scientific; Fremont, CA, USA). Blots were visualized with Pierce ECL Western Blotting Substrate (ThermoFisher Scientific; Fremont, CA, USA) on an ImageQuant LAS 500 (GE Healthcare Life Sciences; Issaquah, WA, USA).

2.10 Dot Blot Exosomes were isolated as previously discussed. 250 µg of isolated exosomes were sent to System Biosciences (Palo Alto, CA, USA) for their ExoCheck service and performed per the company's protocol.

2.11 Exosome Uptake Assay HEK293, U87, HEPG2, and L929 were seeded in 96-well plates. At ~40% confluence, the conditioned media was replaced with 100 µl of UltraCulture serum-free media containing 0.3 µg/µL of isolated exosomes per well. Cells were then imaged at 20x magnification after 6, 24, 48, and 72 hours with either fluorescence or confocal microscopy, as previously outlined.

2.12 Stable Cell Line Development HEK 293 cells were treated with 5 µg/ml puromycin dihydrochloride (ThermoFisher Scientific; Fremont, CA, USA) in DMEM, 10% FBS, and 1%

Pen Strep, 24 hours post-transfection in a 6-well plate at ~80% confluency. After drug resistant cells grew to confluency, the cells were passaged onto 100 mm plates where they were cultured for 8 weeks. Puromycin resistant cells were viewed under fluorescence microscopy as previously outlined to ensure complete GFP or RFP expression in addition to puromycin resistance.

2.13 HOESCHT Stain Cells were cultured for 24, 48, or 72 hours on glass bottom plates. At the desired time, cell culture medium was removed and replaced with 1:1000 Hoescht 33352 (ThermoFisher Scientific; Fremont, CA, USA) in PBS and incubated at 37°C for 10 minutes. The Hoescht solution was removed and cells were washed with PBS before imaging via confocal microscopy as previously described.

2.14 Lysosomal Stain 1 mM LysoTracker Red DND-99 (Invitrogen; Carlsbad, CA, USA) was diluted in cell culture medium to a concentration of 75 nM. Cells were cultured for 48 hours under previously described conditions and culture medium was replaced with the LysoTracker-cell media solution and incubated at 37°C for 30 minutes. The LysoTracker solution was then replaced with fresh cell culture medium or PBS before imaging via confocal microscopy.

2.15 Endosomal Stain Cells were cultured for 24 hours on a 500 µl glass bottom plate before 5-7 µl of CellLight Early Endosomes-RFP, BacMam 2.0 (Invitrogen; Carlsbad, CA, USA) or CellLight Late Endosomes-RFP, BacMam 2.0 (Invitrogen; Carlsbad, CA, USA) was added to the cell culture medium. Cells were then cultured for 16 hours at 37°C before imaging via confocal microscopy.

2.16 Cell Counting Cultured cells were trypsinized and collected before being centrifuged at 1500 rpm for 5 min. Cells were then resuspended and mixed with Trypan Blue 0.4% Solution (MP Biomedicals; Solon, Ohio, USA) before being counted on a C-Chip Disposable Hemocytometers (INCYTO; Convington, GA, USA) and Vista Vision Light Microscope (VWR; Visalia, CA, USA).

RESULTS

3.1 Results

1. *Biogenesis of Exosomes*

Exosomes offer a unique vehicle for intracellular delivery of bioactive cargos to living cells. Being a naturally cell secreted vesicle used in a variety of motifs such as intercellular communication and transport, exosomes can freely travel throughout the body without the threat of an immune response [12]. To harness this property of exosomes, we have developed a chimeric protein via a gene sequence composed of a promoter (CMV), fluorescent reporters GFP or RFP, and either a puromycin resistance gene (Puro) or secretory a *Gaussia* luciferase (Gluc) reporter all anchored on the exosomal membrane by a vesicular stomatitis viral glycoprotein (VSVG) (**Fig 1A, 1B**).

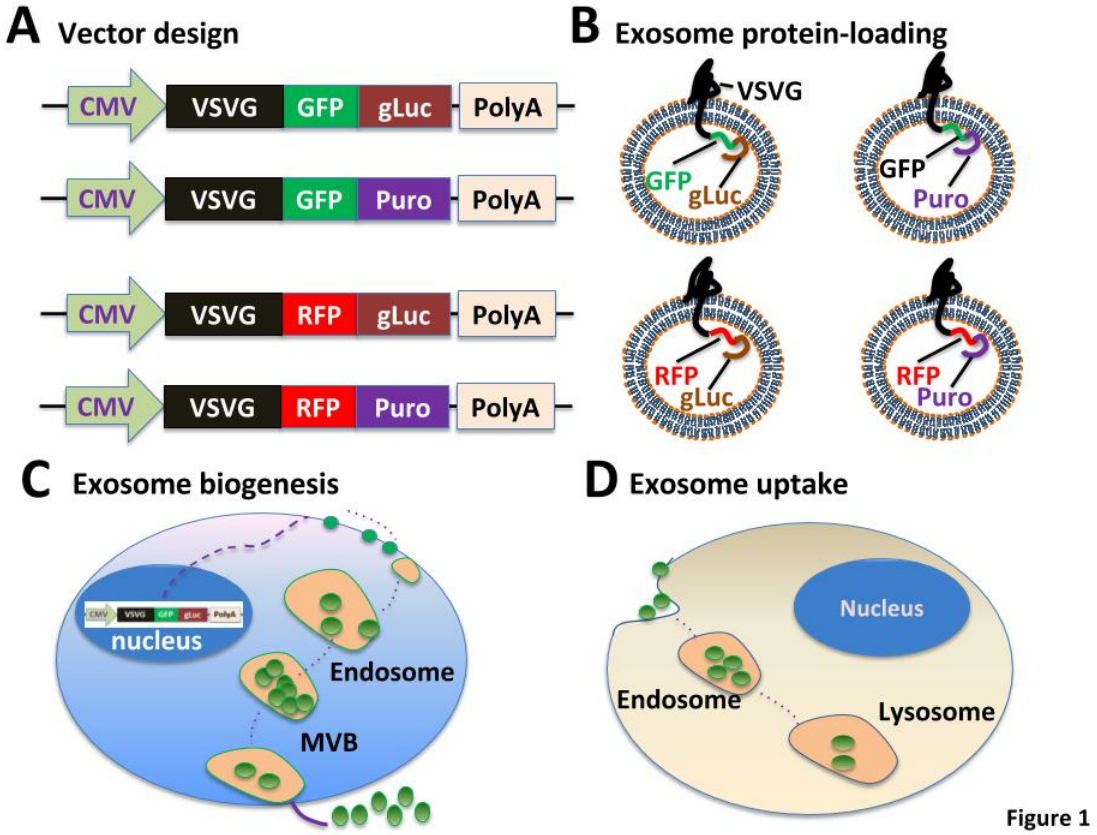

Figure 1. Exosome pseudotyping design

Notes: (**A**) Design of VSVG constructs. For each construct, VSVG is fused with either a GFP or RFP reporter, in addition to either a *Gaussia* luciferase (gLuc) or Puromycin (Puro) resistance gene. (**B**) Illustration of the protein-loaded exosomes based on the four gene constructs. The glycoprotein VSVG (black), reporter GFP (green) or RFP (red), and protein cargo gLuc (gold) or Puro (purple) are shown. (**C**) Model illustrating the biogenesis of modified exosomes in mammalian cells. Upon protein production, VSVG migrates to the plasma membrane via its signaling peptide where inward budding generates early endosomes. A second inward budding within the late endosome forms multivesiclular bodies (MVB). Fusion of the late endosome containing MVBs with the plasma membrane results in extracellular release of exosomes. (**D**) Model illustrating the uptake of modified exosomes in mammalian cells. Modified exosomes are internalized via endocytosis by target cells, resulting in endosome formation, and ultimately either fuse with the lysosomes or cellular membrane for extracellular re-secretion.

Upon translation of the designed gene sequence, the chimeric protein is to the cell membrane by the VSVG signaling peptide where it is anchored by the VSVG protein itself. Invagination of the cell membrane results in endosome formation with the newly anchored chimeric proteins populating the endosomal membrane [15]. A second invagination of the endosomal compartment results in the formation of multiple vesicular bodies (MVB) which upon secretion, produce exosomes containing the designed chimeric VSVG transmembrane protein (**Fig 1C**) [21]. By transfecting our gene constructs into HEK293 cells, we tracked our chimeric protein throughout the exosome biogenesis pathway. After the first 6-24 hours, the GFP reporter can be observed at the outer cell membrane before progressing towards the 1st and 2nd invaginations after 48-72 hours and resulting in punctations throughout the cell (**Fig 2A-L**). These punctations show a stark difference when compared with cytosolic GFP, where a uniform expression is observed, and indicates that reporter GFP expression may be within exosomes (**Fig 2M-O**).

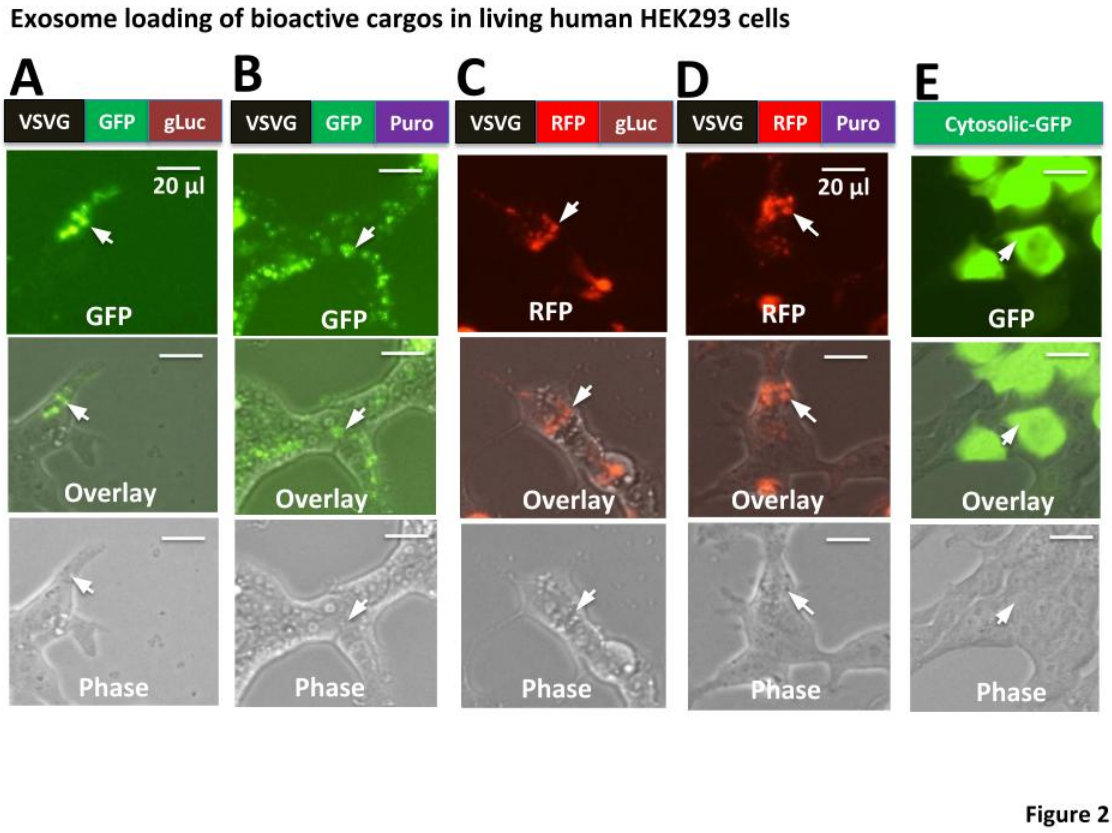

Figure 2

Figure 2. Intracellular tracking of exosome biogenesis
Notes: HEK293 cells were transfected with either each of our four VSVG-based plasmid constructs (**A-D**) or a positive control, EF1-G2F (**E**). Cells were observed by fluorescence microscopy after 24, 48, and 72 hours. VSVG-reporter modified exosomes aggregate into punctated groups after traveling to the cell membrane and invaginating after 48 hours (**A-D**) during biogenesis rather than producing a uniform cytosolic expression profile as seen in the positive control at 48 hours (**E**).

To confirm that our VSVG based chimeric proteins were indeed located in exosomal compartments within the HEK 293 cells, we co-transfected them with known exosomal marker-reporter constructs CD63 and XPACK (**Fig 3**). Co-localized expression of reporter proteins was observed within the intracellular exosomes, confirming our initial observations.

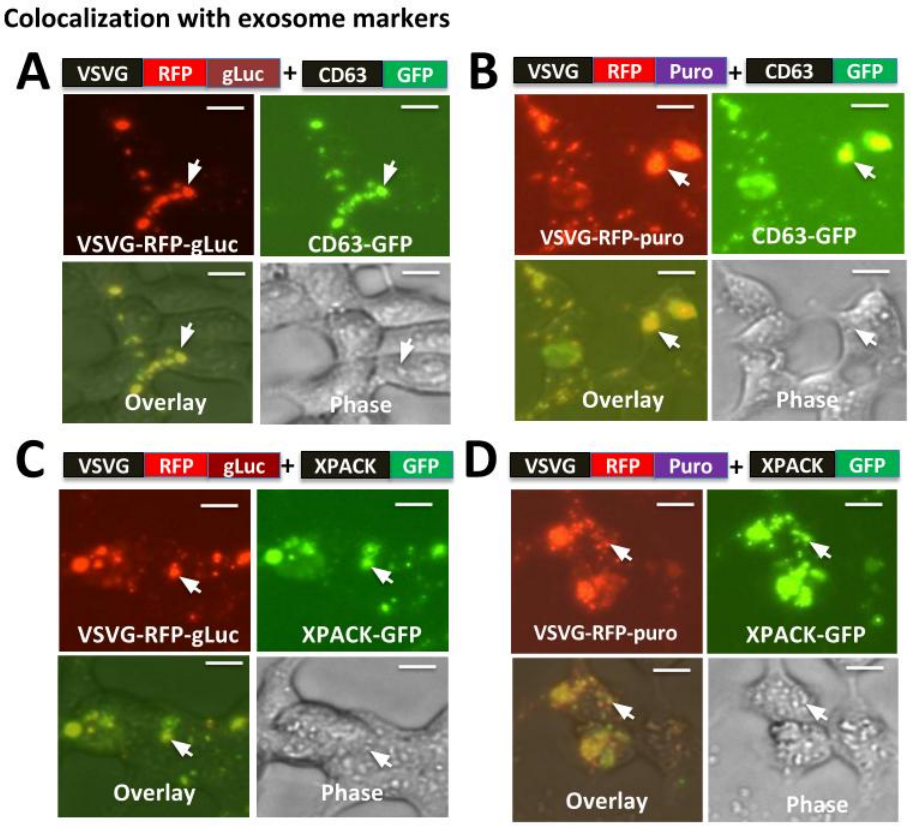

Figure 3

Figure 3. Colocalization of tracking molecules with exosome markers
Notes: HEK293 cells were cotransfected with our VSVG-RFP-gluc (**A,C**) or VSVG-RFP-puro (**B,D**) constructs and known exosomal markers: XPACK-GFP (**C,D)** or CD63-GFP (**A,B**). After 48 hours incubation, modified exosomes were colocalized into subcellular punctations as demonstrated by the yellow overlap within transfected cells. This confirms that our designed constructs produce protein chimeras that travel to the exosomes within producer cells. Cells were observed by fluorescence microscopy.

While the presence of our chimeric protein in exosomes of producer cells was confirmed via imagining, the activity of the bioactive cargos, either secretory Gluc or puromycin resistance within the exosomes were being assayed in parallel. Secretory *Gaussia* luciferase, attached to our GFP/RFP reporter and VSVG transmembrane anchor produces a luminescent signal and can be measured by a microplate reader. To quantitatively measure luminescence expression, a luciferase assay was run, where we observed a dramatic 70-90x increase in both our GFP and RFP transfected cell lysate over

untransfected, parental cell lysate (**Fig 4A**). This indicates that while attached to our VSVG anchoring protein, the bioactivity of the protein cargo is retained within the transfected cells. Separately, the transfected exosome producer cells with puromycin resistance were also assayed to determine cargo activity. By placing the producer cells in cell culture media containing puromycin (3-5 µg/mL) and comparing them visually to untransfected HEK 293 cells, we observed a difference in cell viability and survival (**Fig 4B**). This experiment further confirms that both our protein cargos remain active when inserted into exosomes via a VSVG transmembrane protein.

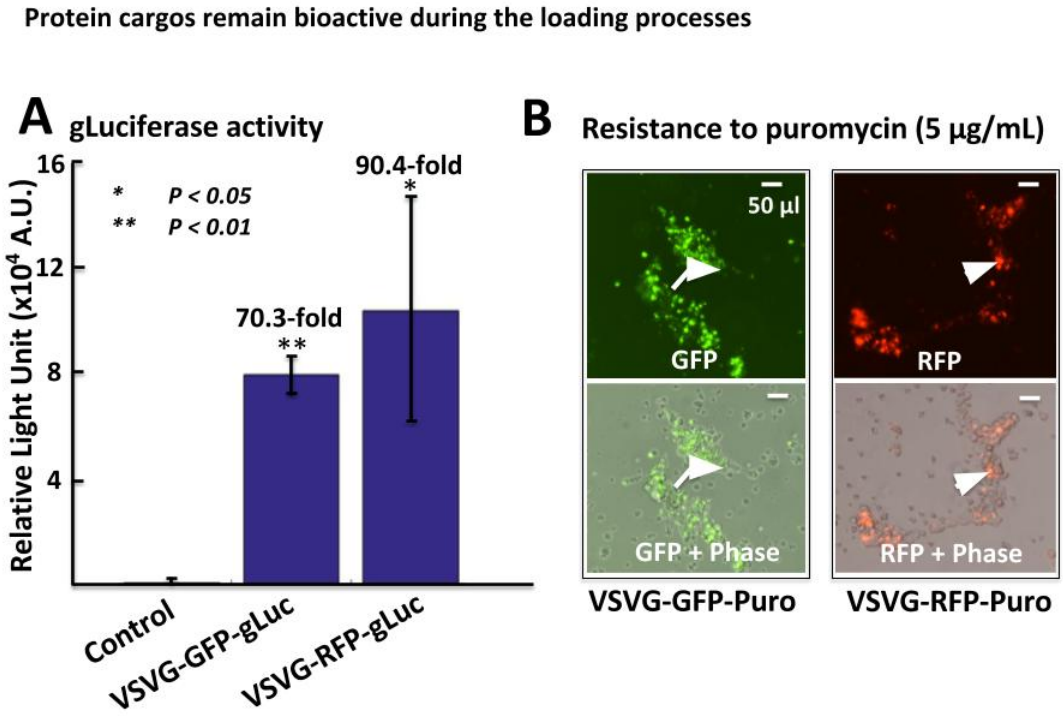

Figure 4. Engineered tracking markers are biologically active
Notes: (**A**) HEK293 cells were transfected with our VSVG-Gluc constructs, lysed at 48 hours, and combined with the luciferin substrate, where luminescence was recorded via plate reader. *Gaussia* Luciferase expression in VSVG-gLuc transfected cell lysate compared to mock transfection of parental HEK293 lysate as a negative control show that the protein cargo is biologically active at roughly a 70 and 90-fold increase for VSVG-GFP-Gluc and VSVG-RFP-Gluc respectively. Data is presented as relative light units from three samples (mean ± standard deviation, n =3). (**B**) At 48 hours post transfection, HEK293 cells previously transfected with VSVG-puro constructs began incubation with 5 µg/ml puromycin media and images were taken at 72 hours post puromycin treatement via fluorescence microscopy. The observed puromycin resistance in VSVG-puro transfected cells shows the biologically active protein cargo was correctly produced and expressed.

2. *Exosome Characterization*

After observing the biogenesis and activity of our engineered exosomes within producer cells, we isolated our engineered exosomes and further characterized their size distribution, concentration, surface markers, and protein cargo activity. An ELISA assay was run with an antibody against a known exosomal marker, CD63 to determine exosome concentration (exosome number/mg protein) of our isolated engineered exosomes compared with parental HEK 293 exosomes (**Fig 5A**). The exosome concentrations are similar between the isolated parental HEK 293 exosomes and that of all of our engineered exosome constructs. In addition to the ELISA, Nanoparticle Tracking Analysis (NTA) was performed comparing parental HEK 293 isolated exosomes and our modified exosomes to observe the particle size and distribution of samples. Once again, similar concentrations (exosome number/mL) are observed between the parental exosomes and modified ones (**Fig 5B**). The NTA also indicates that the particle size is similar between the control exosomes and our engineered nanovesicles (40-100 nm) with a single peak at the correct size being observed. These experiments indicated to us that our engineered exosomes retain their native size and distribution, despite the modifications and presence of a protein cargo.

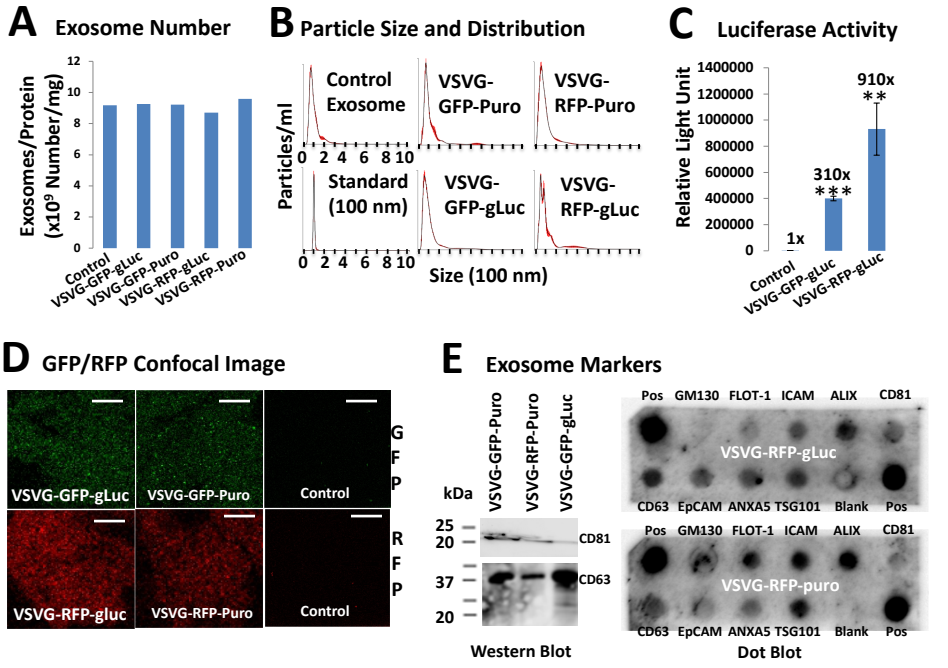

Figure 5

Figure 5. Characterization of exosomes released from producer cells

Notes: Exosomes were isolated from serum-free cell culture media of transfected producer cells with Exo-Quick TC (System Biosciences). (**A**) The total number of exosomes per mg protein of sample was recorded by running an ELISA against a known exosome tetraspanin protein, CD63 antibody. Exosome number per mg protein is similar between all VSVG constructs and the control, unmodified HEK 293 exosomes (~$9x10^9$ exosomes/mg protein). (**B**) Isolated exosomes were analyzed with NTA (Nanosight) indicating similar size distributions between VSVG-based constructs and unmodified HEK 293 exosomes (average size ~105-115 nm). (**C**) Luciferin was combined with isolated exosomes, and luminescence was recorded via plate reader. *Gaussia* Luciferase expression in the isolated exosomes shows a biologically active cargo with significantly higher activity levels of a 310 and 910-fold increase for VSVG-GFP-Gluc and VSVG-RFP-Gluc exosomes respectively. Data is presented as relative light units from three samples (mean ± standard deviation, n =3). (**D**) Isolated modified exosomes were visualized with confocal microscopy. (**E**) Western blot of isolated modified exosomes was performed with known exosome proteins CD63 and CD81 with sizes ~37 and ~22 kDA respectively. A dot blot of isolated modified exosomes from VSVG-RFP-puro and VSVG-RFP-Gluc was performed with known exosome proteins FLOT-1, ICAM, ALIX, CD81, CD63, EpCAM, ANXA5, TSG101 and a negative control protein GM130, showing the presence of correct exosomal molecular markers.

To detect the presence of the bioactive cargo within our modified exosomes, isolated exosomes were observed via confocal microscopy and compared with isolated parental HEK 293 exosomes. GFP and RFP expression was clearly observed in both the puromycin resistance and *Gaussia* luciferase modified exosomes but not in parental HEK 293 secreted exosomes (**Fig 5D**). To quantify the amount of cargo loaded within the isolated exosomes, a luciferase activity assay was run on the isolated modified exosomes containing the Gluc reporter. Luciferase activity was detected at a level of 310, and 910 times in the VSVG-GFP-gLuc and VSVG-RFP-gLuc modified exosomes respectively as compared to parental HEK 293 exosomes (**Fig 5C**). In conjunction, these two experiments indicated to us that the cargo remained active within the isolated engineered exosomes.

Lastly, to further verify that our bioactive cargo was within exosomes, a Dot Blot was performed with known exosomal markers such as CD63, EpCAM, ANXA5, FLOT-1, ICAM, TSG101, ALIX, and CD81, as well as against a negative control marker in GM130. It was observed that both our puromycin resistant and *Gaussia* luciferase containing exosomes had levels of the known exosomal makers, and not that of the negative control maker, GM130 (**Fig 5E**). Furthermore, western blots were performed to not only confirm the presence of known exosomal markers CD63 and CD81, but also determine if those markers were the correct protein size, indicating no change from our VSVG based modifications. A molecular weight of 37-50 kDa, and 20-25 kDa was observed for CD63 and CD81 respectively, which is confirmed from literature as the correct size (**Fig 5E**) [20]. As a whole, these characterization experiments indicate that our protein cargo is active within isolated exosomes, and offer no significant change to particle size, distribution, or expression of native surface proteins.

3. *Exosome Activity in Living Cells*

After characterization of our isolated engineered exosomes for size distribution, cargo bioactivity, and exosomal markers, we investigated the uptake of the engineered exosomes within various living cell lines *in vitro*. In our first uptake experiment, isolated exosomes expressing GFP were introduced with serum-free media into a culture of untransfected living cells and observed for 48 hours. It was noted that the modified exosomes containing GFP were successfully taken in by HEK293, HEPG2, U87, and L929 cells after 24-48 hours incubation time (**Fig 6 A-X**). The presence of our GFP reporter suggests that not only are the exosomes taken up by various cell types, but that the cargo is biologically active for at least 48 hours post-introduction.

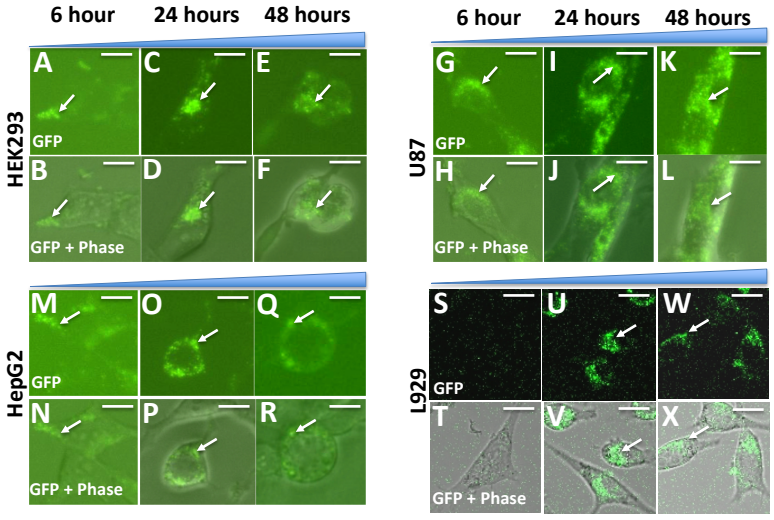

Figure 6

Figure 6. Uptake of protein-loaded exosomes by mammalian cells
Notes: Isolated VSVG-GFP-puro modified exosomes were introduced to HEK293, U87, L929, and HEPG2 cells in culture. After 6, 24 and 48 hours, intracellular localization of the punctated modified exosomes was observed, indicating uptake of the biologically active protein cargo into the target cell. It was hypthoesized that the modified exosomes entered the target cells via endocytosis. Cells were observed via fluorescence microscopy or confocal microscopy.

Next, a stable cell line of modified exosome producer cells was established with both our VSVG-GFP-puro and VSVG-RFP-puro gene constructs in media containing either 3 µg/mL or 5 µg/mL puromycin (**Fig 7A-H**). It has previously been noted by various studies that the cytotoxic nature of VSVG makes it impossible to establish a stable cell line, however, by using a lower concentration of puromycin in cell culture media in conjunction with the inherent robustness of HEK 293 cells, a stable cell line was established [9]. These stable cell lines were used to further ease our research in both the production and uptake of modified exosomes.

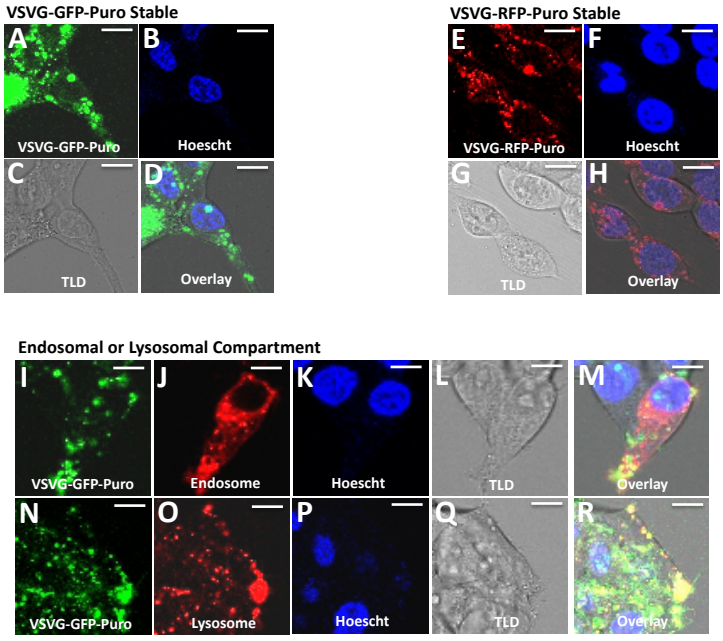

Figure 7

Figure 7. Intracellular localization of exosome biogenesis in a stable cell line
Notes: (**A-H**) HEK 293 cells were transfected with VSVG-based gene constructs containing a puromycin resistance gene sequence. Cell culture media was replaced with media containing 5 ug/ml purmoycin and surviving cells containing the puromycin resistance gene within their genome were grown for 10 weeks. Cells were observed with confocal microscopy. (**I-M**) The VSVG-GFP-Puro stable cell line was transfected with an RFP reporter targeting Rab5a, a biomarker associated with early endosomes. The RFP and GFP reporters were observed to be co-localized within similar cellular compartments via confocal microscopy 48 hours after transfection. (**N-R**) The VSVG-GFP-Puro stable cell line was stained with a red lysosomal dye, and it was observed that the GFP and RFP reporters were co-localized via confocal microscopy 48 hours after plating.

To confirm that the cellular compartment that our engineered chimeric VSVG proteins enter upon biogenesis is initially the endosomes followed by MVBs, our VSVG-GFP-puro stable cell line was transfected with a reporter RFP fused with an early endosomal localizing protein, Rab5a. It was observed that the GFP modified exosomes were co-localized in early endosomal compartments, thus confirming our initial assumption that exosomes are produced from inward budding of endosomes (**Fig 7I-M**). Separately, our VSVG-GFP-puro stable cell line was chemically stained with a red flurorescent dye localizing within lysosomal compartments and observed via confocal microscopy (**Fig 7N-R**). It was noted that our modified exosomes within the stable cell line co-localized with a significant fraction of dyed lysosomal compartments. This indicates that a portion of exosome producing endosomes merge into the lysosomal compartment, which is consistent with previous knowledge of endosomal pathways, where the other portion fuses with the cell membrane, secreting its contents [31].

In a separate line of experiments, exosomes from our VSVG-GFP-puro stable cell line were isolated and incubated with our VSVG-RFP-puro for 48 hours. It was observed over this time period that our GFP expressing exosomes not only were taken up by the stable cells, but also that the isolated GFP modified exosomes were co-localized within the exosome producing compartments of the stable producer cells, indicated by yellow

punctations (**Fig 8A-I**). To further confirm the previous experiment, our VSVG-GFP-puro and VSVG-RFP-puro stable cell lines were co-cultured for 48 hours. It was hypothesized that modified exosomes from both cell lines would be secreted into the surrounding media, and the GFP/RFP expressing exosomes would be taken up by the opposite stable producer cells. This hypothesis was confirmed by confocal images of the co-culture from 24-72 hours, which shows a co-localization of GFP and RFP within stable cells, seen as yellow punctations (**Fig 8J-R**).

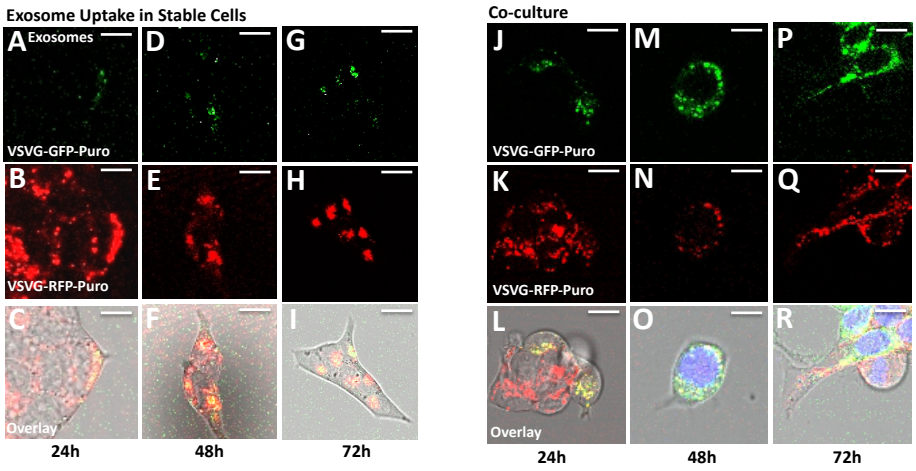

Figure 8

Figure 8. Cellular Uptake and Co-localization of Modified Exosomes
Notes: (**A-I**) Modified exosomes isolated from VSVG-GFP-Puro transfected cells were introduced to the VSVG-RFP-Puro stable cell line and observed for 72 hours. Upon uptake of the modified exosomes, it was observed via confocal microscopy that after 48 hours, the reporter GFP and RFP were co-localized within the stable cell line, indicating that they are within the same cellular compartment, which is presumably the endosomes. (**J-R**) The VSVG-GFP-Puro and VSVG-RFP-Puro stable cell lines were cultured together for 72 hours and observed via confocal microscopy. It was noted that modified exosomes were secreted into the surrounding media and uptaken by the surrounding stable cells. These secreted exosomes were co-localized within the intracellular compartments of the stable cells of the opposite reporter color, further confirming the uptake of modified into the same cellular compartment of stable producer cells.

Through the previous lines of experiments, it was demonstrated that modified exosomes were produced in the endosomal compartments, with a population of exosomes within the endosomes fusing with the lysosomal compartments. Furthermore, it was observed that when isolated modified exosomes were introduced to stable exosome producer cells, they were taken up and localized in the same compartments that produce the modified exosomes, presumably the endosomal compartments. Thus, it was hypothesized that the modified exosomes enter the endosomal and lysosomal compartments upon uptake by non-producer, target cells. To confirm this hypothesis, modified GFP expressing exosomes were incubated with parental HEK 293 cells, and subsequently co-transfected

with a Rab5a based RFP construct targeting the endosomal compartments. It was observed via confocal microscopy that the modified exosomes were localized within the endosomal compartments of non-modified HEK 293 (**Fig9A-D**) and U87 (**Fig9I-L**) cells.

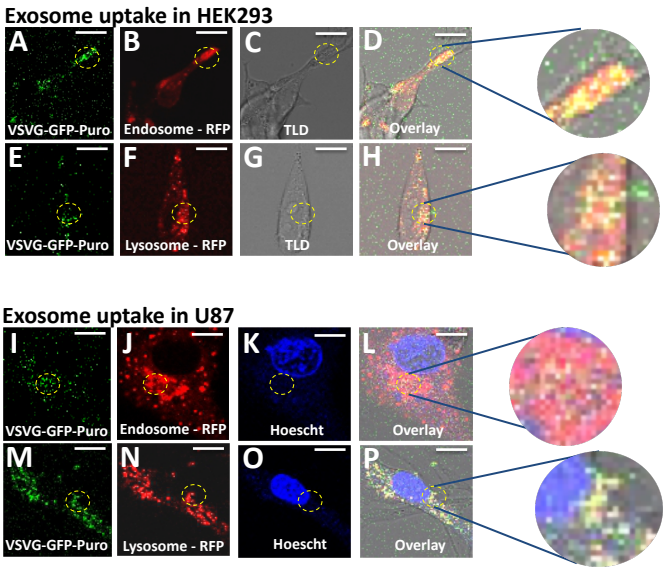

Figure 9

Figure 9. Cellular Compartmentalization of Exosomes upon Uptake
Notes: (**A-D**) Isolated VSVG-GFP-Puro exosomes were introduced to parental HEK293 cells in culture for 24 hours before being transfected with an endosomal Rab5a-RFP reporter. After an additional 24 hours, the cells were observed via confocal microscopy and the GFP and RFP reporters were seen to be co-localized within endosomal compartments. (**E-H**) Again, VSVG-GFP-Puro exosomes were introduced to HEK293 cells in culture for 48 hours before being stained with a red lysosomal dye. The cells were observed via confocal microscopy to have RFP and GFP reporters co-localized within the lysosomal compartments. (**I-P**) The same procedures were then performed with U87 cells, producing similar observations.

Concurrently, modified GFP expressing exosomes were introduced to either non-modified HEK 293 and U87 cells, however in this case, the cells were stained with a red fluorescent dye localized within lysosomal compartments. Once again, it was observed via confocal microscopy that the modified exosomes were localized within those lysosomal compartments in both HEK 293 (**Fig 9E-H**) and U87 (**Fig 9M-P**) cells.

Through the previous experiments, we have demonstrated that isolated modified exosomes enter and localize within endosomal and lysosomal compartments upon incubation with various cell lines. However, although GFP and RFP could be observed, we wish to further quantify the levels of bioactivity of the delivered proteins within these cells. To confirm the bioactivity, unmodified HEK 293 cells were incubated with VSVG-GFP-Gluc modified exosomes for 3-72 hours. At various time points (3, 8, 24, 48 and 72 hours), the HEK 293 cells were washed with PBS to remove modified exosomes not localized within the cells, and subsequently lysed for a luciferase activity assay. Through this time-course luciferase uptake experiment, it was observed that the unmodified HEK 293 cells incubated with modified exosomes presented increasing luciferase activity for 48 hours, before a slight dip in activity at 72 hours (**Fig 10A**). This dip in luciferase activity could be due to the degradation of the Gluc protein within the lysosomes.

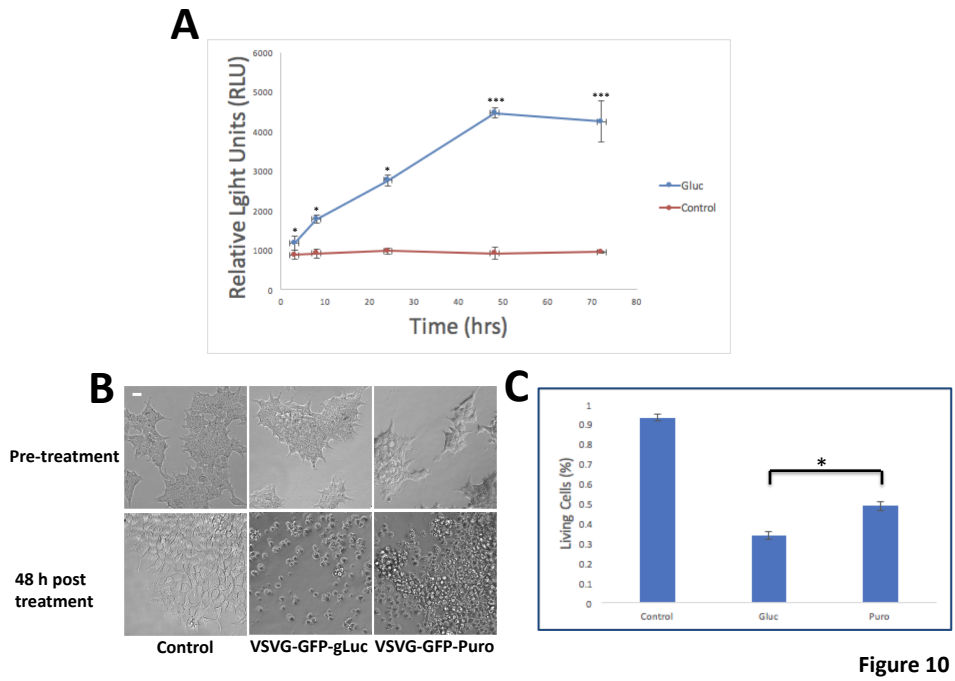

Figure 10. Bioactivity of Cargo upon Uptake of Modified Exosomes
Notes: (**A**) Isolated modified VSVG-GFP-Gluc exosomes were introduced to parental HEK293 cells and at time points: 3, 8, 24, 48 and 72 hours, the cells were washed with PBS to remove free floating modified exosomes and subsequently lysed. A luciferase assay was performed on the cell lysate with results showing an increase in luminescence over time, likely from uptake of the modified exosome and its cargo. At time points 3, 8, 24, 48, and 72 hours a 1.3, 1.9, 2.8, 4.9, and 4.4-fold increase in bioluminescence of cell lysates was observed respectively. Data is presented as relative light units from three samples (mean ± standard deviation, n =3). (**B**) Isolated VSVG-GFP-Puro exosomes, or VSVG-GFP-Gluc were added to parental HEK293 cells and incubated for 48 hours. After which, the media was replaced with 3 µg/mL puromycin cell culture media. Cells were observed via fluorescence microscopy for 48 hours post puromycin treatment. It was noted that cells treated with VSVG-GFP-Puro exosomes had a slight resistance to puromycin. (**C**) After images were taken, the treated cells were collected, stained with Trypan Blue and counted. It was noted that the cells treated with VSVG-GFP-Puro exosomes were ~49% alive as compared to the VSVG-GFP-gLuc negative control which was ~34%. Data is presented as percentage of viable cells from three samples (mean ± standard deviation, n =3).

To further confirm that the modified exosomes delivered bioactive proteins to human cells, VSVG-GFP-puro isolated exosomes were incubated with unmodified HEK 293 cells for 48 hours. After 48 hours, the HEK 293 cell culture media was replaced with media containing 3 µg/mL puromycin. The cells were then observed for 24, and 48 hours for loss of cell viability. Through both cell images, and a Trypan blue cell counting method, it was observed that the cells treated with VSVG-GFP-puro exosomes had greater cell viability than HEK 293 cells that were treated with exosomes that did not contain a puromycin resistance protein (~48% vs. ~34%) (**Fig 10B, C**). Thus, through the previous experiments, it can be deduced that not only do our modified exosomes enter the appropriate cellular compartments, but they also hold the ability to confer their bioactive cargo to target cells upon incubation.

3.2 Discussion

This research has significant implications as it pertains to the development of a novel *in vivo* drug delivery system. As previously mentioned, engineering exosomes can overcome many limitations of current protein drug delivery systems such as intracellular delivery, *in vivo* half-life, immunogenicity, and the crossing of the blood-brain barrier [5]. Furthermore, by using VSVG as an exosomal transmembrane anchor, we can optimize both loading and delivery of therapeutic proteins via exosome delivery [32]. Through our research, we have shown that exosomes can be used as an efficient bioactive carrier system to deliver desired protein therapeutics to various cell types.

We have demonstrated that we are able to direct biological cargo into exosomes using VSVG as a cellular transmembrane anchor. Upon secretion, these modified exosomes can then be collected, isolated, and purified from their producer cells. We have also shown that while the protein cargo within the modified exosomes is still biologically active, it has little effect on the size distribution and native transmembrane proteins of exosomes. Furthermore, it has been demonstrated that these modified exosomes can be taken up and deliver their biological cargo to various mammalian cell types. We have also shown that not only is the cargo still biologically active upon delivery to target cells, but also enters the endosomal and lysosomal compartments of the target cell.

Through the findings of this study, we have multiple avenues of future research to pursue for engineering exosomes as drug delivery vehicles. Because endogenous, non-modified exosomes carry DNA, RNA, and proteins between cells, and we have clearly shown that our modifications do not have an impact on exosome size distribution or molecular markers, it can be extrapolated that we can replace our reporters, GFP/RFP or *Gaussia* luciferase/puromycin resistance, with a similarly sized therapeutic protein for applications such as disease treatment or enzyme replacement therapies [14, 33, 34]. Also, because we have shown that our engineered exosomes enter the endosomal and lysosomal compartments upon uptake, it would make sense to target a lysosomal storage disease with this future enzyme replacement therapy study.

Additionally, rather than using the extracellular domain of VSVG, which is robustly uptaken by a broad spectrum of cell types, we may be able to replace this extracellular region with a targeting ligand, to preferentially direct modified exosomes and thus, its protein cargo to a certain cell type/tissue [35]. Using the example of lysosomal storage disease treatment, an extracellular targeting ligand for macrophages would be physiologically relevant, due to the nature of macrophages in degradation via lysosomal enzymes [36]. In general, with this system, we'd theoretically be able to deliver the necessary bioactive therapeutic to the appropriate tissue *in vivo*. This allows for lower amounts of off-target side effects, which in turn results in a lower necessary dosage required to produce a therapeutic effect [5]. Although we have shown delivery of proteins in this study, it would not be difficult to use our designed constructs to load our engineered exosomes with small molecule drugs, DNA, or RNA, which has far reaching implications in disease treatment of cancer and genetic disease [14, 37].

In future studies, the number of therapeutic proteins within each exosome would need to be determined to estimate the necessary *in vivo* therapeutic dosage. Furthermore, in our next studies, we could use a therapeutic protein within our modified exosomes for a disease model to determine efficacy of the delivery system and treatment. As previously mentioned, we can also remove the extracellular region of VSVG and replace it with a tissue specific targeting ligand to preferentially deliver our cargo to certain cell types, while also eliminates the potential for an immunogenic response to extracellular VSVG. Ultimately, animal studies would also be optimal to give us a

better understanding of an exosome-based drug delivery system *in vivo.* Through these studies, we'd prove the utility of exosomes as a nanocarrier of protein therapeutics and open the door for delivery of other biologically active therapeutics using this system.

CONCLUSION

We have demonstrated that VSVG has the ability to shuttle and anchor bioactive proteins into the secreted exosomal membranes, where, engineered exosomes can be collected and used as a delivery vehicle of desired therapeutic cargo to the endosomal and lysosomal cellular compartments of targeted cells. Our dual reporter system of GFP/RFP and Gluc/puro designed constructs was present in our modified exosomes, allowing us to track their movement visually and perform quantitative analysis. Our modified exosomes overcome many limitations of current protein drug delivery methods such as *in vivo* half-life and immunogenicity to name a few. Because exosomes are used naturally in cellular communication pathways, they offer unique properties *in vivo* that can be levied in drug delivery such as tumor cell penetration, and the ability to cross the blood-brain barrier. Engineered exosomes offer an exciting avenue for drug delivery research but needs to be studied in depth before therapeutic engineered exosomes can find its way into the clinical setting.

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